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Defining the Transcriptome of Accelerated and Replicatively Senescent Keratinocytes Reveals Links to Differentiation, Interferon Signaling, and Notch Related Pathways

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Abstract Epidermal keratinocytes (KCs) undergo highly orchestrated morphological and molecular changes during transition from proliferative compartment into growth arrested early and late differentiation layers, prior to dying in outermost cornified layers of normal skin. Creation of stratum corneum is vital to barrier function protecting against infection. Transcriptional events in KCs regulating complex processes of differentiation and host defense required to maintain constant epidermal thickness and resistance to infection in either young or aged skin are largely unknown. Furthermore, as terminal differentiation is characterized by irreversible loss of replicative potential culminating in dead layers at the skin surface, this process may be viewed as a form of senescence. However, a complete transcriptional profile of senescent (SN) human KCs has not been previously defined to permit delineation of molecular boundaries involving differentiation and senescence. To fill this void, we utilized global transcriptional analysis of KCs maintained in vitro as either cultures of proliferating (PR) cells, early and late confluent (LC) (accelerated senescence) cultures, or KCs undergoing replicative senescence. Global gene expression profiling revealed early confluent (EC) KCs were somewhat similar to PR KCs, while prominent differences were evident when compared to LC KCs; which were also distinct from replicatively SN KCs. While confluent KCs have in common several genes regulating differentiation with replicatively SN KCs, the latter cells expressed elevated levels of genes involved in interferon signaling and inflammatory pathways. These results provide new insights into cell autonomous transcriptional-based programs operative within KCs contributing to replicative senescence, with partial sharing of genes involved in differentiation. In addition, regulation of KC senescence may involve participation of interferon signaling pathways derived from the important role of KCs in protecting skin from infection. Integrating all of the transcriptional data revealed a key role for Notch receptor mediated signaling in the confluency induced differentiation phenotype using this model system. J. Cell. Biochem. 98: 394–408, 2006.

Key words: keratinocytes; differentiation; senescence; interferon; Notch

Keratinocytes (KCs) derived from human skin provide an attractive organ system for understanding the coordinated interplay of genes that impact proliferation, differentiation, senescence, and inflammation [Eckert et al., 1997b; Chaturvedi et al., 1999; Candi et al., 2005]. As a self-renewing tissue, the multiple layers of KCs must be coordinately regulated in space and time to maintain a proper epidermal thickness and create a stratum corneum [Eckert et al., 1997a]. Thus, a delicate balance is operative in which KCs located in the basal layer harboring the proliferative population must then enter a non-replicative state in which

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early differentiation is followed by terminal differentiation, and then cell death to generate and maintain the barrier function of skin (e.g., stratum corneum) [Brattsand and Egelrud, 1999]. We coined the phrase "planned cell death" for these events in epidermis to emphasize the point that KCs must complete the differentiation process prior to onset of cell death to produce corneocytes [Qin et al., 2005]. While there is no question that terminal differentiation ultimately culminates in loss of replicative potential and dead cells; and the final event in the aging process is death, it is unclear whether terminal differentiation and senescence (characterized by irreversible growth arrest) share common molecular mediators or represent entirely distinct processes [Norsgaard et al., 1996; Gandarillas et al., 1999].

The most important function of skin is to provide a lifelong barrier to prevent intrusion of infectious agents, and for KCs to rapidly and effectively generate inflammatory and immunologically relevant responses if the barrier is breached due to trauma, excessive sun exposure, or other stimuli [Barker et al., 1991]. Previous studies clearly demonstrated an impressive array of pro-inflammatory mediators produced by activated KCs [Laduca and Gaspari, 2001], including important roles for cytokines such as interferons. Indeed, KCs actually produce a distinctive type I interferon [LaFleur et al., 2001]. Not only do KCs produce interferons, they can also respond to type I and type II interferons [Arany et al., 1998]. If skin is chronically exposed to sunlight, UV-induced inflammatory mediators produce premature aging [Yaar and Gilchrest, 1998; Sesto et al., 2002]. Conversely, in dermal fibroblasts, the senescent (SN) state mimics inflammatory and repair processes [Shelton et al., 1999]. Thus, there appears to be a link between inflammation and senescence [Kirkwood, 2005], although this connection in KCs remains to be defined at the molecular level. We therefore postulate that interferon signaling-related pathways may play a role in KC senescence [Kulaeva et al., 2003], and in this report evidence is provided to support this notion. Just as senescence may protect against tumorigenesis in young individuals, with the unintended consequence in later years of causing disease secondary to tissue dysfunction [Campisi, 2005], so too that interferon signaling provides protection against infection, but if such signaling is sustained, premature aging of the skin may complicate the overall response.

