Genomic Reprogramming and Skin-Like Maturation of Engineered Human Skin Substitutes

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Background: Cultured skin substitutes (CSS) have been evaluated in clinical trials as an adjunctive treatment for large full-thickness burn wounds. Prepared with autologous fibroblasts, keratinocytes, and biopolymers, CSS can provide permanent wound closure upon engraftment to excised burns.

The Problem: CSS containing only two cell types are limited in anatomy and physiology compared with normal uninjured skin. Identifying deficiencies in CSS can instruct further tissue engineering advances.

Basic/Clinical Science Advances: Expression profiling of CSS during *in vitro* maturation and after transplantation *in vivo* with Affymetrix GeneChip® Arrays was used to characterize pathways that are abnormal or deficient in CSS compared with normal human skin. Examination of the large data set generated from microarray expression analysis revealed similarities between healed CSS and normal skin, particularly in expression of genes involved in epidermal differentiation and barrier function. However, deficiencies in several pathways were also noted, such as the genetic pathways regulating development of adnexal structures, including hair follicles.

Clinical Care Relevance: A deeper understanding of the cellular and molecular events guiding morphogenesis of engineered skin can lead to improvements that will increase clinical efficacy.

Conclusion: The results of GeneChip analysis highlighted the processes that act to regulate tissue development *in vitro* and adaptation to the wound environment and healing *in vivo*. This knowledge can be used to inform modifications to the model that will facilitate incorporation of additional cell types for increased homology with native human skin and improved functional outcome for burn patients treated with engineered skin grafts.

BACKGROUND

BIOENGINEERED SKIN SUBSTITUTES have been developed to meet the medical needs of patients with large skin loss injuries, such as massive full-thickness burns. Skin substitutes for burn wound healing should ideally replace all of the structures and functions of normal skin. Human skin performs a wide range of protective, perceptive, and regulatory functions, but its role in providing a protective barrier is most critical for survival. Cultured skin substitutes

(CSS), evaluated in clinical trials as an adjunctive treatment for large burns, were shown to provide permanent wound closure upon healing and supply barrier function.^{1,2} CSS are comprised of autologous fibroblasts and keratinocytes combined with a bovine collagen-based extracellular matrix (ECM) scaffold. Because CSS contain only two cell types, they cannot replace all of the structures and functions of normal human skin (NHS). Further bioengineering of skin substitutes

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Abbreviation and Acronym

CSS = cultured skin substitutes

ECM = extracellular matrix

GAG = glycosaminoglycan

NHS = normal human skin

STAG = split thickness autograft

TBSA = total body surface area

will likely include incorporation of additional cell types, which will require an in depth understanding of the physiology of CSS. Genomic profiling using gene expression microarrays has been used to characterize CSS, in comparison with its constituent cell types and with NHS, to gain an understanding of the processes involved in morphogenesis of engineered skin at a molecular level.^{3,4} This knowledge can be utilized to aid in the development of next-generation skin substitutes with improved function and greater homology to uninjured NHS.

TARGET ARTICLE

Klingenberg JM, McFarland KL, Friedman A, Boyce ST, Aronow BJ, and Supp DM: Engineered human skin substitutes undergo large-scale genomic reprogramming and normal skin-like maturation following transplantation to athymic mice. *J Invest Dermatol* 2010; **130**: 587.

CLINICAL PROBLEM ADDRESSED

Partial-thickness burns can heal by spontaneous re-epithelialization from epidermal stem cells resident in uninjured skin,⁵ but full-thickness burns require skin grafting for wound closure. The current standard for closure of excised burns is split-thickness autograft (STAG).⁶ For patients with burns covering a large percent of total body surface area (TBSA), insufficient donor sites for STAG can severely delay wound closure, increasing the risk of invasive wound infection and hypertrophic scarring.^{7,8} CSS have been evaluated for permanent skin replacement in patients with >50% TBSA burns.^{1,2,9} Although healed CSS resemble NHS, physiological deficiencies exist that can limit performance and reduce clinical efficacy. Development of skin substitutes with improved functionality requires an in-depth understanding of the processes guiding morphogenesis of CSS. This can be gained by genomic profiling of CSS using GeneChip microarrays.

