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Laser capture microdissection-based *in vivo* genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma

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Keratinocytes undergo a dramatic phenotypic conversion during reepithelialization of skin wounds to become hyperproliferative, migratory, and invasive. This transient healing response phenotypically resembles malignant transformation of keratinocytes during squamous cell carcinoma progression. Here we present the first analysis of global changes in keratinocyte gene expression during skin wound healing *in vivo*, and compare these changes to changes in gene expression during malignant conversion of keratinized epithelium. Laser capture microdissection was used to isolate RNA from wound keratinocytes from incisional mouse skin wounds and adjacent normal skin keratinocytes. Changes in gene expression were determined by comparative cDNA array analyses, and the approach was validated by *in situ* hybridization. The analyses identified 48 candidate genes not previously associated with wound reepithelialization. Furthermore, the analyses revealed that the phenotypic resemblance of wound keratinocytes to squamous cell carcinoma is mimicked at the level of gene expression, but notable differences between the two tissue-remodeling processes were also observed. The combination of laser capture microdissection and cDNA array analysis provides a powerful new tool to unravel the complex changes in gene expression that underlie physiological and pathological remodeling of keratinized epithelium.
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Introduction

Epidermal wounding triggers a dramatic conversion of the epidermal keratinocytes that are located immediately adjacent to the wound margin from a resting state to a hyperproliferative, migratory, and invasive phenotype that is required for cellular invasion of the wound bed and successful reepithelialization of the wound. This phenotypic conversion includes the increased proliferation of basal keratinocytes, dissolution of cell–cell adhesions, detachment of keratinocytes from the basement membrane, lateral migration of keratinocytes into the wounded area, and the invasion of the provisional matrix of the wound bed by keratinocytes (Martin, 1997; Singer and Clark, 1999). In many aspects, this healing response closely resembles the phenotypic events that are observed during squamous cell carcinoma progression, where normal keratinocytes undergo a malignant conversion to acquire a proliferative, migratory, and invasive phenotype. Despite their overall close similarity, however, two crucial characteristics distinguish the two tissue-remodeling processes. First, the phenotypic conversion of wound keratinocytes is driven exclusively by epigenetic events that are extrinsic to the keratinocytes (mechanical disruption of the epithelium, changes in extracellular matrix composition, release of cytokines and growth factors from the wound bed), whereas the phenotypic conversion of keratinocytes during squamous cell carcinoma progression is driven by genomic alterations that are intrinsic to the keratinocytes. Secondly, whereas the transformation of wound keratinocytes to a migratory and invasive state is transient, and a complete phenotypic reversion takes place immediately after reepithelialization is completed, the malignant transformation of keratinocytes during squamous cell carcinoma progression is permanent and devastating to the organism. Tumors have therefore insightfully been described as 'wounds that do not heal', and the comparison of similarities and differences in gene expression in the two processes could considerably advance our understanding of carcinogenesis (Dvorak, 1986). The transcriptome of many types of cancer, including squamous cell carcinoma, has been subjected to systematic analyses in recent years by cDNA array analysis (e.g., Leethanakul *et al*., 2000; Dong *et al*., 2001). In contrast, no similar studies have systematically assessed the overall changes in gene expression that

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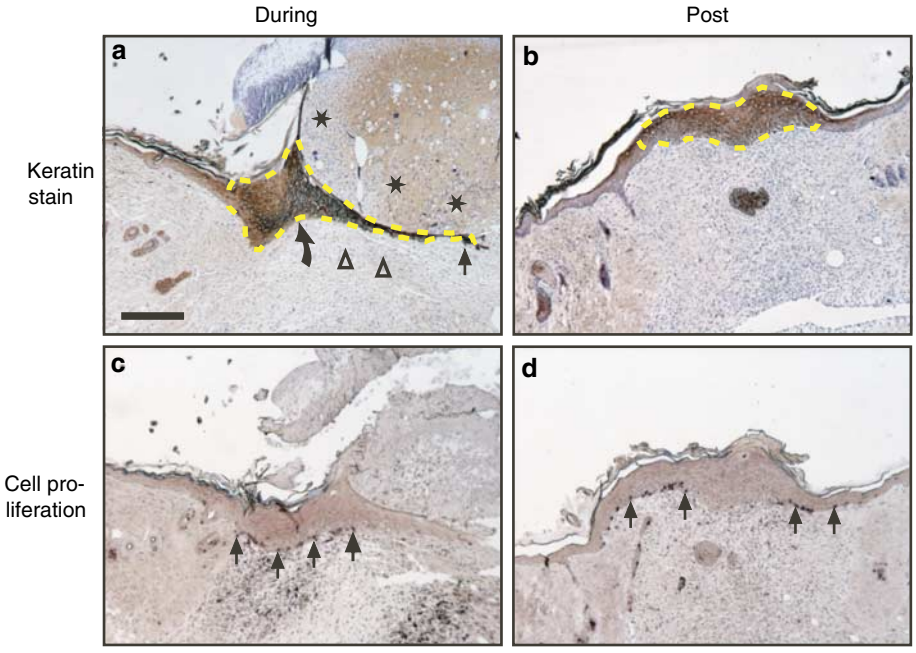

Figure 1 Kinetics of incisional skin wound-healing in mice. Wounds were excised during (day 5) and post (day 9) reepithelialization, and sections perpendicular to the longitudinal direction of the wounds were stained for keratin expression using anti-keratin antibodies (**a** and **b**), or for mitotic activity by *in situ* hybridization using a histone oligonucleotide that detects histones H2B, H3, and H4 mRNA (**c** and **d**). During reepithelialization (**a** and **c**), epidermal wedges (curved arrow in **a**) are migrating through the provisional matrix bisecting the escar (stars) and underlying wound bed (triangles in **a**). The arrow in (**a**) indicates the tip of the migrating keratinocyte wedge. Post reepithelialization (**b** and **d**), the keratinocyte wedges have met and fused to completely reepithelialize the wound. The wound area can be distinguished by the thickened epidermis and by the absence of an underlying dermis. During (**c**) and post (**d**) reepithelialization, high mitotic activity is seen in basal keratinocytes (indicated with arrows), within the newly formed epidermal wedges but only distal to the migration front. The dashed lines delineate the wound edge keratinocytes during (**a**) or post (**b**) reepithelialization as defined in this study. All pictures were taken at the same magnification. Scale bar in A: 200 μm

underlie the phenotype of wound healing keratinocytes *in vivo*. Thus, our knowledge of changes in gene expression during the healing of incisional, excisional, alkali, scrape, and burn wounds has largely been derived from *in situ* hybridization or immunohistochemical studies of the expression of single genes or gene families (e.g., Goliger and Paul, 1995; Martin, 1997; Lund *et al*., 1999; Singer and Clark, 1999; Braun *et al*., 2002).