Inappropriate induction of cell death (e.g., premature cell death), or alterations in the differentiation process adversely influences barrier function and enhances susceptibility to infection, thereby contributing to a wide spectrum of skin diseases, including graft-versus-host disease, toxic epidermal necrolysis, psoriasis, and cancer [Nickoloff et al., 2002a; Murphy and Korngold, 2004; Nickoloff and Nestle, 2004; Quinn et al., 2005]. In addition, the aging process is also characterized by reduced barrier function and increased susceptibility to infection. To gain insight into these pathological conditions, as well as the aging process in skin [Campisi, 1998], we decided to begin by focusing on the identification and comprehensive analysis of genes that regulate human KC behavior in vitro. In an earlier study, we utilized cultured human KCs as a model system and began to define key molecular links between the complex process of proliferation, differentiation, and senescence, by focusing on a relatively small number of proteins [Chaturvedi et al., 1999]. It should be noted that we previously observed that late confluent (LC) KCs expressed many proteins associated with senescence, such as increased levels of cyclin-dependent kinase inhibitors (i.e., p27 and p16; ibid). Thus, we have considered LC KCs as cells that have undergone accelerated senescence as they also share the phenotype of being unable to proliferate after re-seeding [Chaturvedi et al., 1999, 2004b].

As regards regulating terminal differentiation of epidermal KCs in both human and non-human skin, several groups have independently identified a key role for Notch-receptor mediated signaling events [Rangarajan et al., 2001; Nickoloff et al., 2002b; Nicolas et al., 2003; Kolly et al., 2005]. There are four different Notch receptors and several ligands repressed by KCs, and Notch 1 appears to be of particular importance to initiating KC differentiation (ibid). Given a key role for Notch receptor signaling, particular attention was devoted to the delineation of Notch receptor gene levels in this cultured KC model system.

Even though terminal differentiation and replicative senescence both result in suppression of proliferation, the molecular basis for each KC response has not been comprehensively analyzed to delineate classes of genes that

are shared or are distinctive for each process [Gandarillas, 2000]. As mentioned earlier, it has been proposed that terminal differentiation may be linked to senescence, but similarities and differences in a limited number of genes have only been studied in an attempt to resolve this controversy for skin biologists [Gandarillas et al., 1999]. Thus, we undertook a more global gene expression profile approach to address the overlap and/or differences to further advance the understanding of normal and abnormal cutaneous biology with a particular emphasis on KCs undergoing accelerated as well as replicative senescence.

MATERIALS AND METHODS

Keratinocyte Differentiation

The four different stages of KCs examined included: proliferating (PR) cells, early confluent (EC) KCs, LC KCs, and SN KCs. For all cultures, KCs were maintained in a low calcium, serum free medium (KGM, Clonetics Corp., San Diego, CA), and the only variables were the relative degree of confluency, and the overall time in culture. PR KCs were initially expanded in large flasks (T-150) and then transferred to 100 mm dishes. PR KCs were characterized as occupying no more than 30% of the surface area, and hence are regarded as being maintained in a sub-confluent state. By contrast, EC KCs were defined as cultures in which over 90% of the KCs were surrounded by neighboring cells in direct physical contact, and hence virtually covered the entire surface area of the culture dish. Late confluency was defined by feeding the KCs every other day and delaying the harvesting of cells for 4 days following the onset of early confluency, at which time the entire population of KCs was completely surrounded by other KCs (e.g., LC KCs). The PR KC cultures, as well as early and LC cultures were harvested within 3–5 passages, whilst KCs undergoing replicative senescence were continuously passed upon 30–50% confluency for several additional weeks until they failed to divide despite fresh medium changes. KCs that underwent replicative senescence appeared enlarged, flattened, and contained cytoplasmic vacuoles. Since we previously had observed that KCs derived from late confluency had minimal ability to proliferate after reseeding, we have classified these LC KC cultures as having undergone accelerated senescence [Chaturvedi et al., 1999].

