Microarray Analysis of Gene Expression in Cultured Skin Substitutes Compared with Native Human Skin

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Cultured skin substitutes (CSS), prepared using keratinocytes, fibroblasts, and biopolymers, can facilitate closure of massive burn wounds by increasing the availability of autologous tissue for grafting. But because they contain only two cell types, skin substitutes cannot replace all of the functions of native human skin. To better understand the physiological and molecular differences between CSS and native skin, we undertook a comprehensive analysis of gene expression in native skin, cultured keratinocytes, cultured fibroblasts, and skin substitutes using Affymetrix gene chip microarrays. Hierarchical tree clustering identified six major clusters of coordinately regulated genes, using a list of 1030 genes that were the most differentially expressed between groups. These clusters correspond to biomarker pools representing expression signatures for native skin, fibroblasts, keratinocytes, and cultured skin. The expression analysis revealed that entire clusters of genes were either up- or downregulated upon combination of fibroblasts and keratinocytes in cultured skin grafts. Further, several categories of genes were overexpressed in CSS compared with native skin, including genes associated with hyperproliferative skin or activated keratinocytes. The observed pattern of expression indicates that CSS *in vitro*, which display a well-differentiated epidermal layer, exhibit a hyperproliferative phenotype similar to wounded native skin.

Key words: artificial skin/cDNA microarrays/gene expression profiling/tissue engineering/wound healing
J Invest Dermatol 125:1286–1301, 2005

Tissue-engineered skin substitutes have become an important adjunctive therapy for permanent wound closure in patients with massive skin loss because of burn injury. Composed of autologous keratinocytes alone (Carsin *et al*, 2000) or combined with autologous fibroblasts and biopolymers (Boyce *et al*, 1995, 1999, 2002a), skin substitutes can help restore epidermal barrier and facilitate permanent wound closure in patients with insufficient donor sites for autografting. But currently available skin substitutes, which contain one or two cell types, can only replace a subset of the structures and functions of native skin. Cultured skin substitutes (CSS) containing fibroblasts and keratinocytes perform similarly to autograft after healing, but engraftment can be reduced because of inherent limitations in CSS composition (Boyce *et al*, 1995, 2002a). Understanding the physiology of CSS at the molecular level can help guide the engineering of functional improvements for enhanced wound healing.

Expression of a limited number of genes involved in wound healing has been examined in CSS *in vitro*. Multiple growth factors are expressed by CSS, including basic fibroblast growth factor (bFGF), insulin-like growth factor 1, platelet-derived growth factor A, and vascular endothelial growth factor (VEGF) (Goretsky *et al*, 1996; Supp *et al*, 2000a, b; Swope *et al*, 2001). The cytokines interleukin (IL)-1α and IL-6 were shown to be expressed in CSS; IL-1α appeared to have been derived from the keratinocytes and bFGF from the fibroblasts. IL-6 was not highly expressed in either cell type alone, suggesting that it was induced upon combination of fibroblasts and keratinocytes in CSS (Goretsky *et al*, 1996). Collagen IV, collagen VII, and laminin 5 are expressed in CSS cultured in medium containing vitamin C, demonstrating nutrient-dependent basement membrane deposition (Boyce *et al*, 2002b). These studies provide insight into the morphogenesis of CSS, but a deeper understanding can be gained by a comprehensive analysis of gene expression using microarray technology. Expression analysis may highlight deficiencies of CSS that can be addressed using controlled culture conditions or genetic modifications.

Hypothetically, physiological deficiencies in CSS will be reflected by differences in gene expression between native skin and CSS. The goal of this study was to characterize the gene expression profile of CSS, in comparison with the constituent cell types and native human skin (NHS), using gene chip-based cDNA microarrays.

Abbreviations: CSS, cultured skin substitutes; ECM, extracellular matrix; EDC, epidermal differentiation complex; IL, interleukin; MMP, matrix metalloproteinases; NHS, native human skin

Results and Discussion

Microarray analysis and filtering strategy Skin was obtained from healthy adult females undergoing reduction

Table I. RNA samples used for microarray expression analysis

Donor ID#	633	634	Ref	639	651	636
Donor age (y)	52	23	31	40	53	54
Tissue	Abdomen	Breast	Breast	Abdomen	Breast	Breast
Native human skin	✓		✓	✓		✓
Cultured fibroblasts	✓	✓		✓	✓	
Cultured keratinocytes	✓	✓		✓	✓	
Cultured skin substitutes	✓	✓		✓	✓	

mammoplasty or abdominoplasty, and cell cultures were established that were used to prepare CSS (Fig S1). To minimize artifacts because of individual genetic variation, four biological replicates for each sample to be analyzed (NHS, cultured fibroblasts, cultured keratinocytes, and CSS) were isolated from isogenic sources (Table I). Complete sample sets were isolated from two donors. For the remaining samples, the RNA isolated from NHS was not sufficiently intact, based on Agilent Bioanalyzer data, for microarray analysis, and was replaced with RNA from donors with similar demographics.

The Affymetrix Human Genome HU133A gene chip, which was used for microarray expression analysis, represents over 22,000 transcripts from mostly known genes. Examination of the expression data revealed very consistent expression patterns among the biological replicates in each group, indicating relatively little variation because of age, individual genetic differences, or skin biopsy site as compared with relatively large differences between groups.

To analyze relative expression differences between sample groups, the expression levels for each gene were first normalized to the median expression level across the three *in vitro* cultured samples (CSS, fibroblasts, and keratinocytes; see Materials and Methods). By doing so, gene expression differences between the *in vitro* cultured cell types were emphasized and could be compared with expression in NHS. In addition, gene expression differences between the CSS and NHS because of the absence of cell types in CSS (e.g., melanocytes, antigen-presenting cells, nerves, fat, and endothelial cells) could be easily identified, as were genes that are signatures of, or most relevant to, keratinocytes and fibroblasts in the CSS.

A filtering strategy was employed to identify differentially expressed genes in each group; technical details are described in Materials and Methods. Briefly, eight lists were generated, representing the most under- and overexpressed probe sets in each group relative to the other groups. The top ranked probe sets were pooled, resulting in a list of 1720 transcripts (Fig S2). For the purposes of this report, we focused the cluster analysis on genes that were overexpressed, rather than genes that were underexpressed in one group. This allowed us to filter out 690 probe sets that were underexpressed compared with median expression levels (see Fig S2). For example, that list included genes such as plakophilin 1, desmoplakin, and desmocollin 3, which were not expressed in fibroblasts, but were expressed at similar levels in NHS, CSS, and keratinocytes (data not shown). These three genes encode proteins involved in formation of desmosomes, epithelial cell junctions involved in cell adhesion and epidermal morphogenesis (Green *et al*, 1990; King *et al*, 1995; McGrath *et al*, 1997). Similarly, the larger list included several collagen genes, including COL1A1, COL5A1, and COL6A2, which are expressed at similar levels in NHS, CSS, and fibroblasts, but are not expressed keratinocytes (data not shown); genes with this expression pattern were filtered out in the final cluster analysis. In addition, genes overexpressed in the cultured monolayer cells, but underexpressed in CSS and NHS, were in the list of 690 genes filtered out prior to cluster analysis. Examples include genes involved in control of the cell cycle and cytokinesis, such as cyclin A, protein regulator of cytokinesis 1, and kinesin 4A (Page *et al*, 1992; Jiang *et al*, 1998; Zhu and Jiang, 2005) (data not shown).

Signature genes The list of 1030 probe sets, representing 812 non-redundant genes that were relatively overexpressed, was subjected to hierarchical tree cluster analysis resulting in identification of six major gene clusters (Fig 1 and Figs S2 and S3). These clusters correspond to groups of coordinately regulated genes that represent expression signatures for NHS, CSS, fibroblasts, or keratinocytes. Cluster A includes transcripts that were expressed at high levels in both NHS and in CSS, but at low levels in fibroblasts and keratinocytes. Cluster B contains genes that were highly and specifically expressed in CSS. Cluster C includes transcripts that were expressed at the highest levels in NHS, although some of these genes were also moderately expressed in CSS. Cluster C was sub-divided into two smaller clusters, C1 and C2, based on middle-order tree branches (Fig 1). Cluster D contains genes highly expressed in cultured fibroblasts, and Cluster E genes were highly expressed in keratinocytes.