In this study, we used laser capture microdissection (LCM), combined with cDNA array analyses to specifically analyse global changes in gene expression in wound keratinocytes that are proliferating and invading the provisional matrix during incisional skin wound healing *in vivo*. We show that the combination of LCM and cDNA array analysis provides a powerful new tool for the study of the physiology of wound reepithelialization, identify 48 novel candidate reepithelialization-associated genes, and reveal striking similarities as well as distinct differences in gene expression between wound healing keratinocytes and malignant keratinocytes *in vivo*.

Results

Isolation of RNA from wound-healing keratinocytes

The kinetics of the healing of full-thickness mouse incisional skin wounds was first assessed by microscopic analysis of wound sections (Figure 1). At 5 days after wounding, the epidermis that is located at the margins of the wounds was dramatically thickened, and a wedge of keratinocytes was invading the provisional matrix of the wound bed (Figure 1a). The basal keratinocytes of the epidermal wedge displayed hyperproliferation that was most prominent in the part of the wedge that was proximal to the wound edge (Figure 1c). At day 9, the wounds were all completely reepithelialized (Figure 1b), but the newly formed epidermis could still be clearly distinguished from normal epidermis by its increased thickness, and by the distinct hyperproliferation of the basal keratinocytes (Figure 1d).

We performed LCM to specifically isolate wound edge keratinocytes either during (day 5) or post (day 9) reepithelialization to define the changes in gene expression that are associated with the healing process (Figure 2 and data not shown). For comparison, populations of non-wound edge keratinocytes were procured by the identical procedure. In each case, the non-wound edge keratinocytes were obtained from the dorsal region of the same mouse that was subjected to wounding to avoid confounding parameters, such as differences in the stage of the hair cycle, from influencing the analysis. To further ensure that the observed changes in keratinocyte gene expression were reproducibly associated with the reepithelialization process, wound edge and associated non-wound edge keratinocytes were isolated from three different wounds at each of the two time points. For each wound edge or non-

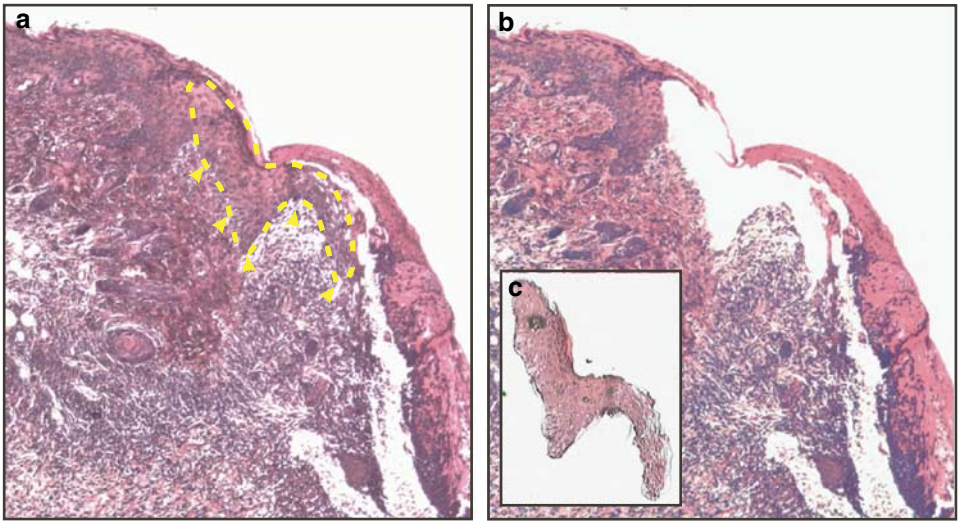

Figure 2 Isolation of wound keratinocytes by LCM. Wound tissue sections (6 μm) were stained with hematoxylin and eosin immediately before performing LCM. The wound prior to LCM (**a**), after LCM (**b**), and the isolated wound edge (**c**) is shown. The arrows indicate the tip of the wound edge keratinocytes. For each sample, LCM was performed on two to four parallel tissue sections and the procured keratinocytes were pooled

wound edge sample, RNA was isolated from approximately 5000 cells and representative high-quality cDNA was generated by RT–PCR from 5 to 9 ng RNA. The length of the cDNA varied from 50 to 2000 bp, as assessed by agarose gel electrophoreses, with the size of the majority of the transcripts being within the 500–1000 bp range, indicating that the integrity of even long mRNA species was preserved by the procedure (data not shown). Taken together, the results showed that the LCM and RT–PCR procedure applied here could reproducibly generate complex cDNA probes from keratinocytes isolated *in situ* from healing wounds.

Identification of genes expressed in wound-healing keratinocytes

The cDNA probes obtained from the various keratinocyte populations were hybridized to Clontech mouse 1.2. cDNA arrays. These cDNA arrays detect a minor fraction of the total number of genes predicted to be expressed in keratinocytes (1176 of approximately 10 000 expressed genes (Jansen *et al*., 2001; van Ruissen *et al*., 2002)), but the arrays were well suited for the purpose, as they are rich in cDNAs of genes that are associated with cell proliferation, adhesion, motility, and migration. Parallel hybridizations were performed with each set of cDNA from wound edge and matching non-wound edge keratinocytes isolated from the same wound (Figure 3). For each array, the expression level of individual genes was determined using a standard global normalization procedure, that is, the signal intensity of each gene was divided by the signal intensity of the entire array. To enable comparisons of the gene expression patterns observed in the parallel array experiments, a fold change ratio for each gene was then calculated by dividing the normalized gene expression level in wound edge keratinocytes with the normalized gene expression level observed in non-wound edge keratinocytes (see Materials and Methods for further details). During reepithelialization, 78–122 genes were reported as being expressed in either wound edge or nonwound edge keratinocytes in the three different wounds that were analysed (Table 1). We decided to focus our analyses only on the 45 genes that were reported as expressed in all the three wounds. Of these 45 reproducibly detected genes, 22 were constitutively expressed in wound edge compared to non-wound edge keratinocytes, whereas 15 were differentially expressed. For the remaining eight genes, the level of expression did not show a consistent pattern in the three different wounds. Using this conservative approach, a total of 37 genes were identified as reproducibly either constitutively or differentially expressed when comparing wound edge and non-wound edge keratinocyte gene expression during reepithelialization. Using the identical procedure to analyse keratinocyte gene expression post reepithelialization, 33 constitutively expressed and 13 differentially expressed genes were identified (Table 1). A comparison of the genes that were expressed during and post reepithelialization showed that 25 genes were expressed in keratinocytes at both stages of the healing process, whereas 12 genes were expressed only during wound reepithelialization, and 21 genes were expressed only post reepithelialization. Thus, the LCM and array procedure facilitated the identification of a total of 58 genes as expressed by keratinocytes in the course of incisional skin wound healing *in vivo* (Table 2).