Affymetrix Array Profiling

Total RNA from cultured KCs in human foreskin at different stages (PR, confluent, or SN) was used for Affymetrix gene expression profiling. Each of the groups (consisting of KCs derived from different foreskins) had two biologically independent replicates in each stage. Total RNA was harvested using the RNeasy kit (Qiagen, Inc., Valencia, CA). For first and second strand cDNA synthesis, SuperScript reverse transcriptase and oligo-dT primer containing T7 RNA polymerase promoter sites were used. In vitro transcription (IVT) reaction was done according to high yield transcription labeling kit from ENZO (ENZO Bio Inc., NY/NY). Fifteen micrograms of cRNA was fragmented and then hybridized to Affymetrix Human array HU-95A chip. Samples were prepared for hybridization according to the Affymetrix Expression Analysis Technical Manual.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

To monitor mRNA expression levels, KC cultures were lysed in a guanidinium containing buffer and RNA extracted using RNeasy (Qiagen). The amount of RNA obtained was determined using Ribogreen (Molecular Probes, Eugene, OR). Thirty nanograms of total RNA from the selected samples of cultured KCs were analyzed by quantitative RT-PCR using LightCycler (Roche Applied Science) following the manufacturer's instructions. All samples were analyzed in duplicate under optimized PCR conditions. The forward, reverse primer sequences and probes used respectively for each gene of interest were as follows: *MCM5* (5′-ACCCTGGGACAGCCAAGTC-3′, 5′-GCCTTTCCCAGACGTGTATACC-3′, and 5′-CTGAAGTTTGTGGAGAAGTGTTCTCCX-3′); *FGFR3* (5′-GGCCATCGGCATTGACA-3′, 5′-GGCATCGTCTTTCAGCATCTT-3′, and 5′-CCGCCAAGCCTGTCACCGTAGCX-3′); *VEGFC* (5′-TCAGGCAGCGAACAAGACCT-3′, 5′-TTCCTGAGCCAGGCATCTG-3′, and 5′-CCCCACCAATTACATGTGGAATAATCACATCTX-3′); *CTGF* (5′-CAGCTCTGACATTCTGATTCGAA-3′, 5′-TGCCACAAGCTGTCCAGTCT-3′, 5′-CACTGTTCAGGAATCGGAATCCTGTCGX-3′); IFI27 (5′-TGTCATTGCGAGGTTCTACTAGCT-3′, 5′-CCCCTGGCATGGTTCTCTT-3′, and 5′-CCTG-

CCCCTCGCCCTGCAX-3′); *Cyclin D2* (5′-ACCATCGAGGAGCGCTACCT-3′, 5′-GCGCATGTAGGGTTGGATGT-3′, and 5′-CAGTGCTCCTACTTCAAGTGCGTGCAGAX-3′). The expression levels were normalized to total cellular RNA. In general our statistical significant array data ($P < 0.05$) shows 90% agreement with RT-PCR or other validation methods.

Statistical Analysis

One-way analysis of variance was conducted and the resulting P values from pairwise comparisons were adjusted using the Benjamini-Hochberg procedure. This procedure controls the false discovery rate (FDR), which is the expected proportion of erroneous significance calls among all the significance calls. A cut-off of FDR ≤ 0.2, which is equivalent to $P \leq 0.007$ in our data, was used to generate 2,343 statistically significant genes. Note that probe sets with the percentage of positive calls by Affymetrix less than 50% in the control group were removed before the statistical filtering.

Pathway Analysis

Probeset IDs that had 0% positive calls for all four stages were filtered out. Thus 7,504 probeset IDs were used for the subsequent analysis. Since the LC KC cultures had the highest induction of differentiation genes, our pathway analysis focused exclusively on comparing PR KCs versus LC KCs. The MetaCore GeneGo database recognized 3,503 of these Affymetrix probeset IDs, corresponding to 2,343 distinct genes in the database.

Immunoblot Analysis

Whole cell extracts were prepared and analyzed as previously described [Chaturvedi et al., 1999]. Detection of activated Notch 1 receptor was accomplished using an antibody from AbCam (ab8925), with anti-actin ab (Santa Cruz Biotechnology, Santa Cruz, CA) serving as a loading control.

RESULTS

Microarray Data Mining Revealed Distinctions Between Proliferating KCs Versus KCs Undergoing Accelerated Senescence and Replicative Senescence

Human epidermis is composed of self-renewing and stratified KCs, which undergo complex biological and morphological changes that are highly coordinated in space and time. Figure 1 is a schematic depiction of human epidermis highlighting five distinct compartments including: first, a basal layer in which proliferation occurs, in-contiguity with a second suprabasal layer in which KCs become growth arrested and undergo early differentiation, followed thirdly by late differentiation, and fourthly, terminal differentiation. Once terminal differentiation is achieved, the outermost layer of KCs undergo cell death in which all DNA, RNA, and cytoplasmic organelles disappear following digestion/degradation to produce corneocytes in the fifth compartment which is responsible for the barrier function of skin.