The cluster analysis revealed dramatic changes in gene expression patterns upon combination of the two cell types, fibroblasts and keratinocytes, in a three-dimensional matrix. Note that genes in Clusters A and B were very low in fibroblast and keratinocyte monolayer cultures, but were upregulated upon combination of the cells in the CSS. Further, genes expressed highly in fibroblast cultures (Cluster D) or keratinocyte cultures (Cluster E) were downregulated in CSS. These changes may reflect shifts in differentiation state and proliferation levels of the cells in the cultured skin compared with monolayer culture. This suggests paracrine interactions between the cells in the context of the CSS, and/or cellular responses to the changes in culture conditions. The 15 most highly expressed genes in each cluster

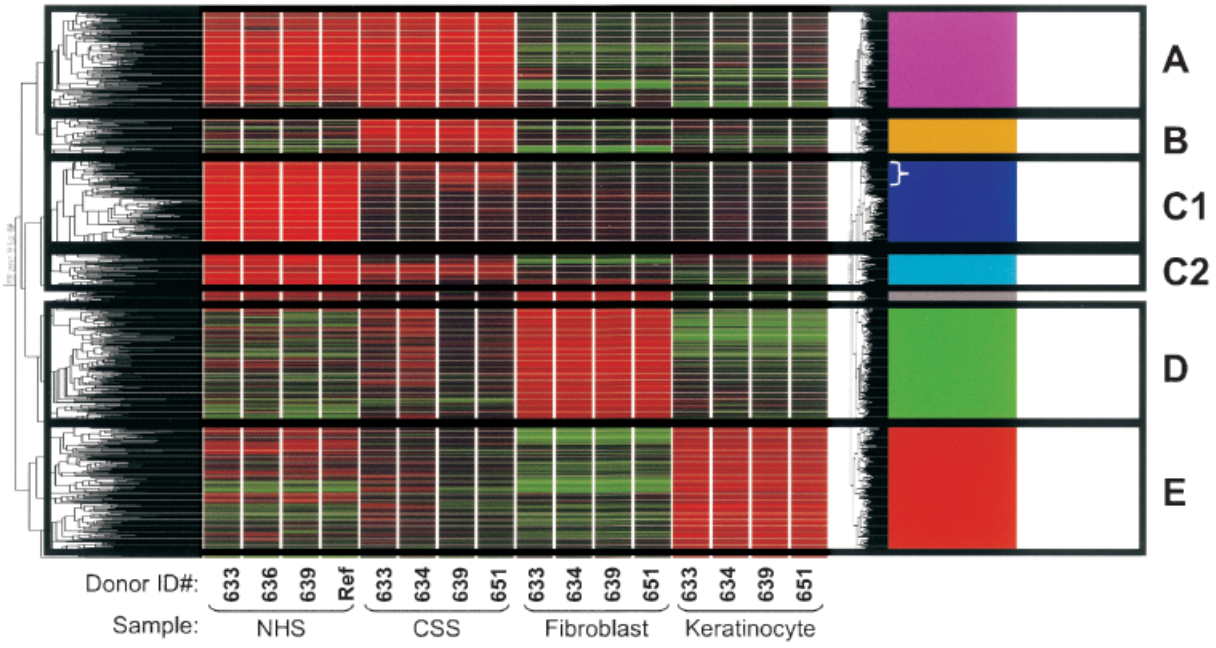

Figure 1
Hierarchical cluster analysis of normalized gene expression in native human skin (NHS), cultured skin substitutes (CSS), fibroblasts, and keratinocytes. Samples from the different donors are indicated on the column labels, and a total of 1030 genes are displayed on the y-axis (see Fig S3 for the list of genes, GenBank Accession Numbers, and quantitative expression data). Clusters A–E are indicated on the right of the figure, and the hierarchal tree is shown at the left. The white bracket indicates genes in Cluster C1 that are increased in CSS samples. Red color indicates high level expression, green color indicates low expression, and black indicates median expression.

are listed in Table II. The complete list of genes identified in the cluster analysis is presented in Fig S3.

To characterize the major biological functions or processes associated with the genes in each cluster, we used the approach of ontological association analysis (see Table S1). For each expression pattern cluster, a significance value was calculated based on the probability of there being a greater number of genes associated with a particular Gene Ontology (http://www.geneontology.org/) grouping in the cluster than would be expected based on random chance. Thus, a low p-value indicates a highly significant association of a particular function and that cluster. For example, in Cluster B, 28 probe sets (representing 18 non-redundant genes) were classified as adhesion related, with a p-value of 1.03×10^{-17}. This indicates that genes within this category are highly overrepresented in Cluster B compared with what would be expected by chance. Other functional categories that were identified in these analyses include apoptosis, cell growth and maintenance, collagen, extracellular matrix (ECM), intermediate filaments, proliferation, proteases, signal transduction, and transport (Table S1). The complete data sets can be accessed online at http://genet.cchmc.org (see Materials and Methods).

Cluster A This cluster contains genes that were highly expressed in CSS and also NHS, but were expressed at very low levels in fibroblasts and keratinocytes. Genes encoding several ECM proteins, which have a critical role in the structural integrity and mechanical strength of skin, were expressed similarly in CSS and NHS. Fibromodulin, decorin, and lumican, all members of the leucine-rich repeat proteoglycan family, were expressed at similar levels in CSS and NHS (Fig S3). These proteins function in collagen network assembly by binding to collagen fibrils (Svensson *et al*, 2000). Also found in Cluster A were matrix metalloproteinases MMP2 and MMP11, proteins involved in degrading ECM, which were both expressed in CSS and NHS, but at higher levels in CSS.

Cluster A was also highly enriched in genes associated with the biological process of signal transduction ($p = 2.25 \times 10^{-10}$; Table S1). Examples of genes in this category include cellular retinoic acid binding protein 2, retinoic acid receptor-related orphan receptor A, signal transducer and activator of transcription 3, src homology 2 domain containing transforming protein C3 (SHC3), IL-8 receptor β (IL8RB/CXCR2), and multiple chemokines (CCL2, CCL8, CCL19, and CXCL12).

Among the genes most differentially upregulated in Cluster A were genes involved in epidermal differentiation. These include several genes (S100A7, SPRR2B, filaggrin, involucrin, and NICE-1) that map to the epidermal differentiation complex (EDC) (Mischke *et al*, 1996; Marenholz *et al*, 2001), a 2-megabase region of chromosome 1q21 containing multiple families of genes that are involved in formation of the cornified envelope of the epidermis or in regulation of keratinocyte terminal differentiation. The EDC contains at least 11 S100 proteins expressed in keratinocytes or in human epidermis, which function as mediators of calcium-associated signal transduction (Eckert *et al*, 2004). The S100A7 gene, also called psoriasin, was very highly expressed in CSS and, to a lesser degree, in NHS. This particular S100 family member is upregulated in psoriatic epidermis and has antimicrobial activity (Madsen *et al*, 1991; Glaser *et al*, 2005). Involucrin and small proline-rich

Table II. Top genes in each cluster based on mean normalized expression level

Normalized expression level:				Gene	Accession	Function/biological process
NHS	CSS	CF	CK			
Cluster A						
34.56	207.90	0.63	1.03	S100A7: S100 calcium binding protein A7/ psoriasin 1	NM_002963	Epidermal differentiation
20.36	49.29	0.90	0.94	ARG1: arginase, type I	NM_000045	Urea cycle
27.44	46.64	0.88	0.93	HAL 1: histidine ammonia-lyase	NM_002108	Nitrogen metabolism
4.93	38.80	0.56	0.94	SPRR2B: small proline-rich protein 2B	NM_006945	Cornified envelope protein; epidermal differentiation
61.55	38.17	0.41	0.98	FLG: filaggrin	AL356504	Promotes keratin filament aggregation in cornified epidermis
17.85	30.39	0.63	0.96	CDSN: corneodesmosin	NM_001264	Cell adhesion molecule; epidermal differentiation; intercellular junction
15.84	28.34	0.58	1.00	NICE-1: NICE-1 protein	NM_019060	Function unknown; upregulated in differentiated keratinocytes
29.09	24.99	0.88	0.55	OSF-2: osteoblast-specific factor 2 (periostin)	NM_006475	Cell adhesion molecule; extracellular matrix; skeletal development
12.89	23.05	0.92	0.96	MAP17: membrane-associated protein 17	NM_005764	Oncogenesis; replication
17.65	20.00	0.65	1.02	LY6G6C: lymphocyte antigen 6 complex, locus G6C	NM_025261	Putative immune related role
13.50	19.74	0.88	0.89	ARS/SLURP1: uPAR-related protein 1	NM_020427	Putative antitumor activity
19.17	19.09	0.67	1.01	KRT23: cytokeratin 23	NM_015515	Intermediate filament protein, histone deacetylase inducible
16.59	17.02	0.97	0.92	PSORS1C1: psoriasis susceptibility 1 candidate 1	NM_014068	Cell adhesion molecule; epidermal differentiation
5.49	16.84	1.01	0.87	CD36: cluster determinant 36	NM_000072	Collagen type I receptor; thrombospondin receptor
9.89	16.78	1.04	0.73	PTGDS: prostaglandin-H2 D-isomerase	NM_000954	Prostaglandin and leukotriene metabolism
1.86	15.13	0.93	0.95	SLC6A14: solute carrier family 6, member 14	NM_007231	Amino acid metabolism; neurotransmitter transport
Cluster B						
1.19	30.19	0.18	0.97	SERPINB4/SCCA2: serine protease inhibitor/ squamous cell carcinoma antigen 2	NM_002974	Serine protease inhibitor
2.13	28.91	0.26	0.95	S100A9: S100 calcium binding protein A9 (calgranulin B)	NM_002965	Cell cycle progression; differentiation
1.07	27.12	0.94	0.92	DEFB4: skin-antimicrobial peptide 1 (formerly defensin β2)	NM_004942	Chemotaxis; immune response
0.87	18.21	0.72	1.04	TCN1: transcobalamin I (vitamin B12 binding protein)	NM_001062	Vitamin B12 transport
1.35	18.21	0.98	0.94	CCL20: chemokine (C–C motif) ligand 20	NM_004591	Chemotaxis; immune response; signal transduction: cell–cell signaling
0.75	18.10	0.76	1.09	MMP10: matrix metalloproteinase 10/stromelysin 2	NM_002425	Zinc ion binding; extracellular matrix
1.59	16.59	0.82	0.99	S100P: S100 calcium binding protein P	NM_005980	Cell cycle progression; differentiation
1.64	14.80	0.89	1.13	MMP9: matrix metalloproteinase 9 (gelatinase B, 92 kDa type IV collagenase)	NM_004994	Zinc ion binding; proteolysis and peptidolysis; extracellular matrix