RELEVANT BASIC SCIENCE CONTEXT

Skin tissue engineering requires selection of nonimmunogenic ECM scaffolds and cellular components, to regenerate as closely as possible the native structure of human skin. The bioengineered ECM must be compatible with the different cell types that will be included, and should promote adhesion, proliferation, and differentiation of skin cells. For CSS, biopolymer sponges fabricated from bovine collagen and glycosaminoglycans (GAGs)

provide a dermal matrix suitable for attachment of keratinocytes with low antigenicity.¹⁰ Dermal fibroblasts inoculated onto collagen-GAG sponges incorporate into pore-like structures of the matrix, attach, and proliferate, and support growth of keratinocytes. CSS are cultured *in vitro* at the air-liquid interface to promote development of a stratified epidermal layer and functional barrier properties.

In spite of the clinical benefits of CSS, limitations in anatomy and physiology remain. For example, healed CSS are characterized by deficient pigmentation due to absence of melanocytes, the pigment-producing cells of the epidermis. Incorporation of melanocytes into preclinical models of CSS demonstrated restoration of pigmentation, but was complicated by inefficient retention of melanocytes in the basal epidermis.^{11,12} This was associated with aberrant expression in CSS of appropriate cell surface receptors for melanocytes, including integrins.¹² Another example of an anatomic deficiency of CSS is lack of a vascular plexus *in vitro*, which delays vascularization after grafting and can contribute to reduced engraftment. Incorporation of endothelial cells for engineering of a microvascular system in CSS *in vitro* was hindered by low retention and poor *in vitro* organization of endothelial cells.¹³ Hypothetically, incorporation of melanocytes, endothelial cells, and other cell types into CSS for increased homology with NHS will require a complete characterization of CSS at the molecular level. This was undertaken using genomic profiling to characterize the changes in gene expression that occur during *in vitro* morphogenesis of CSS and *in vivo* adaptation after transplantation.⁴

EXPERIMENTAL MODEL OR MATERIAL: ADVANTAGES AND LIMITATIONS

The experimental model used for this study⁴ consists of CSS, prepared with cells from adult human donors, cultured *in vitro*, and transplanted as skin grafts to athymic mice.¹⁴ Athymic mice lack T cells, permitting permanent engraftment of human cells. To gain an understanding of the changes that occur in CSS during incubation in culture and after transplantation, multiple *in vitro* and *in vivo* time points were analyzed. RNA samples isolated from biopsies of CSS, and from NHS for comparison, were analyzed using Affymetrix Human GeneChips®, followed by bioinformatics analysis. Previous studies using the athymic mouse model for studies of CSS showed substantial homology of

healed CSS in mice with CSS grafted to burn patients.^{9,14} Other advantages of this system include the ability to analyze cells isolated from multiple different donors, reducing artifacts caused by person-to-person variability, and the ability to collect biopsies at multiple different time points, by grafting large numbers of mice. Thus, dynamic changes can be investigated without the need for collection of biopsies from patients. A limitation of this model is that, because human GeneChips are analyzed, genes expressed in cells from the mouse host are not characterized. This permits identification of gene expression in cells from the CSS without complications from host-derived cells, but not characterization of the host response to grafted CSS.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

There were 10,349 probe sets, representing 6,688 individual transcripts, that were expressed consistently across all samples of at least one group or time point.⁴ The raw data generated by scanning the GeneChips were converted to relative values by referencing each gene's expression to the level in CSS at day 3 *in vitro*. In this way, the relative level for each probe set, or gene, in day 3 *in vitro* was equal to 1, and the probe sets that were expressed at higher or lower levels in each of the other samples were higher or lower than 1, respectively. This allowed for comparisons of gene expression levels among different sample groups. Hierarchical clustering was used to permit observation of expression behaviors displayed by different groups of coordinately regulated genes, and resulted in a gene tree. Eleven clusters of genes, which each exhibited different patterns of expression across groups, were identified (Fig. 1). Each of the 11 clusters was subjected to gene set enrichment analysis¹⁵ to identify biological processes, molecular functions, and cellular components significantly associated with each cluster. This analysis identified functions associated with different patterns of gene expression behavior.