Initial studies aimed at defining factors that could influence KC proliferation and differentiation used cultures of either mouse or human KCs, revealing an important role for extracellular calcium ion gradients [Rosenthal et al., 1991]. More recently, investigative skin biologists turned to transgenic mice to define individual genes that can regulate normal epidermopoiesis [Koster et al., 2002; Eckert et al., 2004]. While notable insights have been gained by either eliminating a gene or overexpressing a gene at various epidermal levels, progress has been slow using the one gene one phenotype approach. In addition, mouse and human KCs may not yield identical results in response to various stimuli [Chaturvedi et al., 2004a]. Moreover, given the complexity of processes likely to regulate KC behavior, a systems biology approach was employed to facilitate simultaneous examination of thousands of genes in a population of KCs that were maintained in a uniform environment. To dissect relevant informational pathways, we combined our expertise in KC biology; with our ability to analyze the transcriptome using validated computational methods.

To minimize the number of confounding variables in this analysis, we examined the transcriptome of foreskin-derived KCs in which the only difference amongst each KC population of interest was their proliferation status, degree of confluency, and replicative potential. In other words, KCs from an identical donor were fed every other day using a low calcium, serum-free medium, and the four different types of cultures included: (1) early passage rapidly PR KCs in which no more than 30% of the surface area was occupied by KCs (designated as PR KCs); (2) cultures in which more than 90% of the KCs