Table II. Continued

Normalized expression level:				Gene	Accession	Function/biological process
NHS	CSS	CF	CK			
0.62	13.46	0.78	0.89	CXCL1: chemokine, cxc motif, ligand 1/GRO1 oncogene	NM_001511	Cell proliferation; chemotaxis
1.33	11.87	0.16	1.01	SERPINB3/SCCA1: serine/cysteine proteinase inhibitor, clade B, member 3/squamous cell carcinoma antigen 1	NM_006919	Serine protease inhibitor; tumor antigen
1.06	11.72	0.42	1.05	LCN2: lipocalin 2/neutrophil gelatinase-associated lipocalin	NM_005564	Transporter activity; dysregulated epidermal differentiation marker
1.07	11.35	0.74	1.00	ATP12A: ATPase, Na + K + transporting, α-1 polypeptide like	NM_001676	Potassium ion transport; metabolism
1.04	11.16	0.98	0.97	S100A12: S100 calcium binding protein A12/ calgranulin-related protein	NM_005621	Calcium-dependent signal transduction
0.91	10.97	1.03	0.81	IL-6: interleukin 6 (interferon, β2)	NM_000600	Cell proliferation; humoral immune response; cell surface receptor linked signal transduction
0.41	10.35	0.09	0.99	PI3: protease inhibitor 3, skin-derived (SKALP)/ elafin precursor	NM_002638	Immune response; antimicrobial
Cluster C1						
150.82	68.73	0.93	0.95	LOR: loricrin	NM_000427	Structural constituent of cytoskeleton; cell shape and size control
88.35	54.27	0.84	0.98	KRT2A: keratin 2A	NM_000423	Intermediate filament; epidermal differentiation
86.51	1.36	1.01	1.02	HLA-DRB3: major histocompatibility complex, class II, DR β3	NM_002124	Immune response; antigen presentation; antigen processing
84.69	1.07	1.00	1.27	TYRP1: tyrosinase-related protein 1	NM_000550	Melanin biosynthesis from tyrosine
71.49	0.98	1.06	0.97	HLA-DRA: major histocompatibility complex, class II, DR α	NM_019111	Immune response; antigen presentation; antigen processing
70.49	1.10	0.83	0.97	CD74/HLA-DR-γ: invariant γ chain of class II antigens	NM_004355	Immune response; chaperone activity
62.79	46.43	0.90	0.97	SPRL1B/LEP10: small proline rich-like 1B/late envelope protein 10	NM_014357	Epidermal differentiation
53.25	1.28	2.66	0.78	HLA-DPA1: major histocompatibility complex, class II, DP α-1	NM_033554	Immune response; antigen presentation; antigen processing
49.24	0.97	0.94	1.02	FCER1A: high affinity immunoglobulin epsilon receptor α-subunit	NM_002001	Immune response; receptor signaling protein activity; IgE binding
43.60	0.96	1.00	1.07	DCT: dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	NM_001922	Melanin biosynthesis
42.04	3.10	1.06	0.83	DF: D component of complement (adipsin)	NM_001928	Serine-type peptidase; complement activation
39.43	24.89	0.64	1.06	CALML5/CLSP: calmodulin-like 5 (calmodulin-like skin protein)	NM_017422	Epidermal differentiation; regulation of transcription, DNA-dependent; signal transduction
37.94	1.00	0.99	1.03	HLA-DQA1: major histocompatibility complex, class II, DQ α-1	NM_002122	Pathogenesis

36.89	9.32	0.72	1.01	IGFBP5: insulin-like growth factor binding protein 5	NM_000599	Signal transduction; regulation of cell growth
30.16	0.96	1.00	0.99	LYZ: lysozyme precursor	NM_000239	Carbohydrate metabolism; inflammatory response; cytolysis; cell wall catabolism; antimicrobial
Cluster C2						
63.18	2.69	0.91	0.93	SPARCL1: SPARC-like 1 (mast9, hevin)	NM_004684	Calcium-binding EF-hand; serine protease inhibitor, Kazal type; extracellular matrix
37.04	0.92	3.61	0.86	CRIP2: cysteine-rich protein 2	NM_001312	Zn-binding protein LIM domain protein; transcription regulation
20.23	1.05	0.80	1.45	HLA-DQB2: major histocompatibility complex, class II, DQ β2	NM_182549	Immune response; antigen presentation; antigen processing
13.72	1.31	3.56	0.22	TNA: tetranectin (plasminogen-binding protein)	NM_003278	C-type lectin; sugar binding; extracellular matrix
13.32	1.30	2.04	0.48	AQP1: aquaporin-1 (channel-forming integral protein, 28 kDa)	NM_198098	Water transport; excretion
10.68	1.79	0.11	1.28	CXCL14: chemokine (C–X–C motif) ligand 14	NM_004887	Immune response; signal transduction; cell–cell signaling; inflammatory response
10.58	1.16	2.04	0.72	PDGFRL: platelet-derived growth factor receptor-like protein	NM_006207	Platelet activating factor receptor activity; tumor suppressor
9.90	1.07	0.67	1.37	WNT4: wingless-type MMTV integration site family, member 4	NM_030761	Signal transduction; cell fate regulation
9.88	1.22	2.16	0.82	ABCA8: ATP-binding cassette, sub-family A member 8	NM_007168	Nucleotide binding; ATP binding
9.60	7.45	1.29	0.55	DPT/TRAMP: dermatopontin/tyrosine-rich acidic matrix protein	NM_001937	Cell adhesion molecule activity: extracellular matrix
7.21	3.40	0.85	0.84	NEBL: nebulette	NM_006393	Regulation of actin thin filament length; tropomyosin binding; structural constituent of muscle
6.70	1.02	0.48	2.56	NFIB: nuclear factor I/B	NM_005596	Transcription factor
6.57	1.03	0.22	1.19	GATA3: GATA-binding protein 3	NM_002051	Zn-finger transcription activating factor
6.27	0.99	0.83	1.49	PER2: period circadian protein 2	NM_022817	Circadian rhythm
6.10	1.92	1.00	0.55	ANK2: ankyrin-2, nonerythrocytic	NM_001148	Structural constituent of cytoskeleton
Cluster D						
1.53	1.46	15.48	0.52	GLIPR1: glioma pathogenesis-related protein	NM_006851	Extracellular
0.99	1.47	15.03	0.75	CNR1: cannabinoid receptor 1 (brain)	NM_001840	G-protein signaling, coupled to cyclic nucleotide second messenger; behavior
2.21	1.39	11.44	0.67	HRB2: HIV-1 rev binding protein 2	NM_007043	Rev binding protein
0.57	1.03	11.09	0.41	IGFBP2: insulin-like growth factor binding protein 2, 36 kDa	NM_000597	Regulation of cell growth
2.30	1.64	10.57	0.31	TPM2: tropomyosin 2 (β)	NM_003289	Structural constituent of muscle; actin binding; muscle development
1.03	1.42	9.65	0.53	BAG-2: BCL2-associated athanogene 2	NM_004282	Protein binding; chaperone activity; apoptosis regulator activity
2.76	2.12	9.37	0.65	GNG11: guanine nucleotide binding protein 11	NM_004126	Heterotrimeric G-protein GTPase, γ-subunit; signal transduction
1.55	1.35	8.62	0.35	EPS8: epidermal growth factor receptor pathway substrate 8	NM_004447	Cell proliferation; signal transduction
1.44	1.99	8.48	0.59	PLAT: plasminogen activator, tissue	NM_000930	Proteolysis and peptidolysis; blood coagulation; protein modification