Comparison of LCM and cDNA array analysis to *in situ* hybridization analysis and validation of data

We next performed *in situ* hybridization of mRNA expression in wound keratinocytes with a subset of the

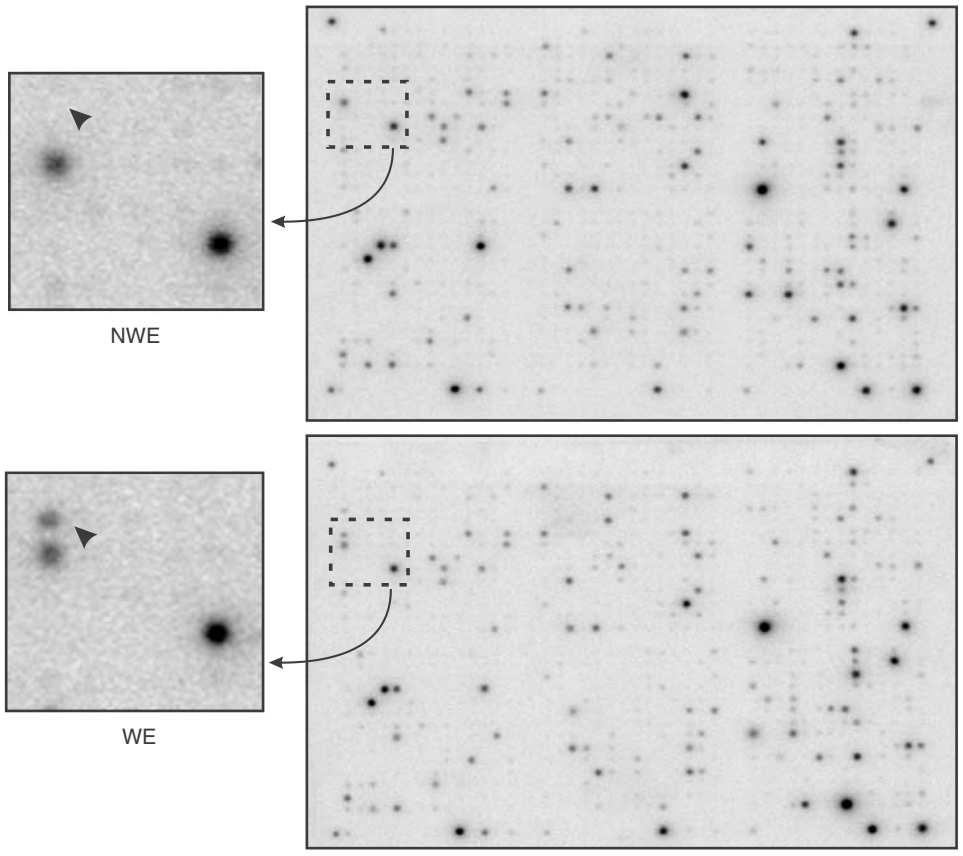

Figure 3 Representative analysis of cDNA array hybridization. Clontech mouse 1.2 cDNA expression arrays were used to investigate the expression pattern of 1176 mouse genes in wound edge (WE) and non-wound edge (NWE) keratinocytes isolated by LCM. A representative result of array hybridization of ^{32}P-labeled cDNA isolated from WE (lower panel) and NWE (upper panel) keratinocytes isolated from post reepithelialization wounds is shown. In the enlarged frames, an example of a gene whose expression is only detected in WE keratinocytes (Connexin 26) is indicated with an arrow

Table 1 Reproducibility of array analyses

	During	*Post*
Expressed genes	78–122	92–140
Expressed in all 3 experiments	45	47
Non-reproducible expression pattern	8	1
Reproducible expression pattern in all three experiments	37	46
Constitutive expression	22	33
Differential expression	15	13

Array analyses comparing wound edge and non-wound edge keratinocyte expression patterns were performed on samples isolated from three wounds during reepithelialization and three wounds post reepithelialization. Of the identified genes, 25 were identical in wounds during and post reepithelialization, thus identifying a total of 58 genes expressed by keratinocytes in the course of incisional skin wound healing

genes identified by LCM and cDNA array analysis, to compare the two methods of analyzing gene expression in wound healing and to validate the novel procedure. This method was chosen for the validation of the LCM and array approach for two specific reasons. First, the material analysed in this study was microdissected from heterogeneous tissue raising the issue of sample contamination. *In situ* hybridization is the only validation procedure that provides spatial resolution of gene expression within the wound field to accurately identify the source of mRNA expression (see below). Secondly, previous systematic comparative analyses have demonstrated that cDNA arrays detect relative changes in gene expression as accurately as quantitative RT real-time PCR or Northern blot hybridization (Taniguchi *et al*., 2001; Yuen *et al*. 2002). Specifically, we analysed the expression of mRNA for HB-EGF, cystatin C, and cellular retinoic acid binding protein II (CRABP-II) both during and post reepithelialization (Figure 4 and data not shown). HB-EGF and CRABP-II represented genes that were upregulated in wound edge keratinocytes, and cystatin C a gene that was moderately downregulated in wound edge keratinocytes during reepithelialization and then constitutively expressed post reepithelialization. The *in situ* hybridization analysis confirmed that HB-EGF, CRABP-II, and cystatin C were all expressed in wound edge keratinocytes (Figure 4a-i and data not shown). HB-EGF and CRABP-II mRNA could clearly be discerned as upregulated in wound edge as compared to non-wound edge keratinocytes by the *in situ* hybridization procedure. Cystatin C mRNA was detectable in both wound edge and non-wound edge keratinocytes by*in situ* hybridization, but the relative expression level of cystatin C in wound edge as compared to non-wound edge keratinocytes was

more difficult to gauge by*in situ* hybridization, because of the dramatic difference in the tissue morphology of wounded and unwounded epidermis (Figure 4d-f and data not shown). Notably, CRABP-II mRNA was found in wound edge keratinocytes both during (Figure 4g-i) and post (data not shown) reepithelialization by the *in situ* hybridization analysis. In contrast, the LCM and cDNA array analyses reported the CRABP-II gene as expressed in all the three wounds post reepithelialization, but only in two of three wounds during reepithelialization.

Surprisingly, our array analyses indicated that the mesenchymal marker decorin was heavily downregulated in wound edge as compared to non-wound edge keratinocytes (not listed in Table 2). Decorin is known to be very highly expressed in the dermis, but has never been reported in keratinocytes (Danielson *et al*., 1997). In order to investigate whether the identification of this gene in our array analyses was an artifact, we performed decorin *in situ* hybridization analysis of wounds during and post reepithelialization (Figure 4j-l and data not shown). Decorin mRNA was extremely abundant in the dermis, whereas no specific signal was observed in any keratinocytes (Figure 4j-l). Thus, our detection of this highly expressed mesenchymal protein by the LCM and cDNA array analysis procedure probably stems from the contamination of the keratinocyte samples with small quantities of the underlying dermis. Consequently, the decorin data were not included in Tables 1 and 2.