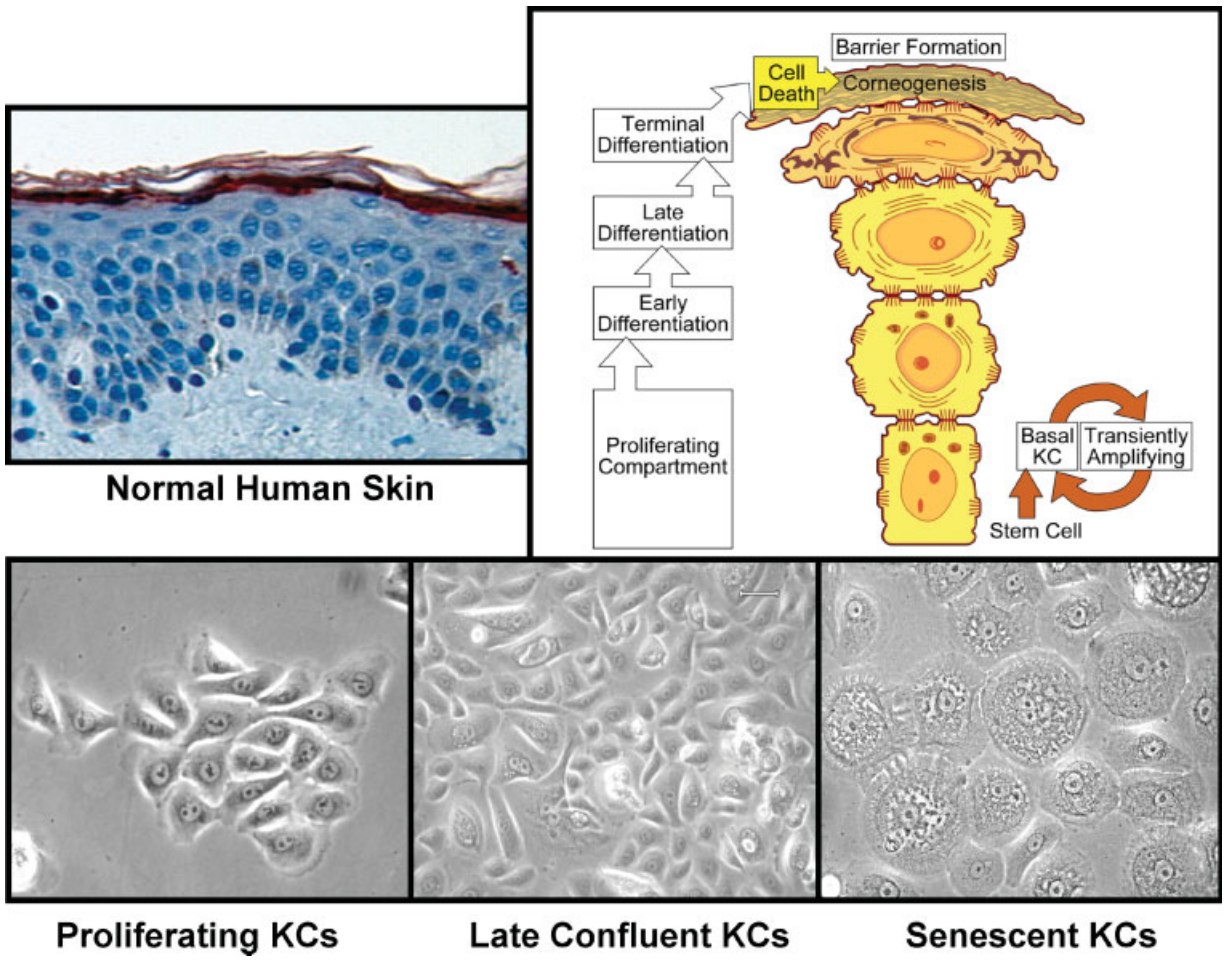

Fig. 1. Upper Panels: Histological appearance of normal human skin and adjacent cartoon highlighting different layers beginning with proliferating (PR) cells in the basal layer, followed by KCs undergoing early and late differentiation, and ultimately cell death to form the stratum corneum. **Lower Panels**: Phase contrast microscope appearance of KCs present under various culture conditions including: PR KCs, late confluent (LC) KCs, and senescent (SN) KCs. Note the different morphological appearance of cultured KCs in which PR KCs grow as small refractile cells with open chromatin in the nucleus including nucleoli. By contrast, LC KCs appear less refractile with increased cytoplasm and more condensed nuclear chromatin. KCs undergoing replicative senescence are enlarged, flattened with cytoplasmic vacuoles.

were in contact with adjacent cells (designated: EC KCs); (3) cultures in which KCs had achieved confluency and were then fed every other day for four more days (designated: LC KCs); and (4) cultures in which several passages of sub-confluent cells was performed until KCs ceased to proliferate despite medium changes reflecting replicative exhaustion (designated: replicative senescence).

The phase contrast microscopic appearance of each KC population is portrayed in Figure 1. Note the PR KCs are relatively small and refractile with prominent nucleoli, whereas LC KC cultures are characterized by being less refractile and larger with less conspicuous nuclei. The SN KCs are even larger than confluent KCs and are flattened with cytoplasmic vacuoles.

Unsupervised hierarchical clustering reveals distinct groupings of gene expression patterns for KCs under four different culture conditions (e.g., PR, EC, LC, and SN; Fig. 2). In this figure the height of each link between members of the cluster is relatively proportional to the average squared distance of each leaf in the sub-tree as measured from the mean value for that subtree. These data reveal distinct clustering of genes that are differentially expressed in a constitutive fashion for KCs maintained under each of the four culture conditions, and was consistently observed in a second independent set of KC cultures.

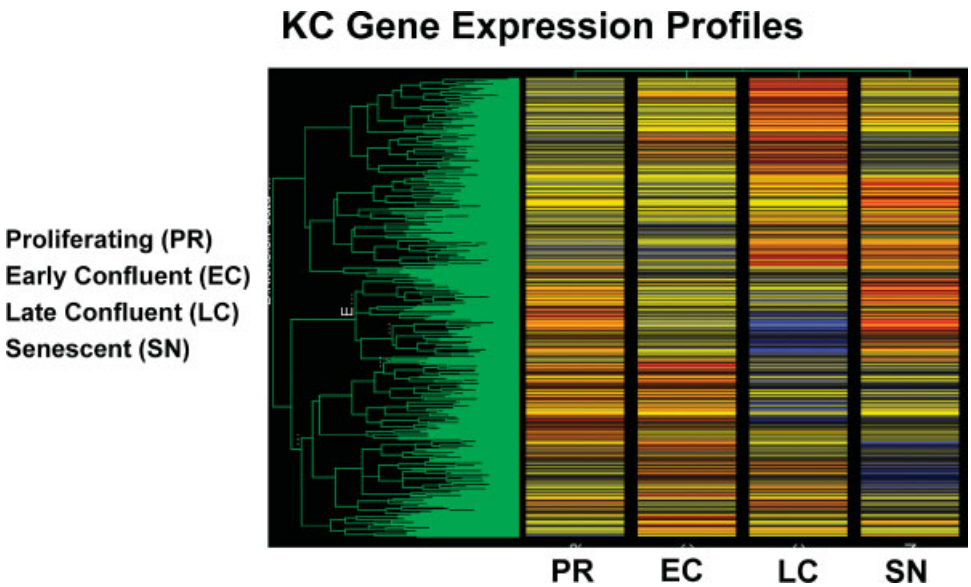

Fig. 2. Hierarchical clustering of microarray data generated from human KC cultures maintained under various conditions, including: PR KC, early confluent (EC) KC, LC KC, and SN KC cultures. Major changes in gene expression are seen in LC and SN KC cultures compared to PR KC cultures. Data are graphically displayed with color to represent quantitative changes. Increases in mRNA levels are shown as shaded red, and decreases in mRNA levels are represented by shaded blue. Black and gray colors illustrate that the gene expression for a given gene is close to mean values across samples.