Table II. Continued

Normalized expression level:				Gene	Accession	Function/biological process
NHS	CSS	CF	CK			
1.32	1.06	7.91	0.72	KCNK2: potassium channel, subfamily K, member 2	NM_014217	Sensory perception
0.87	1.35	6.91	0.49	PTGER2: prostaglandin E receptor 2, EP2 subtype, 53 kDa	NM_000956	G-protein coupled receptor protein signaling pathway; signal transduction
1.10	1.19	6.72	0.66	CRLF1: cytokine receptor-like factor 1	NM_004750	Signal transduction
1.06	1.29	6.52	0.54	KDELR3: KDEL (Lys–Asp–Glu–Leu) endoplasmic reticulum protein retention receptor 3	NM_006855	ER lumen protein retaining receptor; intracellular protein transport
0.93	1.78	6.50	0.40	NID2: nidogen 2 (osteonidogen)	NM_007361	Collagen binding; calcium ion binding; cell adhesion; basement membrane
0.68	1.40	6.50	0.48	PRKCM: protein kinase C, mu	NM_002742	Signal transduction
Cluster E						
0.67	1.20	0.72	8.75	PTHLH: parathyroid hormone-like protein	NM_002820	cAMP generating peptide; peptide hormone; epidermal differentiation
1.29	1.02	0.49	7.66	GCLC: glutamate-cysteine ligase, catalytic subunit	NM_001498	Circulation; glutathione biosynthesis
5.04	0.88	0.05	7.50	KRT15: cytokeratin 15	NM_002275	Structural constituent of cytoskeleton; epidermal differentiation; intermediate filament
0.69	1.14	0.28	7.36	DSG2: desmoglein 2	NM_001943	Calcium-dependent cell adhesion molecule
0.84	0.96	0.95	6.89	NRG1: neuregulin 1	NM_013957	Signal transduction; cell growth and differentiation
0.77	1.04	0.37	6.85	FLRT3: fibronectin leucine rich transmembrane protein 3	NM_198391	Cell adhesion receptor; signal transduction
0.60	1.02	0.46	6.80	PKP2: plakophilin 2	NM_004572	Cytoskeleton; integral to membrane; desmosome
1.11	1.21	0.20	6.57	SORL1: sortilin-related receptor, L(DLR class) A repeats containing	NM_003105	Lipid transport; receptor mediated endocytosis; cholesterol metabolism; intracellular protein transport
1.75	0.99	0.72	6.33	TACSTD1: tumor-associated calcium signal transducer 1	NM_002354	Lymphocyte antigen; tumor antigen; calcium-independent cell adhesion
0.39	0.97	0.51	6.23	ARTN: neurotrophic factor artemin isoform 1	NM_057091	Neuroblast proliferation; signal transduction
0.65	1.31	0.45	5.82	LAMC2: laminin, $\gamma 2$	NM_005562	Heparin binding; structural molecule; cell adhesion; epidermal differentiation
3.93	1.02	0.56	5.79	ADRB2: β-2 adrenergic receptor	NM_000024	G-protein coupled receptor; signal transduction
0.34	0.88	0.53	5.33	SLC7A5: solute carrier family 7, member 5	NM_003486	Amino acid transport; amino acid metabolism
0.12	0.92	0.11	5.17	AREG: amphiregulin (schwannoma-derived growth factor)	NM_001657	Growth factor; cell proliferation; cell–cell signaling
0.35	1.23	0.61	5.00	TNFRSF21: tumor necrosis factor receptor superfamily, member 21 precursor	NM_014452	Apoptosis

The mean expression levels for each sample group, gene symbols and names, accession numbers, and brief functional summaries for the 15 most highly expressed genes in each cluster are shown. NHS, native human skin; CSS, cultured skin substitutes; CF, cultured fibroblasts; CK, cultured keratinocytes.

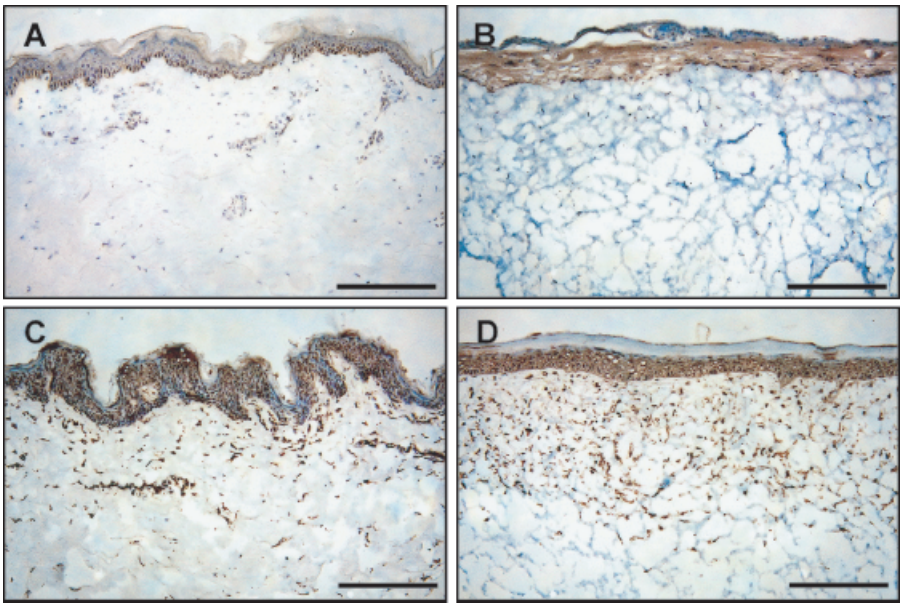

Figure 2
Localization of MMP10 and TIMP3 in native human skin (NHS) and cultured skin substitutes (CSS). (*A*, *B*) Immunostaining of NHS and CSS, respectively, with anti-MMP10 antibody. (*C*, *D*) Anti-TIMP3 staining in NHS and CSS, respectively. Epidermis is at the top of each panel; *scale bar* = 0.2 mm.

proteins (SPRR), including SPRR2B, are precursors of the cornified envelope and are critical for skin barrier function. Both of these genes were expressed at relatively higher levels in CSS compared with NHS, and were higher in both tissues compared with cultured fibroblasts or keratinocytes (Table II and Fig S3). Also linked to the EDC are multiple NICE proteins; NICE-1 was upregulated in CSS compared with fibroblasts and keratinocytes, and was also highly expressed in NHS. The function of NICE-1 is unclear, but it is thought to have a role in epidermal differentiation because it is upregulated in differentiated keratinocytes (Marenholz *et al*, 2001).

There were additional differentiation-associated genes in Cluster A that are unlinked to the EDC, including the keratin intermediate filament genes keratins 1, 10, and 23, the cornified envelope glycoprotein corneodesmosin (Simon *et al*, 1997), and the psoriasis susceptibility locus PSORSC1C (Holm *et al*, 2003). Further, the ARS/SLURP-1 gene (secreted Ly-6/uPAR related protein) was highly expressed in both CSS and NHS. SLURP-1 is believed to function in skin homeostasis, and is mutated in the autosomal recessive skin disorder Mal de Meleda, which is characterized by diffuse palmoplantar keratoderma (Chimienti *et al*, 2003; Eckl *et al*, 2003).

Cluster B This cluster contains genes that were highly expressed in CSS and were expressed at low levels or absent from NHS, fibroblasts, and keratinocytes. Thus, Cluster B represents genes that were upregulated upon combination of fibroblasts and keratinocytes for preparation of CSS, and were relatively specific to CSS compared with NHS. Examination of the functions of genes present in this cluster

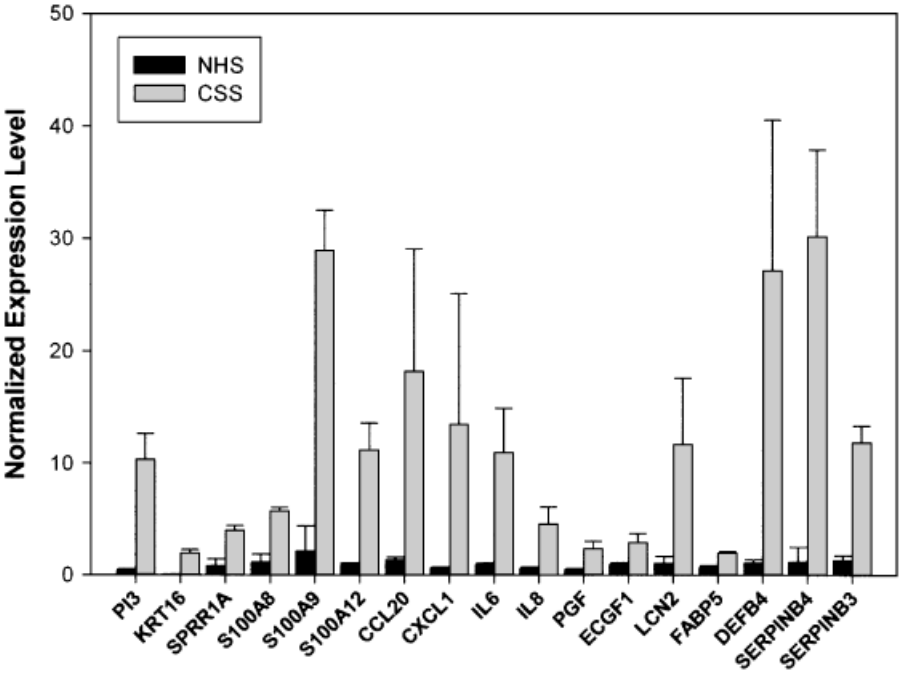

Figure 3
Relative expression of genes involved in epidermal differentiation and hyperproliferation. Shown are mean normalized expression levels; error bars represent ± standard deviations. All genes shown were expressed at significantly higher levels (*t* test) in cultured skin substitutes (CSS) compared with native human skin (NHS) ($p < 0.05$).