Novel reepithelialization-associated genes

An exhaustive screening of the published literature revealed that 27 of the 58 genes (47%) that were identified in the study have been previously reported to be expressed in either primary keratinocyte cultures or in the epidermis, clearly underscoring the validity of the LCM-based approach (Table 2 and data not shown). Interestingly, however, 48 of the genes identified here (83%) either have not been previously reported to be expressed in keratinocytes during wound healing, or have not been reported to be differentially expressed during wound healing, and, thus, represent novel reepithelialization-associated genes. In all, 14 of these genes were associated with transcriptional regulation, 12 genes were associated with either extra- or intracellular signaling, eight genes encoded proteins involved in cell cycle progression or apoptosis, two genes were associated with the formation of cell–cell junctions, two genes encoded structural proteins, and 10 genes had miscellaneous or unknown functions (Table 2). A comprehensive description of the putative function of each of the novel reepithelialization-associated genes in wound healing is beyond the scope of this paper, but a few of the genes identified here merit mentioning. Klf4, or Krüppel-related factor 4, is a transcription factor that is normally expressed in the suprabasal epidermis. Klf4 expression was strongly upregulated during reepithelialization. Klf4 plays an essential role in epidermal development and is required for the establishment of epidermal barrier function (Segre *et al*., 1999). The specific upregulation of Klf4 in wound edge keratinocytes during reepithelialization suggests that the transcription factor may have an important role in both epidermal development and wound reepithelialization. Galectin 7 is a β-galactoside-binding protein with proposed functions in both cell–matrix interaction and apoptosis (Bernerd *et al*., 1999). The ectopic application of galectin 7 was recently reported to accelerate corneal wound healing (Cao *et al*., 2002). The endogenous expression of galectin 7 in keratinocytes and the specific upregulation of galectin 7 expression in wound edge keratinocytes during reepithelialization identified here strongly implicate galectin 7 as critical to wound reepithelialization. The Fas I receptor has an essential role in regulating apoptosis of keratinocytes in response to genotoxic stress, and the receptor is expressed in both basal and suprabasal keratinocytes of the normal epidermis (Oishi *et al*., 1994; Lee *et al*., 1998; Wehrli *et al*., 2000). The specific downregulation of the Fas I receptor in wound edge keratinocytes identified here may be critically related to wound reepithelialization by desensitizing wound keratinocytes to the many apoptosis-inducing stimuli that are associated with wounding. Cyclin D2 was the only one of the cyclin genes represented in the array that was identified as differentially expressed in our study, and mRNA for this cyclin was strongly reduced in wound edge as compared to non-wound edge keratinocytes both during and post reepithelialization, implying a function in the reepithelialization process. The specific functions of the D-type cyclins in keratinocyte biology have not been investigated in detail. However, transgenic overexpression of cyclin D2 has been reported to lead to epidermal hyperproliferation (Rodriguez-Puebla *et al*., 2000).

Similar changes in gene expression underlie phenotypic alterations of keratinocytes during wound healing and early squamous cell carcinogenesis

Gene expression in squamous cell carcinoma, and to a lesser extent basal cell carcinoma, has been extensively investigated in previous studies by Northern and Western blotting, immunohistochemistry, *in situ* hybridization, and cDNA array analysis (e.g. , Rundhaug *et al*., 1997; Leethanakul *et al*., 2000; Dong *et al*., 2001). The healing response underlying epidermal reepithelialization closely resembles the phenotypic events that are observed during squamous cell carcinoma progression (see Introduction). The systematic delineation of changes in gene expression in wound keratinocytes performed in this study permitted the first direct comparison of global gene expression patterns in the two tissue-remodeling processes. Overall, of the 58 genes reported as expressed in wound keratinocytes, 21 (36%) were previously reported expressed in squamous or basal cell carcinoma (columns SCC and BCC in Table 2). Interestingly, of the 15 genes that were found to be differentially expressed in wound keratinocytes in this study, and for which information was available on their expression in squamous or basal cell carcinoma, nine (60%) showed an identical change in gene expression

Table 2 Genes identified as constitutively and/or differentially expressed when comparing wound edge and nonwound edge keratinocyte gene expression patterns

Expression in wound edge as compared to non-wound edge keratinocytes – this study			*Epidermal expression – previous studies*				*References*
	During	*Post*	*N*	*W*	*SCC*	*BCC*	
Transcription factors							
NF-E2-related factor (Nrf2)	↑[a]	∼	+	+	↑[c]	–	Braun et al. (2002) Moi et al. (1994) Leethanakul et al. (unpublished)
Klf4	∼	↑[a]	+	–	–	–	Segre et al. (1999)
HMG-14	↓ 0.68	→	–	–	↓[d]	–	Ding et al. (1994) Dong et al. (2001)
Prothymosin α	↓ 0.40	↓ 0.29	+	–	–	–	Karetsou et al. (2002) Moll et al. (1996)
NAB2 (NGFI-A binding protein 2)	→	∼	–	–	–	–	Anderson et al. (1993) Svaren et al. (1996)
OBF-1	→	∼	–	–	–	–	Knoepfel et al. (1996) Strubin et al. (1995)
Hepatocyte nuclear factor 4	→	→	+	–	–	–	Sladek et al. (1990) Taraviras et al. (1994)
Insulin promoter factor 1	→	→	–	–	–	–	Kaneto et al. (1997) Ohlsson et al. (1993)
Msh-like homeobox protein 3	→	→	–	–	–	–	Shimeld et al. (1996)
Short stature homeobox protein 2	→	→	–	–	–	–	Rao et al. (1997) Semina et al. (1998)
Transcription termination factor 1	→	→	–	–	–	–	Evers et al. (1995)
Fragile X mental retardation syndrome 2 homolog FMR2	∼	→	–	–	–	–	Gecz et al. (1996) Gu et al. (1996)
Zinc finger protein GLI3	∼	→	–	–	↓[c,f]	→[c,f]	Kinzler et al. (1987) Dahmane et al. (1997)
Ret finger protein	∼	→	–	–	–	–	Cao et al. (1996)
CREBP-1	∼	→	+	–	–	–	Montminy and Bilezikjian (1987) Westergaard et al. (2001)
Signalling							
Heparin binding EGF-like growth factor	↑[a]	↑[a]	+	+	↑[d]	–	Higashiyama et al. (1991) Tokumaru et al. (2000) Xiao et al. (1999) Rundhaug et al. (1997)
CRABP-II	↑[g]	↑[a]	+	–	↓[c]	↓[c]	Eller et al. (1994) Eller et al. (1995)
IL-1 receptor type II	↓[b]	∼	+	–	–	–	Deyerle et al. (1992) Freedberg et al. (2001) McMahan et al. (1991)
Interferon γ receptor	∼	↓ 0.79	+	–	–	↓[c]	Fountoulakis et al. (1990) van den Oord et al. (1995) Kooy et al. (1998)
TGF-β receptor I	→	↓ 0.69	+	+	Early → or ↑ Late ↓[c]	–	Ebner et al. (1993) Frank et al. (1996) Gold et al. (1997) Kubo et al. (2001)
Oxytocin receptor	→	→	–	–	–	–	Ivell et al. (2001) Kimura et al. (1994)
GM-CSF receptor α subunit	→	→	–	–	–	–	Gearing et al. (1989)
Laminin receptor	→	→	+	–	↑[c]	–	Kopf-Maier and Flug (1996) Leethanakul et al. (unpublished)
Inhibin β-C precursor	→	→	–	–	–	–	Chen et al. (2002) Schmitt et al. (1996) Vale et al. (1986)
Calmodulin	∼	→	+	+	↓[d]	–	Lansdown et al. (1999) Dong et al. (2001)
Radical fringe homolog precursor	∼	→	+	+	–	–	Thelu et al. (1998) Thelu et al. (2002)
Fibroblast growth factor 14	∼	→	–	–	–	–	Wang et al. (2000) Yamamoto et al. (2000)
MKK3	∼	→	–	–	–	–	Derijard et al. (1995)
MKK4	∼	→	–	–	–	–	Derijard et al. (1995)
RAB23	∼	→	–	–	–	–	Eggenschwiler et al. (2001) Olkkonen et al. (1994)
B-raf proto-oncogene	∼	→	–	–	–	–	Hagemann and Rapp (1999)
Cell cycle/apoptosis							
Galectin 7	↑ 1.97	∼	+	–	↓[c,d]	↓[c,d]	Bernerd et al. (1999) Madsen et al. (1995) Magnaldo et al. (1998)
Fas I receptor	∼	↓ 0.74	+	–	–	–	de Panfilis et al. (2002) Lee et al. (1998) Wehrli et al. (2000)
Cyclin D2	↓[b]	↓[b]	+	–	↓[d,h]	–	Rodriguez-Puebla et al. (2000) Dong et al. (2001)
TNF α-induced protein 3 (A20)	→	∼	–	–	↑[c]	–	Lee et al. (2000) Opipari et al. (1990) Codd et al. (1999)
Nucleoside diphosphate kinase B	→	∼	–	–	–	–	
Defender against death 1	→	↓ 0.70	–	–	–	–	Hong et al. (2000) Nakashima et al. (1993)