The data were further analyzed by principal component analysis (PCA). The axes represent the two leading principal components that contain 21.1% and 14.3% of total variance, respectively (Fig. 3). Each marker in the two-dimensional plot corresponds to the gene expression pattern of each sample. To enhance the validity of our conclusions, duplicate transcriptome profiles were generated using parallel sets of KCs maintained under each culture condition, and two different human foreskins were utilized to generate the respective KC cultures using identical protocols. Note the tight clustering for individual KC population amongst both parallel sets of KCs, and between KCs derived from different donor foreskins. While the gene expression profile of EC KC cultures (red circles, squares) were somewhat similar to PR KC cultures (green circles, squares), prominent differences were clearly evident when compared to late KC cultures (blue circles, squares). Interestingly, the KC cultures undergoing accelerated senescence (late confluency) were also characterized by a gene expression profile distinct from the KCs that underwent replicative senescence (black circles, squares).

Figure 4 portrays Venn diagrams summarizing the number of genes either upregulated (left side panel) or downregulated (right side panel) when comparing PR KC cultures to cultures of EC, LC, and SN KCs. Note the large number of genes (both upregulated and downregulated) which are not shared amongst the different KC populations. However, there are genes that are shared in common as depicted in the areas of overlapping circles for both upregulated as well as downregulated genes.

Validation of Differential mRNA Expression in KC Cultures

Given the large number of genes either upregulated or downregulated when comparing PR KC cultures to either cultures of LC or SN KC cultures, an initial validation survey of differential mRNA express was conducted using quantitative RT-PCR for eight representative genes. These genes influence a broad spectrum of cell behavior including cell cycle, proliferation, differentiation, and inflammation/immune responses. Thus, the genes selected for qualitative RT-PCR analysis include: minichromosome maintenance protein 5 (MCM5), fibroblast growth factor receptor 3 (FGFR3), vascular endothelial growth factor C (VEGFC), epithelial membrane protein 3 (EMP3), connective tissue growth factor (CTGF), interferon-alpha