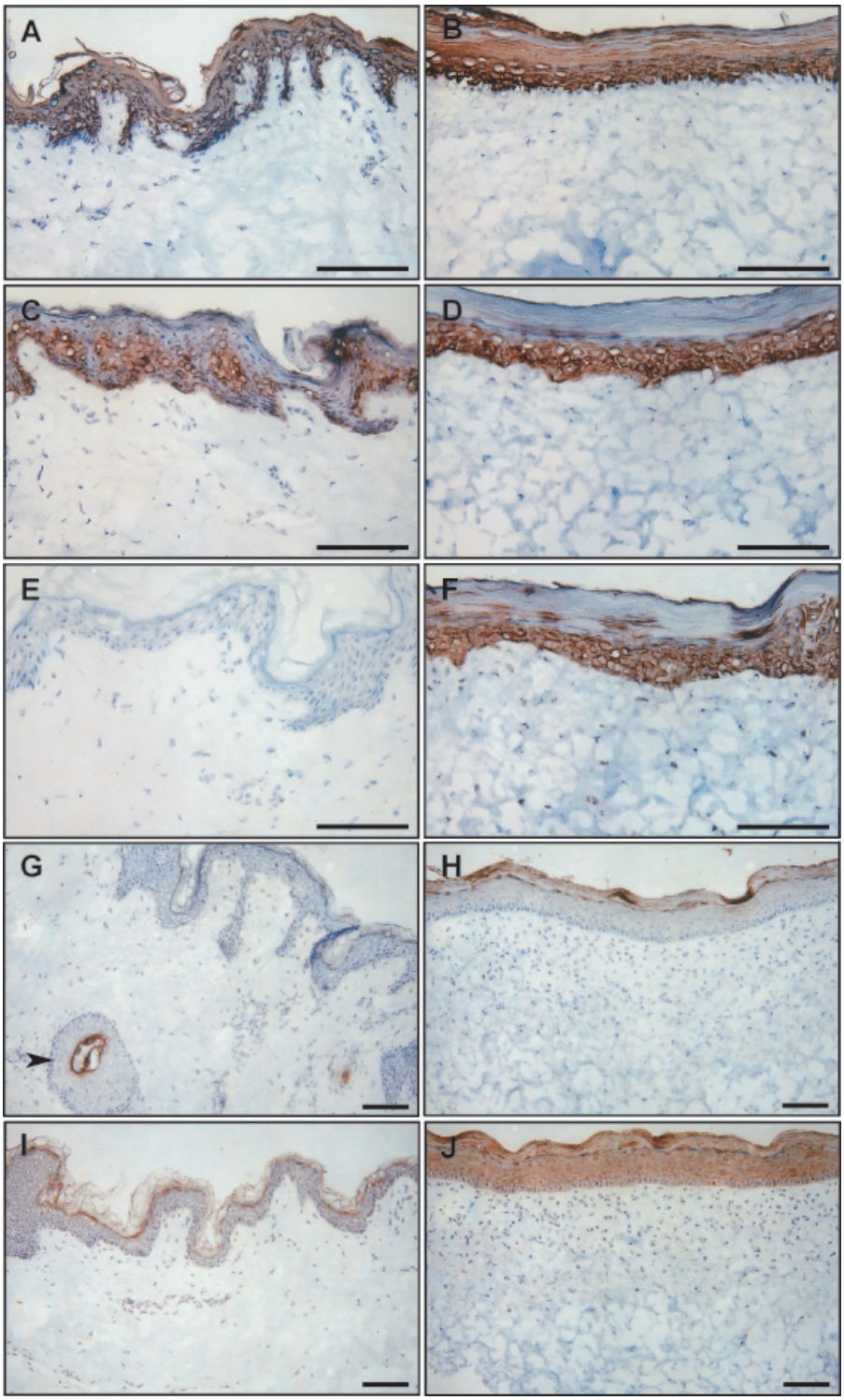

Figure 4
Immunohistochemical localization of keratins and differentiation markers in native human skin (NHS) and cultured skin substitutes (CSS). Shown are sections of NHS (*left column*; *A*, *C*, *E*, *G*, *I*) and CSS (*right column*; *B*, *D*, *F*, *H*, *J*), stained with antibodies against human keratin 10 (*A*, *B*), keratin 5/8 (*C*, *D*), keratin 16 (*E*, *F*), lipocalin 2 (*G*, *H*), or SERPINB3/B4 (*I*, *J*). Arrow in panel *G* indicates positive lipocalin 2 staining in hair follicle. *Scale bars* = 0.1 mm.

reveals important information about the physiology of CSS, and characteristics that distinguish CSS from NHS.

Genes involved in cell proliferation were highly enriched in Cluster B ($p = 4.01 \times 10^{-9}$; Table S1), and keratinocyte mitogens were highly expressed in CSS. For example, IL-6 was among the most highly expressed transcripts in Cluster B (Table II). IL-6 is a proinflammatory cytokine that has been shown to stimulate the proliferation and migration of keratinocytes, and it is expressed at high levels in psoriasis (Grossman *et al*, 1989; Olaniran *et al*, 1995). Also expressed at high levels in CSS was the endothelin-1 receptor EDNRA (Fig S3), which participates in autocrine signaling of keratinocyte proliferation (Bagnato *et al*, 1995; Venuti *et al*, 1998).

Proteases involved in digestion of the ECM were among the most highly expressed genes in Cluster B. MMP9 and MMP10 were expressed at high levels in CSS (Table II); a third related gene in Cluster B, MMP3, was also elevated in CSS compared with NHS (Fig S3). The MMPs make up a large family of enzymes that, as a whole, are capable of

degrading essentially all ECM components. MMP3 and MMP10 (also called stromelysin 1 and stromelysin 2, respectively) degrade proteoglycans and are expressed during wound repair (Madlener *et al*, 1998). MMP3 expression has been reported in fibroblasts (Sirum and Brinckerhoff, 1989) as well as keratinocytes (Madlener *et al*, 1998), whereas MMP10 is limited to keratinocytes and is increased in wounds at the tip of the migrating epithelium (Madlener *et al*, 1998; Krampert *et al*, 2004). MMP9, also called gelatinase B, degrades denatured collagen and is expressed in wounds in both the granulation tissue and in the hyperproliferative migrating epithelium (Madlener *et al*, 1998). Consistent with previous reports, MMP10 protein in CSS was localized to the epidermal layer, and was also observed in the epidermis of NHS (Fig 2). Interestingly, TIMP-3, a physiological inhibitor of MMP, was found by immunohistochemistry to be expressed in NHS and CSS (Fig 2). The expression of MMP and TIMP-3 in CSS suggests that the cells are actively remodeling the ECM to facilitate cell proliferation, migration, and differentiation into a stratified epithelium.