Striking changes in gene expression were observed during *in vitro* incubation and after transplantation to mice.⁴ Genes involved in biosynthetic pathways, such as RNA processing, biopolymer modification, and protein folding, were highly expressed early during morphogenesis of CSS *in vitro*. Many of the genes that were most highly upregulated during *in vitro* incubation were genes involved in epidermal differentiation, including keratin 2, loricrin, beta defensin 2, and multiple

Figure 1. Heat map depicting expression levels of 10,349 probe sets expressed in cultured skin substitutes *in vitro* or *in vivo*. Expression levels are depicted by color: red for high expression (>1), blue for low expression (<1), and black for mean expression (day 3 *in vitro* = 1). Each vertical column represents the mean for all samples in each group ($n = 3$), as indicated at the bottom of the figure. Each row represents a separate probe set, as indicated in the gene tree at the left side of the figure. Eleven clusters of genes were identified using hierarchical clustering; these clusters are color-coded and aligned with the right side of the heat map. As an example, cluster 1, the largest cluster, is identified by the yellow color block and contains genes that have the following expression pattern: expressed at low to moderate levels *in vitro*, increasing *in vitro* and after grafting, peaking at day 28 *in vivo* and then decreasing, and expressed at relatively low levels in NHS. Adapted from target article.⁴ d., day; C1–C11, 11 clusters; NHS, normal human skin.

S100 calcium binding proteins.¹⁶ This reflects the important processes of epidermal differentiation and stratification that occur during *in vitro* maturation of CSS. Massive changes in gene expression were observed after transplantation of CSS to mice, even as early as 3 days after grafting. This early period *in vivo* corresponds to a period of angiogenesis of the skin substitutes, marked by the beginning of vessel ingrowth from the wound bed.¹⁷ The fact that gene expression changes were observed so quickly after grafting suggests that the local wound environment exerts a strong influence on graft physiology. Immediately after grafting, genes involved in the stress response, response to external stimuli, and cell motility were upregulated, consistent with a wound healing

environment. Later after grafting, genes involved in regulation of developmental processes, anatomical structure morphogenesis, and apoptosis were upregulated, suggesting that tissue remodeling and maturation were occurring. Multicellular organismal development genes were highly represented in cluster 1 (see Fig. 1), indicating that these genes are upregulated *in vivo* and expressed at highest levels at days 14 and 28 after grafting. This suggests that genes normally involved in embryonic development are also regulating morphogenesis and adaptation of transplanted skin substitutes. Structural genes involved in epidermal barrier were among the most highly upregulated in CSS *in vivo*, particularly at later time points. Many of these were expressed at similar levels in CSS and NHS, consistent with the development of functional epidermal barrier in CSS after grafting.

In addition to similarities between CSS and NHS, microarray analysis demonstrated many important differences in gene expression.⁴ Some of these differences were due to cell types, such as endothelial cells or cells of the immune system, that are absent from CSS. Other differences have greater functional significance, and may reveal physiological deficiencies in CSS. For example, genes expressed in adherens junctions and tight junctions were expressed at significantly lower levels in healed CSS compared with NHS, suggesting deficiency of these structures in CSS *in vivo*. The beta defensin 2 gene, which was significantly upregulated during *in vitro* incubation, was downregulated after grafting, and remained very low at all *in vivo* time points. Defensin genes encode antimicrobial proteins that help the body fight infection.¹⁸ Hypothetically, resistance of CSS to microbial infection could be enhanced by increasing expression of beta defensin 2 and related genes. Other differences between CSS and NHS were observed for genes involved in development of adnexal structures, such as hair follicles. For example, bone morphogenetic protein 2, which is involved in hair follicle development, was expressed at lower levels in CSS than in NHS.⁴ The engineering of adnexal structures in CSS may require modification of the gene expression profiles of the constituent cell types to provide a permissive environment to accommodate the differentiation of cells into functional units, such as follicles.