Proliferation-associated protein 1	~	→	–	–	–	–	Radomski and Jost (1995)
Cytoplasmic dynein light chain	~	→	–	–	–	–	Dick et al. (1996)
Cell–cell junction proteins							
Connexin 26	↑[a]	~	+	+	Early ↑		Butterweck et al. (1994) Goliger and Paul (1995) Zhang and Nicholson (1989) Rundhaug et al. (1997)
	–				Late ↓[d]		
Connexin 31.1	→	→	+	+	↓[d]	–	Goliger and Paul (1994): Goliger and Paul (1995) Haefliger et al. (1992) Budunova et al. (1995)
E-cadherin	→	→	+	+	↓[c]		Hirai et al. (1989) Nagafuchi et al. (1987) Schipper et al. (1991)
δ-Catenin	→	→	–	–	–	–	Lu et al. (2002) Lu et al. (1999) Paffenholz and Franke (1997)
Connexin 40	~	→	+	–	–	–	Butterweck et al. (1994) Richard (2000)
Structural proteins							
Cytokeratin 1	↑ 7.7	↑ 2.63	+	–	↓[d]	–	Rothnagel et al. (1992) Rundhaug et al (1997)
Cytokeratin 14	↑ 2.7	~	+	–	↑[e]	–	Coulombe et al. (1991) Lloyd et al. (1995) Gimenez-Conti et al. (1990)
Miscellaneous							
Thymosin β 4	↓ 0.44	↓ 0.35	–	–	↓[d]	–	Low et al. (1981) Malinda et al. (1999) Dong et al. (2001)
AnnexinII p11 subunit	↓ 0.51	↓ 0.28	+	+	↑[c]	–	Kim and Hajjar (2002) Munz et al. (1997) Robinson et al. (1997) Wu et al. (2002)
Cystatin C	↓ 0.40	→	+	–	–	–	Huh et al. (1999) Ono et al. (2000)
Apolipoprotein-E	↓ 0.20	~	+	–	–	–	Barra et al. (1994) Feingold et al. (1995) Grehan et al. (2001)
Heat shock protein 27	↓ 0.51	~	+	+	↓[d]	–	Laplante et al. (1998) Kiriyama et al. (2001)
Sentrin (SUMO)	→	↓ 0.61	–	–	↑[d]	–	Mahajan et al (1997) Okura et al. (1996) Wilson and Rangasamy (2001) Dong et al. (2001)
105 kDa heat shock protein	→	↓[d]	–	–	–	–	Yasuda et al. (1995)
Galanin precursor	→	~	+	–	–	–	Pincelli et al. (1990) Tatemoto et al. (1983)
Klotho protein	→	→	–	–	–	–	Kuro-o et al. (1997) Mian (1998)
PW29/HR21spA	~	→	–	–	–	–	Sadano et al. (2000) Yu et al. (1995)
SA2 nuclear protein	~	→	–	–	–	–	
Deleted in split hand/split foot gene	~	→	–	–	–	–	Crackower et al. (1996)

Arrows in the 'during' and 'post' columns indicate either constitutive (→), upregulated (↑) or downregulated (↓) expression in wound edge keratinocytes as compared to non-wound edge keratinocytes. Numbers adjacent to arrows indicate the mean fold ratio up- or downregulation in wound edge as compared to non-wound edge keratinocytes in three different wounds either during or post reepithelialization. ~ Indicates that the gene was not detected at the given time point according to the definitions specified in materials and methods. [a]Only expressed in wound edge keratinocytes. [b]Only expressed in non-wound edge keratinocytes. In the 'epidermal expression' columns, arrows indicate constitutive (→), upregulated (↑) or downregulated (↓) expression during progression of either basal cell carcinoma (BCC) or squamous cell carcinoma (SCC). + Indicates expression of a given gene in normal epidermis (N) or during wound healing (W). - Indicates that no data regarding expression of the gene in the given tissue is available. Genes in bold show similarities in gene expression patterns between the present wound healing study and previous BCC and/or SCC studies. [c]Human study. [d]Mouse study. [e]Hamster study. [f]Expression of GLI3 was detected in 76% of the investigated human BCC's and in none of the investigated SCC's. [g]Expressed in wound edge keratinocytes in two out of three wounds during reepithelialization in array analyses and by ISH (Fig. 4 G-I). [h]In an independent study, Western blots of total tumor lysates were used to show that cyclin D2 is upregulated in papilloma as opposed to normal skin in chemically induced skin carcinoma (Balasubramanian *et al*., 1998)