Consistent with the increased MMP expression observed in CSS, examination of other genes in Cluster B reveals a profile of gene expression suggestive of epidermal differentiation and hyperproliferation, resembling a wound-healing phenotype. Several genes that encode proteins of the cornified envelope, inflammatory mediators, angiogenic factors, and markers of abnormal keratinocyte differentiation were expressed at significantly higher levels in CSS compared with NHS (Fig 3). For example, one of the most highly expressed genes in Cluster B was protease inhibitor 3 (PI3), also called skin-derived antileukoproteinase (SKALP) or elafin, which is expressed at very low levels in normal skin but is upregulated in psoriasis (Nakane *et al*, 2002). PI3 is a constituent of the keratinocyte cornified envelope, where it co-localizes with involucrin. Similarly, the gene encoding keratin 16 (KRT16), an intermediate filament that contributes to the structural scaffold of epidermal keratinocytes, was expressed at very low levels in native skin but was significantly higher in CSS (Fig 3). This keratin is upregulated during a process termed keratinocyte activation, which occurs during wound healing, and is markedly overexpressed in epithelia that display abnormal differentiation, such as psoriatic epidermis (Coulombe *et al*, 1995; Padilla *et al*, 1996; Coulombe, 1997). At the protein level, immunohistochemistry showed that in contrast to keratins 5 and 10, which are not known to be increased in activated keratinocytes, keratin 16 was dramatically increased in CSS compared with NHS (Fig 4). Small proline-rich protein SPRR1A, which encodes a major precursor of the cornified envelope, is linked to involucrin in the EDC on chromosome 1q21 (Marenholz *et al*, 2001). Like PI3 and KRT16, SPRR1A is highly expressed in differentiating epithelia and in psoriasis (Hohl *et al*, 1995; Fujimoto *et al*, 1997), and was also expressed at significantly higher levels in CSS compared with NHS (Fig 3). Other members of the EDC, such as S100A8, S100A9, and S100A12, were also present in Cluster B and expressed at very high levels in CSS (Table II; Fig 3). These calcium-binding proteins are expressed minimally in normal skin but are upregulated in psoriasis (Eckert *et al*, 2004).

Multiple chemokines and cytokines with putative roles in the pathogenesis of psoriasis were expressed at significantly higher levels in CSS compared with NHS (Fig 3). For example, CCL20 expression was 13.5-fold higher in CSS than in NHS. This member of the C–C chemokine family, also called macrophage inflammatory protein-3α (MIP-3α), is expressed in skin after barrier disruption (Schmuth *et al*, 2002), in psoriatic epidermis (Homey *et al*, 2000), and in differentiating cultured keratinocytes (Pernet *et al*, 2003). CCL20 has been shown to direct the migration of dendritic cells and lymphocytes to sites of antigen invasion, and was proposed to play a role in immunosurveillance in inflammatory skin conditions such as psoriasis (Homey *et al*, 2000; Schmuth *et al*, 2002). The C–X–C chemokine CXCL1, also referred to as growth-related oncogene α, and IL-8 are both overexpressed in psoriatic epidermis where they are proposed to influence neutrophil migration and activation (Gillitzer *et al*, 1996). CXCL1 and IL-8 were elevated 21.6- and 7.7-fold, respectively, in CSS compared with NHS (Fig 3).

Cluster analysis indicates that angiogenic genes, which have been implicated in cutaneous inflammation and psoriasis, were also increased in CSS compared with NHS. Endothelial cell growth factor 1 (ECGF1, or platelet-derived ECGF) is overexpressed in psoriatic epidermis, which may contribute to the microvascular changes observed in lesional skin (Creamer *et al*, 1997). Placental growth factor, a member of the VEGF family, has also been associated with pathologic angiogenesis and has a proposed role in the control of cutaneous inflammation (Oura *et al*, 2003). These angiogenic growth factors were both present in Cluster B, and were significantly overexpressed in CSS compared with NHS (Fig 3).

Increased or abnormal epidermal differentiation is a hallmark of psoriasis, and several genes that are associated with keratinocyte differentiation and are increased in psoriasis were present in Cluster B. For example, lipocalin 2 (LCN2), also called neutrophil gelatinase-associated lipocalin (NGAL), a marker for dysregulated differentiation in keratinocytes (Mallbris *et al*, 2002), was among the most highly expressed genes in Cluster B. LCN2 expression in normal skin is restricted to hair follicles, but strong epidermal expression is observed in lesional skin from psoriasis patients (Mallbris *et al*, 2002). In CSS, high levels of LCN2 protein were found in the epidermis (Fig 4), but in the corresponding NHS, expression was found only in hair follicles, consistent with previous reports (Mallbris *et al*, 2002). Serine protease inhibitors SERPINB3 and SERPINB4, also referred to as squamous cell carcinoma antigens SCCA1 and SCCA2, were expressed at approximately 9- and 25-fold higher levels, respectively, in CSS compared with NHS. These proteins were identified in sera of psoriasis patients and localized to the epidermis of lesional skin (Takeda *et al*, 2002a). Immunohistochemistry with an antibody that recognizes both SERPINB3 and SERPINB4 showed expression in both CSS and NHS, but at relatively higher levels in CSS (Fig 4). Epidermal fatty acid binding protein (FABP5), which is involved in lipid transport and metabolism in the epidermis is also overexpressed in differentiated keratinocytes in normal and psoriatic epidermis (Siegenthaler *et al*, 1994), was expressed at significantly higher levels in CSS than NHS (Fig 3). Interestingly, FABP5 has been shown to

co-localize with S100A7 (psoriasin), a Cluster A gene that is also overexpressed in psoriasis, and this complex may target FABP5 to specific subcellular locations (Ruse *et al*, 2003).

The S100A7 gene, which was identified in Cluster A and thus was elevated in both NHS and CSS, has been shown to have antimicrobial activity (Glaser *et al*, 2005). In Cluster B, the antimicrobial peptide gene DEFB4 (formerly called human β-defensin-2) was among the most highly expressed genes. Expression of DEFB4 is regulated by cytokines, including IL-1 and IL-6, and epidermal differentiation (Erdag and Morgan, 2002; Liu *et al*, 2002). Originally identified from psoriatic skin, DEFB4 peptide plays an important role in the innate cutaneous immune system (Harder *et al*, 1997; Schroder and Harder, 1999). A previous study demonstrated expression of DEFB4 protein in CSS (Supp *et al*, 2004). In this study, microarray analysis showed that expression of DEFB4 RNA in CSS was approximately 25-fold higher than in NHS, and 29-fold higher than in fibroblasts or keratinocytes (Fig 3 and Table II).

The dramatic increases in expression of Cluster B genes in CSS compared with monolayer cultures suggests that the upregulation may result from combination of fibroblasts and keratinocytes. Additional influences on the expression profiles of cells in the context of CSS may include the composition of the nutrient medium, which has a higher calcium concentration (1.8 mM) compared with keratinocyte growth medium (Boyce, 1999; Swope *et al*, 2001), and the culture format, composed of a stratified three-dimensional air-exposed tissue. These culture conditions have been optimized to enhance epidermal differentiation and stratification (Boyce, 1999; Swope *et al*, 2001; Boyce *et al*, 2002b). Because differentiated epidermal keratinocyte cultures have been shown to upregulate genes involved in terminal differentiation, barrier formation, and host protection (Jansen *et al*, 2001), the state of keratinocyte differentiation in the CSS may account for some of the observed gene expression changes. Further, the relatively high cell density in the CSS may facilitate cell–cell interactions within and between cell types that could modulate gene expression.

Cluster C1 Cluster C contains genes that are the most highly and specifically expressed in NHS, although some of these genes are also expressed in CSS, CF, or CK. To facilitate the analysis, Cluster C was sub-divided based on middle-order hierarchical tree branches (Fig 1). An overview of the genes represented in Cluster C1 reveals a significant number of genes involved in pathogenesis and immunity ($p = 8.01 \times 10^{-21}$; Table S1), including eight of the 15 most highly expressed genes in this cluster: HLA class II genes HLA-DRB3, HLA-DRA, HLA-DR-γ (CD74), HLA-DPA1, and HLA-DQA1; FCER1A, the α subunit of the IgE receptor; and the antimicrobial agent lysozyme (LYZ) (Table II). Two additional genes in the top 15, tyrosinase-related protein 1 and dopachrome tautomerase, are expressed in melanocytes and are required for biosynthesis of the pigment melanin. These 10 genes are specific for NHS because they are normally expressed in cells that are not present in CSS and they would not be expected to be expressed in fibroblasts or keratinocytes. The apparent low level of expression of these genes in the cultured samples probably reflects a very low level persistence of contaminating cell types in the fibroblast and/or keratinocyte primary cultures. This has been documented previously for melanocytes, which can persist as "passengers" in keratinocyte cultures and result in foci of pigmentation in cultured skin grafts prepared with these cells (Swope *et al*, 1997).

Among genes in Cluster C1 that are not involved in either immune response or pigmentation were a number of genes associated with epidermal differentiation, including four of the top 15 most highly expressed genes (Table II). The genes encoding loricrin (LOR), keratin 2A (KRT2A), small proline-rich-like 1B (SPRL1B), and calmodulin-like skin protein (CALML5) are expressed at relatively high levels in NHS and also in CSS (Table II). LOR and SPRL1B both map to the EDC on chromosome 1q21, and are major components of the epidermal cornified envelope (Marenholz *et al*, 2001). The product of the CALML5 is localized to the stratum corneum (Mehul *et al*, 2000) and KRT2 is only found in suprabasal epidermal cell layers (Collin *et al*, 1992). These genes fall at the top of Cluster C1; note that many of the genes at the top of this hierarchical cluster are moderately expressed in CSS as well as highly expressed in NHS (Fig 1 and Fig S3). Further, CSS from cell strains 633 and 634 have slightly different patterns of expression for multiple genes involved in epidermal differentiation, compared with 639 and 651 (Fig 1, *white bracket*; see also Fig S3). The results suggest that, although there were no significant histological differences that distinguished these grafts (Fig S1), and their expression profiles were highly similar overall, for some groups of genes there were subtle expression differences.