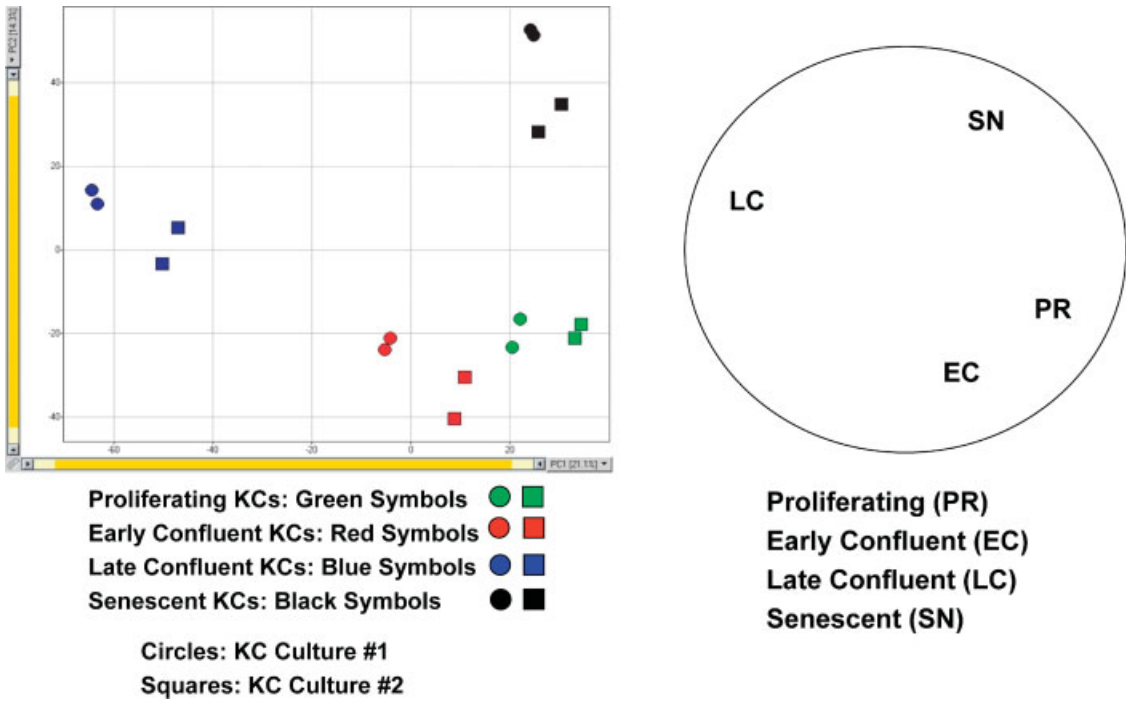

Fig. 3. Principal component analysis (PCA) (2D). PR KC cultures (green circles, squares), EC KC cultures (red circles, squares), LC KC cultures (blue circles, squares), and replicative senescence (black circles, squares). The PCA clearly demonstrates distinct patterns of overall gene expression in which LC KC cultures are different than EC/PR KC cultures and SN KC cultures are very different from LC as well as EC/PR KC cultures.

inducible protein 27 (IFI27), small proline-rich protein 1A (SPRR1A), and cyclin D2. The relative expression values for each selected gene determined by quantitative RT-PCR is presented (Fig. 5) for PR KCs (open bars), LC KCs (shaded bars), and SN KCs (black bars).

As can be seen, some mRNA levels were relatively high in PR KC cultures (e.g., MCM5,

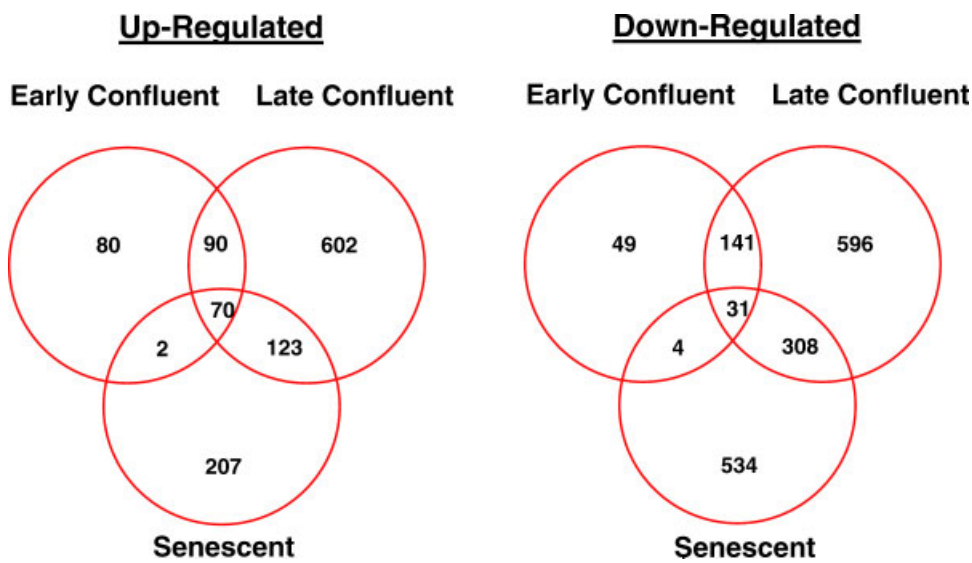

Fig. 4. Venn diagram summarizing genes either upregulated (**left side panel**) or downregulated (**right side panel**) in either EC, LC, or SN cultures compared to PR KCs. Note that while numerous genes are shared in their up or downregulated patterns amongst various culture conditions, there are between 49 and 602 different genes that are either up or downregulated when comparing/contrasting PR KCs against EC, LC, or SN KC cultures.