during early stage squamous cell carcinogenesis and wound-healing (marked with bold in Table 2). Thus, with the exception of TGF-β receptor 1, annexinII p11 subunit, and sentrin, all of the genes detected as being downregulated in wound edge as compared to non-wound edge keratinocytes, that is, HMG-14, IFN-γ receptor, cyclin D2, thymosin β4, and hsp27, have previously been reported to be downregulated during squamous or basal cell carcinoma progression. Similarly, NF-E2-related factor, HB-EGF, connexin 26, and cytokeratin 14 have all been reported to be overexpressed during epidermal carcinogenesis and were all found to be upregulated in wound edge keratinocytes in this study (Table 2). These results unequivocally demonstrate that the phenotypic similarities between wound healing keratinocytes and neoplastic keratinocytes are reflected at the level of gene expression. Also of interest, the irreversible loss of keratinocyte differentiation during squamous cell carcinoma progression as opposed to the maintenance of keratinocyte differentiation during wound healing is also reflected at the level of gene expression. Thus, expression of three genes believed to be associated with keratinocyte differentiation, that is, CRABP-II, galectin 7, and cytokeratin 1 (Eller *et al*., 1994; Magnaldo *et al*., 1998; Freedberg *et al*., 2001), is reduced in squamous cell carcinoma, whereas we detected increased expression of all the three genes in wound edge as compared to non-wound edge keratinocytes (Table 2). Of other notable differences, GLI3, laminin receptor (67 kDa), calmodulin, TNF-α-induced protein 3 (A20), connexin 31.1, E-cadherin, and sentrin, were all constitutively expressed in keratinocytes during and/or post reepithelialization, whereas expression levels for these genes have been shown to be either up- or downregulated in squamous cell carcinoma (Table 2). In conclusion, the expression of all the genes for which discrepancies in expression patterns exist between wound healing and squamous cell carcinoma could be critically associated with the irreversible loss of growth control and invasiveness that distinguishes malignant keratinocytes from wound keratinocytes.

Discussion

In this paper, we describe the first use of LCM combined with cDNA array analysis to systematically analyse changes in gene expression in keratinocytes during skin wound healing *in vivo*. We used the procedure to identify 48 genes not previously specifically implicated in wound reepithelialization, and to illuminate similarities and differences between gene expression in malignant and wound healing keratinocytes. The procedure described here is very simple and can immediately be applied to study other physiological and pathophysiological remodeling processes of the keratinized epithelium of the skin, oral cavity, and urogenital tract, including excisional, chemical, freeze, and burn injuries, keloid formation, psoriasis, excema, chronic ulcers, blistering diseases, and inherited skin disorders. The procedure is equally well suited for the analysis of tissue samples derived from experimental animals as from frozen archival human tissue.

The many advantages of being able to directly perform global mRNA expression analysis of defined keratinocyte subpopulations *in vivo* are self-evident and are not in need of extensive discourse. In contrast to cell or organ culture models of wound healing, all the environmental cues that combine to determine the wound keratinocyte transcriptome are preserved, including stromal cell interactions, extracellular matrix interactions, wound bed-produced inflammatory mediators and growth factors, hypoxia, and circulating systemic factors.

However, the method, as presented here, is also endowed with some limitations that warrant mentioning. The analysis, by its inherent design, is limited to studying changes in steady-state levels of mRNA, and changes in wound healing gene expression that manifest at the level of mRNA translation or post-translational protein modification will go unnoticed. However, the method can be easily extended to also address changes in protein expression levels and post-translational modifications (Knezevic *et al*., 2001; Curino *et al*., 2002; T Pedersen and A Curino, unpublished data). The LCM and cDNA array analysis successfully identified multiple genes whose mRNA levels have previously been shown to be altered during skin wound healing. However, some well-established wound healing-associated genes were not reported by the analysis, despite being represented in the cDNA arrays used in this study. This was particularly evident for the sizeable group of extracellular matrix-degrading proteases and their receptors and inhibitors, whose expression is known to be strongly upregulated (although stoichiometrically still being present in minute amounts) in migrating keratinocytes at the wound edge. These included the urokinase plasminogen activator, urokinase plasminogen activator receptor, plasminogen activator inhibitor-1, matriptase/MT-SP1, neuropsin, and matrix metalloproteinases 3, 9, 10, and 13 (Romer *et al*., 1996; Madlener *et al*., 1998; Kitayoshi *et al*., 1999; Lund *et al*., 1999, unpublished data). The improvement of methods for RNA isolation, reverse transcription, and hybridization, and the use of more sensitive arrays should overcome such limitations in sensitivity. An additional potential problem was revealed by the artifactual reporting of mRNA for the very highly expressed mesenchymal marker, decorin, in the analysis, revealing that the procured samples were contaminated with trace amounts of the underlying mesenchyme. It is important to emphasize, however, that we do not believe that mesenchymal contamination of keratinocyte samples represented a general problem in the interpretation of our array results, because decorin is expressed in extremely high quantities in the dermis, and because mRNA for no other mesenchymal cell markers were reported in the array analysis. Nevertheless, the use of *in situ* hybridization analyses for validation of the expression data in our array experiments permitted the identification of the specific cells in which a given mRNA was produced.