Cluster C2 This cluster contains genes that were highly expressed in NHS, including a number of genes expressed at intermediate levels in keratinocytes, CSS, or fibroblasts. In contrast to Cluster C1, fewer of the genes in Cluster C2 were involved antigen presentation or the immune system.

Among the most highly expressed genes in Cluster C2 were the ECM genes SPARCL1, tetranectin (TNA), and dermatopontin (DPT) (Table II). SPARCL1, a secreted glycoprotein widely expressed in multiple tissues, is considered a matricellular protein because it modulates cell–ECM interactions but does not directly contribute to ECM architecture (Hambrock *et al*, 2003; Brekken *et al*, 2004). Although the exact function of SPARCL1 in skin is unclear, it has been shown to inhibit cell adhesion *in vitro* (Hambrock *et al*, 2003). TNA is a C-type lectin protein that binds plasminogen in the ECM. It is widely expressed, although its expression may be elevated in some tumors, and it has been suggested to function in tissue remodeling (Ibaraki *et al*, 1995; Iba *et al*, 2001). Like SPARCL1 and TNA, DPT is widely distributed throughout the body. DPT is associated with decorin in the ECM, where it functions in cell adhesion, collagen fibril formation, and regulation of skin elasticity (Takeda *et al*, 2002b).

Other noteworthy genes present in Cluster C2 include the secreted factors CXCL14 and Wnt4 (Table II). The chemokine CXCL14 is expressed in many normal human tissues, but expression is absent in many tumor cell lines and cancers. Originally cloned from oral epithelial cells, CXCL14 was shown to inhibit angiogenesis, suggesting its loss may be associated with tumor vascularization

(Shellenberger *et al*, 2004). Because many pro-angiogenic genes are upregulated in CSS, it is not surprising that CXCL14 is expressed at relatively lower levels in CSS compared with NHS. Wnt-4 is a member of the highly conserved Wnt family of secreted glycoproteins, which have been shown to regulate cell fate and differentiation. Wnt-4 signaling is involved in the developmental cascade leading to sex determination in humans (Jordan *et al*, 2001), is required for kidney development in mice (Stark *et al*, 1994), and is important for epithelial–mesenchymal interactions in the skin (Saitoh *et al*, 1998; Bhatia and Spiegelman, 2005). Interestingly, the transcription factor Tcf4, which participates in the Wnt/β-catenin/Tcf signaling pathway, is also present in Cluster C2 and was expressed at relatively higher levels in NHS compared with CSS (Fig S2). TCF genes act downstream of Wnt to regulate transcription of Wnt target genes. Recent studies showed that mouse skin tumors express high levels of Wnt4, and inhibition of Tcf4 signaling inhibited growth of skin cancer cells, suggesting that these genes may play a role in skin carcinogenesis (Bhatia and Spiegelman, 2005).

Cluster D This cluster contains genes that were very highly expressed in fibroblast cultures. These genes were expressed at relatively low levels in most other samples, although slightly elevated relative expression of many Cluster D genes was observed in CSS from strains 633 and 634 compared with strains 639 and 651 (Fig 1 and Fig S3). This may be because of individual genetic differences or to subtle differences in the rates of differentiation or development of the CSS, rather than age- or tissue-specific differences, because these variables did not correlate with differences in gene expression levels (Table I). Nearly half of the most highly expressed genes in Cluster D are involved in signal transduction, including guanine nucleotide binding protein gamma 11 (GNG11), epidermal growth factor receptor pathway substrate 8 (EPS8), prostaglandin E receptor 2 (PTGER2), cytokine receptor-like factor 1 (CRLF1), and protein kinase C, mu (PRKCM) (Table II). The gene that was most highly expressed in fibroblasts, relative to keratinocytes and CSS, was glioma pathogenesis-related protein (GLIPR1) (Table II). The GLIPR1 gene is highly expressed in human brain tumors (glioblastomas and astrocytomas), but expression was not previously identified in adult tissues or primary cell cultures, suggesting a possible role in tumor cell growth (Murphy *et al*, 1995). Human GLIPR1 has structural similarities with plant pathogenesis-related proteins that are induced in response to infection (Murphy *et al*, 1995), and the mouse homolog has been proposed to promote cell apoptosis (Ren *et al*, 2002). Another gene in Cluster D with putative pro-apoptotic activity is the cannabinoid receptor 1 gene (CNR1), which was highly expressed in fibroblasts compared with lower expression in CSS, NHS, and keratinocytes (Table II). CNR1 is highly expressed in the brain, where it is responsible for the psychoactivity of cannabinoids (Casanova *et al*, 2003). It was found in the skin, and activation of CNR1 was shown to induce skin tumor regression by inhibiting tumor angiogenesis and promoting tumor cell apoptosis (Casanova *et al*, 2003). Interestingly, BAG-2, which encodes a chaperone protein associated with BCL2, was also highly expressed in fibroblasts. BAG-family genes are overexpressed in many cancers, and can increase cell survival and decrease apoptosis. The high level of expression of both anti- and pro-apoptotic genes, as well as several genes involved in cell proliferation and signal transduction, suggests a balance of positive and negative regulators to maintain the proliferative potential of fibroblasts in monolayer culture, which is shifted upon combination with keratinocytes in the three-dimensional culture model.

Cluster E This cluster contains genes that were the most highly expressed in sub-confluent keratinocyte monolayer cultures, relative to fibroblasts and CSS. Functional groups highly enriched in Cluster E include cell communication ($p = 2.51 \times 10^{-27}$), signal transduction ($p = 1.56 \times 10^{-28}$), and structural proteins ($p = 4.98 \times 10^{-25}$), among others (Table S1). In Cluster E, the most highly expressed gene relative to fibroblasts and CSS was parathyroid hormone-like protein (PTHLH), which regulates epithelial–mesenchymal interactions during development of the teeth and mammary glands and differentiation of keratinocytes in adult skin (Foley *et al*, 1998). Two growth factors that regulate keratinocyte proliferation and differentiation, neuregulin 1 (NRG1), and amphiregulin (AREG), were also highly expressed in keratinocytes (Table II). Other investigators observed expression of these genes in conditioned medium from primary keratinocytes and have proposed roles for these factors in cell migration and growth regulation (Schelfhout *et al*, 2002). Expression levels of PTHLH, NRG1, and AREG are decreased in CSS compared with keratinocytes in monolayer culture, suggesting that their expression may be downregulated by factors produced by fibroblasts or by the three-dimensional culture format.

Conclusions

The microarray expression analysis described here revealed that many CSS signature genes are also expressed at similar levels in NHS (Cluster A), but there are also clusters of genes, specifically Clusters B, C1, and C2, which are differentially expressed. In contrast, other groups of investigators have observed relatively similar gene expression profiles in native skin compared with reconstituted epidermis (Bernard *et al*, 2002; Gazel *et al*, 2003). The cultured skin model utilized in those studies, SkinEthic, contains only keratinocytes, suggesting that some of the differences observed between CSS and NHS in this study may be because of the presence of fibroblasts.

Over 200 genes were identified that were overexpressed in NHS compared with CSS. We utilized a normalization scheme that de-emphasized the majority of genes expressed in structures absent from CSS, such as blood vessels and nerves. But, because the CSS are prepared with low-passage primary cell cultures, some residual non-fibroblast or non-keratinocyte cells may have been present. Many of the genes expressed specifically in NHS function in immune regulation, but we hypothesized that the cluster analysis would identify molecular deficiencies of the CSS compared with native skin. Thus, genetic modification may be useful for targeted overexpression of transcripts that are

missing from CSS, and may enhance the morphogenesis of skin substitutes in culture.

A major finding from this study was that the gene expression profile of CSS was very different than the sum of genes expressed in fibroblasts and keratinocytes. There were hundreds of genes identified that were either down- or upregulated upon combination of the two cell types in the context of cultured skin. This suggests an influence of mesenchymal–epithelial interactions between the fibroblasts in the dermal matrix and the epidermal keratinocytes on gene expression, although culture conditions may also be important. Skin replacements composed only of keratinocytes will form a stratified epidermal layer in culture, but their clinical performance is variable (Carsin *et al*, 2000). The importance of a dermal component in skin substitutes, not only for ease of transplantation but also for optimal *in vitro* epidermal development, is now well recognized. The role played by fibroblasts has been supported by other investigators, who showed that diffusible factors released by fibroblasts regulate epidermal morphogenesis (El Ghalbzouri and Ponec, 2004).