INNOVATION

This study marked the first comprehensive analysis of gene expression of an engineered tissue

both *in vitro* and after transplantation *in vivo*. This extends a previous report from our laboratory that compared gene expression in CSS at the end of the *in vitro* incubation period with NHS, fibroblasts, and keratinocytes.³ In that study, global shifts in gene expression were observed between CSS and its constituent cell types, fibroblasts, and keratinocytes. However, that study only analyzed CSS at a single time point. To extend that study, we characterized the gene expression profile of CSS at multiple different points during *in vitro* development and after transplantation.⁴ These time points correspond to discrete phases of the morphogenesis of CSS *in vitro* and *in vivo*. By combining the hierarchical clustering of expression data with pathway analyses using bioinformatics approaches, the critical events guiding *in vitro* morphogenesis and *in vivo* adaptation of CSS can be appreciated. This wealth of data represents an atlas of gene expression for skin tissue engineering. These data can be mined and compared with existing or new data sets to identify new relationships between groups of genes or molecular pathways. Importantly, analysis of this data reveals networks of genes that interact to regulate tissue development of skin substitutes, which include many of the same gene networks involved in embryonic development, and can be used to guide future modifications of CSS for improved functional outcome.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

The study described here⁴ compared CSS with NHS, which represents the gold standard for skin tissue engineering. However, to date, the adaptation of grafted normal skin after transplantation has not been fully characterized using a genomic approach. Grafting with split-thickness skin is currently the standard of care for treatment of excised burn wounds, yet the gene expression changes that occur during the healing of grafted STAG have not been investigated. This represents an important gap in knowledge about the healing of skin grafts, and would have been an important, though technically more challenging, comparison for the genomic profiling of engineered skin grafts.

Also lacking in the literature is analysis of other types of tissue-engineered constructs after transplantation to host animals. Analysis of *in vitro* expression patterns have been described for engineered oral mucosa¹⁹ and kidney,²⁰ but *in vivo* analyses after transplantation were not reported. Understanding the adaptation of different types of

tissues to the host physiology may reveal similar mechanisms and increase our understanding of tissue regeneration. Further information may be obtained by comparison with developmental processes, eventually leading to processes that permit fabrication of engineered tissues based on mimicking embryonic tissue development.

FUTURE DEVELOPMENT OF INTEREST

Skin contains multiple cell types, but the most advanced skin substitutes currently available contain only two cell types. Future advances in the development of engineered skin replacements will likely include increased complexity for greater homology with NHS. For example, engineering of a vascular plexus would likely involve incorporation of multiple different cell types in CSS, such as microvascular endothelial cells, perivascular cells, and perhaps even endothelial progenitor cells. Modification of the gene expression profile of CSS, either by adjusting culture conditions, fabrication methods, or via genetic modification of cells, will likely be required to facilitate these additional engineering steps. However, this type of development will lead to improved skin substitutes that function more like uninjured human skin, and will represent a major advance in the treatment of burns and other skin loss injuries.

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TAKE-HOME MESSAGE

Basic science advances

- CSS comprised of autologous fibroblasts, keratinocytes, and a collagen-GAG biopolymer matrix have been evaluated in clinical trials as an adjunctive therapy for patients with massive burn injuries.
- CSS provide permanent wound closure but still exhibit anatomical and functional deficiencies compared with NHS.
- Gene expression profiling using GeneChip technology, combined with bioinformatics analysis, was used to characterize the gene expression profile of CSS during *in vitro* maturation and after transplantation to excisional wounds in athymic mice.
- Analysis of the rich data sets obtained from gene expression profiling revealed the complexity of interactions between cells, and between CSS and the wound environment, which underlie the morphogenesis of engineered skin.

Clinical science advances

- CSS that only contain two cell types are limited in composition and function compared with NHS.
- Analysis of the gene expression profile of CSS in comparison to NHS revealed differences that can be addressed by further engineering to increase homology with normal skin.
- Based on the results of gene expression analysis, it may be necessary to modify the expression profile of CSS to enable incorporation of additional cell types for development of adnexal structures or enhanced function.

Relevance to clinical care

- For patients with extremely large burn wounds or other full-thickness skin injuries, skin substitutes represent an alternative approach to wound closure that can reduce hospitalization and improve functional outcome. However, a deeper understanding of the molecular events that guide tissue development in skin substitutes is required to regulate the process for optimal structural design and functional outcome.