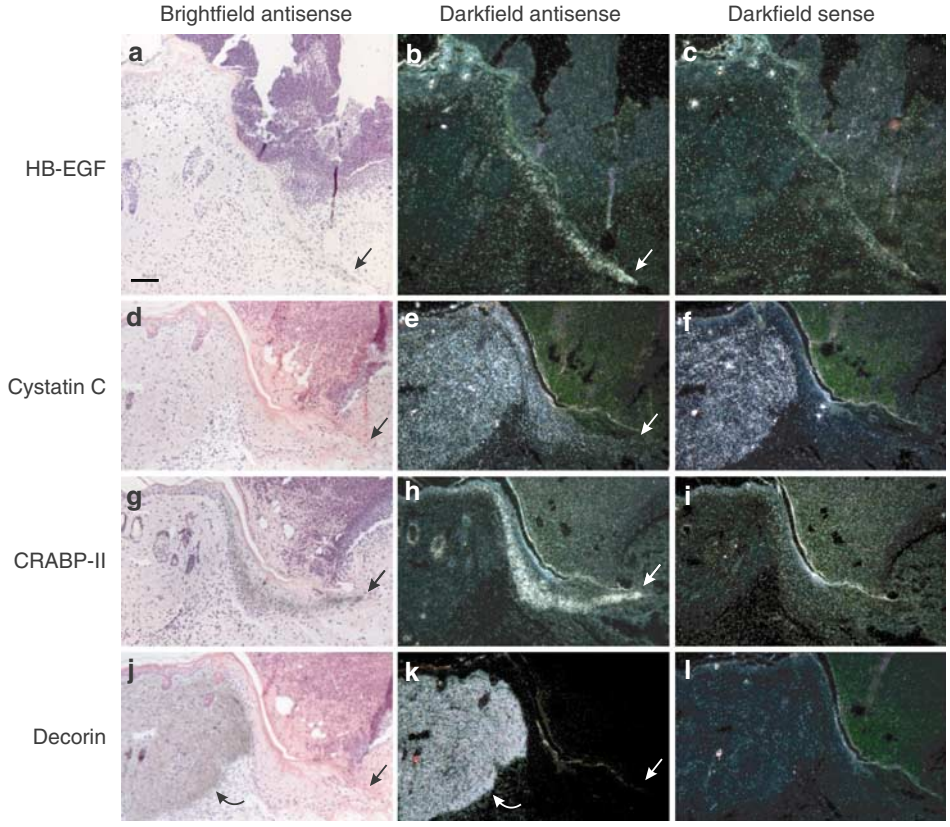

Figure 4 Validation of the LCM and cDNA array procedure by *in situ* hybridization. *In situ* hybridization with ^{35}S-labeled cDNA performed during reepithelialization with antisense probes (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**) or sense probes (**c**, **f**, **i**, **l**) complementary to HB-EGF (**a**–**c**), cystatin **c** (**d**–**f**), CRABP-II (**g**–**i**) or decorin (**j**–**l**) mRNA. Representative H & E stained bright field (**a**, **d**, **g**, and **j**) and dark field (**b**, **c**, **e**, **f**, **h**, **i**, **k**, and **l**) sections are shown. Similar analyses were performed on wounds post reepithelialization (data not shown). The arrows indicate the tip of the migrating keratinocytes (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**). The curved arrows indicate the dermis-specific decorin signal (**j**, **k**). All pictures were taken at the same magnification. Scale bar in (**a**): 100 μm

The analysis performed here identified an intriguing array of candidate reepithelialization-associated genes encoding transcription factors, cytokines, growth factors, intracellular signaling molecules, apoptosis, and cell cycle-associated proteins. Molecular probes, such as neutralizing antibodies, specific inhibitors, and gene-manipulated mice are available for many of these genes, facilitating a straightforward analysis of their causal involvement in incisional wound healing in future studies.

One of the most appealing aspects of the analysis presented here was the opportunity to directly compare global changes in keratinocyte gene expression during skin wound healing and squamous cell carcinoma progression. The phenotypic similarities of the two tissue-remodeling processes, as well as parallel malignant and physiological remodeling of other organs (e.g., mammary gland involution versus ductal mammary carcinoma and normal shedding of colonic epithelium versus colon cancer), have been noted previously, and the similarities have been proposed to be reflected at the level of gene expression (Dvorak, 1986; Dano *et al*., 1999). This paradigm has been particularly well investigated in the context of extracellular matrix-degrading enzymes, where striking similarities between the two tissue-remodeling processes have been documented (Johnsen *et al*., 1998; Dano *et al*., 1999). Importantly, the data presented here unequivocally demonstrate that these similarities extend to other groups of molecules including transcription factors, structural proteins, growth factors, and signal transduction molecules. However, the specific differences in gene expression between the two processes that were unraveled in this study may be even more relevant to enhancing the understanding of squamous cell carcinogenesis. Thus, the constitutive or increased expression of GLI3, CRABP-II, calmodulin, galectin-7, connexin 26, connexin 31.1, E-cadherin, and cytokeratin 1 in wound keratinocytes, as opposed to the frequent loss of expression of these genes in advanced squamous cell carcinoma, may help explain the fundamental differences between controlled and reversible versus uncontrolled and irreversible keratinocyte proliferation and invasion.

Materials and methods

Mice and tissue preparation

All animal studies were performed in a pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care International-certified facility, according to

Institutional guidelines, and under an approved animal study proposal. Young adult C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were anesthetized prior to surgery by inhalation of 2% isoflurane (Ohmeda PPD, Liberty Corner, NJ, USA), or by subcutaneous administration of a 1 : 1 mixture of Dormicum (Roche A/S, Basel, Switzerland) and Hypnorm (Janssen-Cilag Ltd., High Wycombe, UK) as previously described (Lund *et al*., 1999). Full-thickness incisional skin wounds (15 mm) were made in the mid-dorsal area as described previously (Romer *et al*., 1996). Mice presenting with growth phase (anagen) hair follicles were discarded. The wounds were left undressed and unsutured. For LCM analysis, the mice were anesthetized by inhalation of 2% isoflurane and perfused intracardially with 10 ml ice-cold phosphate-buffered saline (PBS). The wound field was excised, bisected in the midtransversal plane, embedded in OCT and snap-frozen in isopentane that was cooled by liquid nitrogen. Mice for which tissue was to be used for immunohistochemistry and *in situ* hybridization were anesthesized by subcutaneous administration of a 1 : 1 mixture of Dormicum and Hypnorm, and perfused intracardially with 10 ml PBS followed by 10 ml 4% paraformaldehyde in PBS. The wounds were excised and processed into paraffin as described previously (Romer *et al*., 1996).

LCM

The isolation of keratinocytes from histological sections by LCM was performed essentially as described (Bonner *et al*., 1997; Simone *et al*., 1998; Leethanakul *et al*., 2000; Curino *et al*., 2002). Cryostat sections (6 μm) were prepared perpendicular to the longitudinal direction of the wound, and the sections were kept on dry ice, or at -80°C, until they were subjected to LCM. Just prior to the procedure, the sections were fixed in 70% EtOH for 10 s and stained with hematoxylin and eosin by immersion using the following protocol: 10 s deionized H_2O, 30 s hematoxylin, 2×10 s deionized H_2O, 10 s 70% EtOH, 1 min Eosin Y (alcoholic), 2×10 s 95% EtOH, and 2×10 s 100% EtOH, and 30 s xylene. LCM was performed using an Arcturus PixCell II apparatus, with a 15 μm laser beam, power settings of 50–90 mW, and a laser pulse duration of 6–7 mS.

RNA extraction and assessment of RNA quality

RNA samples were isolated from wound edge and non-wound edge keratinocytes from six individual wounds, that is, three wounds isolated during reepithelialization (healing day 5) and three wounds isolated post reepithelialization (healing day 9). The RNA was purified from microdissected keratinocytes using a modified version of the Stratagene RNA microisolation kit (Stratagene, La Jolla, CA, USA), as described in detail at the web site http://dir.nichd.nih.gov/lcm/LCM_Werbsite_Introduction.htm (Leethanakul *et al*., 2000). After DNase treatment, the RNA was precipitated and redissolved in 5 μl H_2O. The integrity of all RNA samples was verified by RT–PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using the Promega Access one-step RT–PCR kit (Promega, Madison, WI, USA), and the GAPDH specific primers; 5′ primer; 5′-CACAGTCAAGGCCGAGAATG-3′ (bp 204–223 of the murine GAPDH gene - GenBank accession no. M32599 (Sabath *et al*., 1990)) and 3′ primer; 5'-GCTGTTGAAGTCGCAGGAGA-3' (bp 885–904). The amount of total RNA from six individual samples was quantitated with the VersaFluor Fluorometer system (Life Technologies, Rockville, MD, USA) as described previously (Leethanakul *et al*., 2000).