The expression profile of CSS showed overexpression, relative to cultured monolayer cells and normal human skin, of clusters of genes that are known to be overexpressed in hyperproliferative skin disorders and during wound healing. These genes include cytokines, growth factors and receptors, antimicrobial proteins, and differentiation markers. The analysis suggests that the keratinocytes in CSS are present in an activated state, similar to wounded human skin. Combined with the expression patterns of MMP observed in CSS, these findings are consistent with remodeling of the dermal matrix and differentiation of a stratified epidermal layer, processes that are observed during wound healing. Although many of the genes that were upregulated in CSS are also increased in psoriasis, the CSS do not display a psoriatic phenotype, either *in vitro* (Fig S1) or after grafting to patients (Boyce *et al*, 2002a). This indicates a balance of proliferation and differentiation factors in the CSS that may shift during *in vitro* culture and after grafting *in vivo*. Future studies will be required to examine the kinetics of the gene expression changes, and to determine how expression is regulated after grafting to wounds.

Materials and Methods

Preparation of CSS Human skin samples were obtained with Institutional Review Board approval and in accordance with the Declaration of Helsinki Principles, from breast and abdominal tissue of healthy adult females. Informed consent was not required because the skin was obtained from discarded surgical tissues. Biopsies of the skin were stored in RNAlater (QIAGEN Inc., Valencia, California) for subsequent RNA purification, and additional biopsies were frozen and embedded for histological sections. One of the skin samples is a reference sample (Table I, "Ref") used as a calibration standard for all microarray analyses done in our laboratory. Fibroblasts and keratinocytes were isolated from fresh skin, cultured separately in growth medium specific for each cell type (Boyce and Ham, 1985; Boyce, 1999), and expanded for a maximum of two passages prior to harvest for preparation of CSS. CSS were prepared with cells from four independent donors ($N = 8$ total; two per donor) as described in detail elsewhere (Boyce, 1999; Boyce *et al*, 2002a). Briefly, fibroblasts were grown to confluence (95%–100%) and were harvested and inoculated onto collagen–glycosaminoglycan polymer substrates (Boyce *et al*, 1988) at a density of 5×10^5 per cm^2. Keratinocytes were harvested at subconfluence (85%–90%) and inoculated onto the dermal substrates 1 d following fibroblast addition, at a density of 1×10^6 per cm^2. Samples of both cell types to be used for RNA isolation were collected when cells were harvested for CSS inoculation. CSS were cultured at the air–liquid interface for 2 wk *in vitro*, using specific CSS maturation medium described in detail elsewhere (Boyce *et al*, 2002b). A 2-wk *in vitro* incubation has been shown to be optimal for development of mature epidermal barrier and basement membrane deposition (Boyce *et al*, 1996, 2002b), and is comparable with the culture period utilized for preparation of CSS for grafting to burn patients (Boyce *et al*, 1999, 2002a). Epidermal barrier development *in vitro* was evaluated by measuring surface electrical capacitance (SEC) using a dermal phase meter (NOVA DPM 9003; NOVA Technology Corp., Gloucester, Massachusetts); a decrease in SEC during *in vitro* incubation indicates reduced surface hydration and increased epidermal barrier (Boyce *et al*, 1996, 2002b). Biopsies of CSS were collected at the end of the culture period for histological examination to ensure optimal morphology (Fig S1), and for RNA isolation and immunohistochemistry.

Microarray analysis Quantitative analysis of RNA expression was performed using Affymetrix gene chip cDNA microarrays (Affymetrix, Santa Clara, California). Samples of fibroblasts and keratinocytes, as well as biopsies of the NHS and CSS *in vitro* ($N = 4$ independent donors each), were used for preparation of total RNA samples using the RNeasy Mini Kit (QIAGEN Inc.), followed by phenol and chloroform extraction and ethanol precipitation. RNA quality was measured using the Agilent Bioanalyzation system (Agilent Technologies Inc., Palo Alto, California), to ensure the integrity of the RNA. cDNA synthesis and hybridization to the Affymetrix Human Genome HU133A gene chip was performed by the Affymetrix Gene Chip Core Facility at Cincinnati Children's Hospital Medical Center (Cincinnati, Ohio) using standard protocols. To check the reproducibility of the hybridization data, two of the RNA samples were analyzed in duplicate; results of these duplicate chips were essentially identical (data not shown), confirming the fidelity of the analysis.

The hybridized arrays were scanned using Microarray Suite (MAS) Software (Affymetrix), and were analyzed with GeneSpring 7.1 (Silicon Genetics, Redwood City, California) using Affymetrix MAS 5.0 cel files subjected to the RMA (Irizarry *et al*, 2003) cel file pre-processor built in to GeneSpring 7.1. The raw data from this study have been deposited into the NCBI GEO database (Accession number GSE3204). To analyze the data, the values for RNA expression were first converted to relative levels by referencing the RMA-generated per-chip and per-array series expression value data to the median expression values of the *in vitro* samples (fibroblasts, keratinocytes, and CSS). To identify differentially expressed genes from each group, an initial four-way analysis of variance (ANOVA) using Student's *t* test with a false discovery rate cutoff of 0.05 using the Benjamini Hochberg test was used. This identified a total of 12,483 probe sets, which were then consistently filtered based on rank most under- and overexpressed compared with the median for each of the four sample groups. This led to a list of 1720 probe sets, which were finally filtered for those probe sets that were overexpressed in any of the four sample types (Fig S2). This final list was comprised of 1030 probe sets, with the remaining 690 probe sets representing genes that are much less expressed in one or two samples compared with the median expression in the cultured samples. We concentrated our characterizations on the 1030 probe set list to allow us to focus on genes that are highly expressed in one or two sample groups, rather than genes that are in common between groups. This list of 1030 probe sets was subjected to hierarchical clustering using standard correlation metric to obtain clusters of coordinately regulated genes representing the transcriptional signatures of human skin relative to keratinocytes, fibroblasts, and CSS. Top-level hierarchical tree branches were used to form six major clusters (Fig 1).

Ontological data mining was performed using tools in GeneSpring 7.1. The functional data obtained in this analysis, including the functional categories and lists of genes referred to in Table S1, are available online at http://genet.cchmc.org. To access the data from this website, login as a guest, choose the option "HG-U133," open the "Experiments" folder, and select the file named "Smiley-EtAl_2005."

Histology and immunohistochemistry For immunohistochemistry, biopsies of NHS and CSS *in vitro* were imbedded frozen using M1 embedding matrix (Lipshaw, Pittsburgh, Pennsylvania). Immunostaining was performed using the following antibodies: rabbit anti-human MMP10, 1:200 dilution (Abcam Inc., Cambridge, Massachusetts); rabbit anti-TIMP3, 1:40 dilution (Abcam Inc.); mouse anti-human cytokeratin 10, 2 μg per mL (US Biological, Swampscott, Massachusetts); mouse anti-human cytokeratin 5/8, 2 μg per mL (US Biological); mouse anti-human cytokeratin 16, 1 μg per mL (US Biological); mouse anti-human NGAL (lipocalin 2), 25 μg per mL (Accurate Chemical & Scientific Corp., Westbury, New York); rabbit anti-human SCCA1/2, 1 μg per mL (Santa Cruz Biotechnology Inc., Santa Cruz, California). Sections were incubated with primary antibodies for 1 h at room temperature in a humidified chamber. Antibody detection utilized the Vectastain Elite ABC Universal Kit with DAB substrate (Vector Laboratories, Burlingame, California), and sections were counterstained using Hematoxylin QS (Vector Laboratories). For negative controls, serial sections were incubated in parallel with either non-immune Rabbit IgG or non-immune mouse IgG (R&D Systems, Minneapolis, Minnesota), or without antibody (data not shown). Sections were examined using a Microphot-FXA microscope (Nikon, Melville, New York) and were photographed using a Spot-Jr. Digital Camera (Diagnostic Instruments Inc., Sterling Heights, Missouri).

The authors thank Jodi Miller and Sherry Liles for media preparation, Christopher Lloyd for collagen membrane fabrication, and Deanna Snider in the Shriners Hospital Histology Core Facility for preparation of histological sections. In addition, we thank the staff of the Affymetrix and Bioinformatics Core Facilities at Cincinnati Children's Hospital Medical Center for their assistance. This research was supported by a Medical Research grant from the Shriners Hospitals for Children.

Supplementary Material

The following material is available online for this article.
Figure S1 Histology of cultured skin substitutes (CSS).
Figure S2 Filtering strategy used for microarray analysis.
Figure S3 Hierarchical cluster analysis of normalized gene expression of 1030 probe sets expressed in native human skin (NHS), cultured skin substitutes (CSS), fibroblasts, and keratinocytes.
Table S1 Functional profiles of gene clusters by ontological data mining.

DOI: 10.1111/j.0022-202X.2005.23971.x

Manuscript received April 26, 2005; revised July 19, 2005; accepted for publication August 12, 2005

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