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REFERENCES

- Boyce ST, Kagan RJ, Yakuboff KP, Meyer NA, Rieman MT, Greenhalgh DG, and Warden GD: Cultured skin substitutes reduce donor skin harvesting for closure of excised, full-thickness burns. *Ann Surg* 2002; **235**: 269.
- Boyce ST, Kagan RJ, Greenhalgh DG, Warner P, Yakuboff KP, Palmieri T, and Warden GD: Cultured skin substitutes reduce requirements for harvesting of skin autograft for closure of excised, full-thickness burns. *J Trauma* 2006; **60**: 821.
- Smiley AK, Klingenberg JM, Aronow BJ, Boyce ST, Kitzmiller WJ, and Supp DM: Microarray analysis of gene expression in cultured skin substitutes compared with native human skin. *J Invest Dermatol* 2005; **125**: 1286.
- Klingenberg JM, McFarland KL, Friedman A, Boyce ST, Aronow BJ, and Supp DM: Engineered human skin substitutes undergo large-scale genomic reprogramming and normal skin-like maturation following transplantation to athymic mice. *J Invest Dermatol* 2010; **130**: 587.
- Yan X and Owens DM: The skin: a home to multiple classes of epithelial progenitor cells. *Stem Cell Rev* 2008; **4**: 113.
- Brusselaers N, Pirayesh A, Hoeksema H, Richters CD, Verbelen J, Beele H, Blot SI, and Monstrey S: Skin replacement in burn wounds. *J Trauma* 2010; **68**: 490.
- Xiao-Wu W, Herndon DN, Spies M, Sanford AP, and Wolf SE: Effects of delayed wound excision and grafting in severely burned children. *Arch Surg* 2002; **137**: 1049.
- Garner WL: Epidermal regulation of dermal fibroblast activity. *Plast Reconstr Surg* 1998; **102**: 135.
- Boyce ST, Greenhalgh DG, Kagan RJ, Housinger T, Sorrell JM, Childress CP, Rieman M, and Warden GD: Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. *Plast Reconstr Surg* 1993; **91**: 632.
- Boyce ST, Christianson DJ, and Hansbrough JF: Structure of a collagen-GAG dermal skin substitute optimized for cultured human epidermal keratinocytes. *J Biomed Mater Res* 1988; **22**: 939.
- Swope VB, Supp AP, and Boyce ST: Regulation of cutaneous pigmentation by titration of human melanocytes in cultured skin substitutes grafted to athymic mice. *Wound Repair Regen* 2002; **10**: 378.
- Swope VB, Supp AP, Schwemberger S, Babcock G, and Boyce S: Increased expression of integrins and decreased apoptosis correlate with increased melanocyte retention in cultured skin substitutes. *Pigment Cell Res* 2006; **19**: 424.
- Supp DM, Wilson-Landy K, and Boyce ST: Human dermal microvascular endothelial cells form vascular analogs in cultured skin substitutes after grafting to athymic mice. *FASEB J* 2002; **16**: 797.
- Boyce ST, Foreman TJ, English KB, Stayner N, Cooper ML, Sakabu S, and Hansbrough JF: Skin wound closure in athymic mice with cultured human cells, biopolymers, and growth factors. *Surgery* 1991; **110**: 866.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, and Mesirov JP: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005; **102**: 15545.
- Marenholz I, Zirra M, Fischer DF, Backendorf Z, Ziegler A, and Mischke D: Identification of human epidermal differentiation complex (EDC)-encoded genes by subtractive hybridization of entire YACs to a gridded keratinocyte cDNA library. *Genomics* 2001; **11**: 341.
- Supp DM, Supp AP, Bell SM, and Boyce ST: Enhanced vascularization of cultured skin substitutes genetically modified to overexpress vascular endothelial growth factor. *J Invest Dermatol* 2000; **114**: 5.
- Ganz T: Defensins and other antimicrobial peptides: a historical perspective and an update. *Comb Chem High Throughput Screen* 2005; **8**: 209.
- Alaminos M, Garzon I, Sanchez-Quevedo MC, Moreu G, Gonzalez-Andrades M, Fernandez-Montoya A, and Campos A: Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa. *J Tissue Eng Regen Med* 2007; **1**: 350.
- Rosines E, Sampogna RV, Johkura K, Vaughn DA, Choi Y, Sakurai H, Shah MM, and Nigam SK: Staged *in vitro* reconstitution and implantation of engineered rat kidney tissue. *Proc Natl Acad Sci USA* 2007; **104**: 20938.