Construction of ^{32}P-labeled cDNA probes and cDNA array analysis

The Clontech Switch Mechanism at the 5′ end of mRNA (SMART) technology (Clontech, Palo Alto, CA, USA) was used to construct cDNA probes with flanking SMART tags. High specific activity ^{32}P-labeled cDNA probes were generated by performing PCR using SMART primers in the presence of ^{32}P-dCTP, as described previously (Leethanakul *et al*., 2000). RNA (2.5 μl) (approximately 5–9 ng total RNA) was used for the generation of each probe. The successful generation of representative probes was verified by the formation of a DNA smear ranging in size from 50 to 2000 bp after agarose gel electrophoresis of aliquots of the probe. The radiolabeled probes were used to screen Clontech mouse 1.2 cDNA expression arrays (Clontech). The complete list of genes represented on the array can be found on the following web site http://www.clontech.com/atlas/genelists/index.shtml. For each analysis, cDNA samples from wound edge and non-wound edge keratinocytes were analysed in parallel. The arrays were prehybridized with 0.75 mg denatured salmon sperm DNA in 10 ml hybridization solution (Clontech) for 1 h at 68°C. The hybridizations were performed after adding 1×10^6 cpm/ml denatured cDNA probe and 5 μl denatured C_0t DNA (1 mg/ml) directly to the prehybridization mix. The two arrays were incubated overnight with rotation at 68°C, washed 4×20 min in $2 \times$ SSC, 1% SDS, and 2×20 min in $0.1 \times$ SSC, 0.5 % SDS, wrapped in Saran wrap and subjected to PhosphorImage analysis using ImageQuant software from Molecular Dynamics (Molecular Dynamics, Sunnyvale, CA, USA). The expression level of each gene on the arrays was determined with Clontech ATLAS IMAGE software (Clontech), as recommended by the manufacturer, using global normalization, and defining the background as the median intensity of the nonspotted areas of the entire membrane. Both during and post reepithelialization, a gene was defined as being expressed if the adjusted signal intensity of the gene (i.e., the measured signal intensity minus the background) was at least twice the background level in keratinocyte populations isolated from three different wounds. Wound edge and non-wound edge keratinocyte gene expression patterns were compared in a total of six wounds; three wounds isolated during and three wounds isolated post reepithelialization. For all genes expressed in both wound edge and non-wound edge keratinocytes, a fold change ratio was calculated by dividing the normalized gene expression level in the wound edge keratinocytes with the normalized gene expression in the non-wound edge keratinocytes. Previous comparative systematic analyses of methods for detecting mRNA in tissues (Taniguchi *et al*., 2001; Yuen *et al*., 2002) have demonstrated that cDNA array analysis detects qualitative changes in gene expression as accurately as quantitative RT real-time PCR or Northern blot hybridization. However, compared to the aforementioned methods of detecting gene expression, array analysis systematically underestimates the magnitude of changes of expression of individual genes. We therefore defined a gene as upregulated if the fold change ratio of the gene was at least above 1.15 in all the three wounds. Likewise, we defined a gene as downregulated if the fold change ratio of the gene was at least below 0.85 in all the three wounds.

In situ *hybridization and immunohistochemistry*

Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol/water solutions. Radioactive *in situ* hybridization was performed essentially as described previously (Kristensen *et al*., 1991; Engelholm *et al*., 2001). The tissue

sections were treated with 0.25 μg/ml proteinase K in 50 mM tris[hydroxymethyl]-aminomethane-HCl (Tris), pH 8.0, 5 mM ethylenediaminetetraacetic acid at 44°C for 5 min, dehydrated, and air dried. A total of 30 μl hybridization solution with 3×10^6 cpm of the appropriate denatured ^{35}S-labeled probes were added to each section, and the hybridization was carried out overnight at 55°C. ^{35}S-labeled probes were generated using IMAGE clones that were all verified for the correct insert by sequencing. The IMAGE clone 1179316, that contains the cDNA for mouse heparin binding epidermal growth factor-like growth factor (HB-EGF) cDNA, was linearized with *Eco*R1 and transcribed with T3 polymerase for antisense probes and with *Not*I and T7 polymerase for sense probes. IMAGE clone 3981285, containing the cDNA for mouse decorin was linearized with *Xma*I and transcribed with T7 polymerase to produce antisense probes, and linearized with *Hind*III and transcribed with Sp6 polymerase to produce sense probes. IMAGE clone 3660521, containing the mouse cellular retinoic acid binding protein II (CRABP-II) cDNA, was linearized with *Sal*I and transcribed with T7 polymerase to generate antisense probes, and linearized with *Not*I and transcribed with Sp6 polymerase for sense probes. IMAGE clone 3968413, containing the mouse cystatin C cDNA was linearized with SalI and transcribed with T7 polymerase for antisense probes, and linearized with *Not*I and transcribed with Sp6 polymerase for sense probes. The linearized plasmids were phenol–chloroform extracted and/or purified on microspin S-300 HR columns (Amersham-Pharmacia, Piscataway, NJ, USA).

Tissue sections were stained for mitotic activity by nonradioactive *in situ* hybridization, using NovoCastra histone oligonucleotide probes (NovoCastra, Newcastle upon Tyne, UK) that detect histone H2B, H3, and H4 mRNA, and the NovoCastra *in situ* hybridization detection kit (NovoCastra), according to the manufacturer's instructions. Immunohistochemistry was performed with the DAKO streptABComplex HRP duet kit (DAKO, Carpinteria, CA, USA), according to the manufacturer's instructions with the following modifications. Antigens were retrieved by proteolytic digestion with 0.025% trypsin for 8 min at 37°C, and endogenous peroxidase activity was blocked by incubation in 1% H_2O_2 for 18 min at RT. The sections were incubated overnight at 4°C with primary polyclonal rabbit anti-cow pan-keratin antibody (Z0622 DAKO, Copenhagen, Denmark) diluted 1:500 in 50 mM Tris-buffered saline/0.25% bovine serum albumin (Tris/BSA). Sections were incubated with biotinylated swine-anti rabbit antibody (E353, DAKO) diluted 1 : 100 in Tris/BSA for 30 min, developed with NOVA-RED stain (Vector Laboratories, Burlingame, CA, USA) and counterstained with Mayers hematoxylin.

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