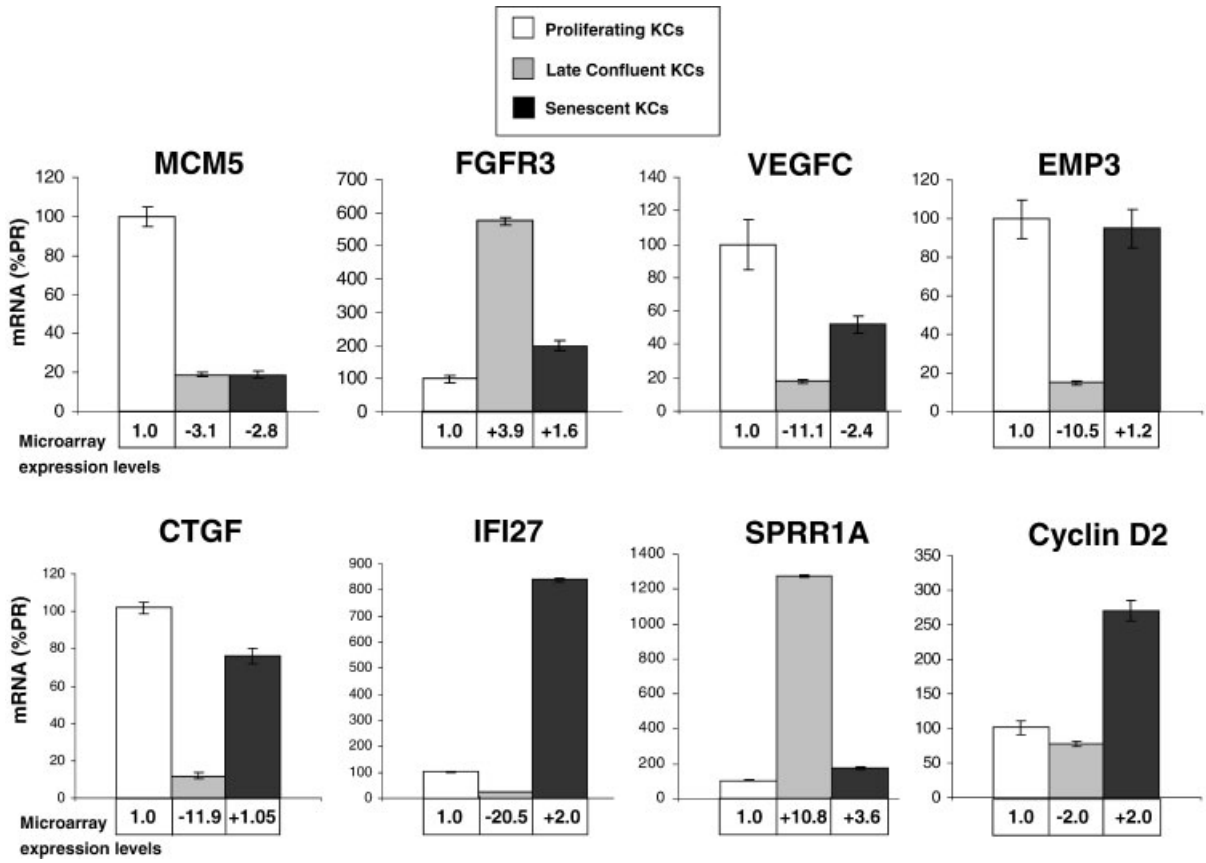

Fig. 5. Verification of selected genes (MCM5, FGFR3, VEGFC, EMP3, CTGF, IFI27, SPRR1A, and Cyclin D2) regulating KC behavior by comparing mRNA levels using quantitative RT-PCR-based results versus microarray-based results. RNA was obtained from cultures of PR KCs (open bars), LC KCs (shaded bars), or SN KCs (black bars), and relative mRNA expression levels determined by quantitative RT-PCR (y-axis). Relative mRNA levels based on Affymetrix based array analysis are provided in boxes immediately beneath each bar. Note the qualitatively similar relative mRNA expression levels for all eight genes comparing quantitative RT-PCR with microarray based data.

VEGFC); whereas other mRNA levels were relatively low in PR KC cultures and increased in LC KC cultures (e.g., FGFR3, SPRRIA) or in SN KC cultures (e.g., IFI27, cyclin D2); and still other genes displayed additional patterns of up or downregulation depending upon the culture conditions. To confirm and extend these quantitative RT-PCR results, the relative expression levels for these eight genes was also detected by the Affymetrix array profiling. The microarray-based relative expression levels are presented in boxes immediately beneath the bar graph for each KC culture condition (Fig. 5). For each gene of interest, the relative expression values were qualitatively similar when comparing the quantitative RT-PCR data with the Affymetrix microarray based data. Based on this independent validation of differential gene expression, we explored additional genes for their up and downmodulation amongst specific groups of genes participating in the regulation of distinct KC responses involving: cell cycle; differentiation; and inflammation/immune responses.

Expression Profile for Genes Regulating Cell Cycle

To continue to dissect individual gene levels in this experimental system, we first analyzed cell cycle-related genes because KCs contained within LC and SN populations are characterized as being growth arrested, and therefore likely to have numerous components of the transcriptome altered relative to PR KC cultures. Indeed, many genes that promote cell division such as CDC2, Ki67, cyclin A2, cyclin B1, and cyclin B2 were similarly reduced by at least twofold in both LC and SN KCs relative to PR KCs (Fig. 6). Several kinases, phosphatases, and cyclins are known to regulate the cell cycle, and either promote or inhibit cell division. In addition to the aforementioned gene regulating cell cycle, cyclin D2 mRNA levels were reduced

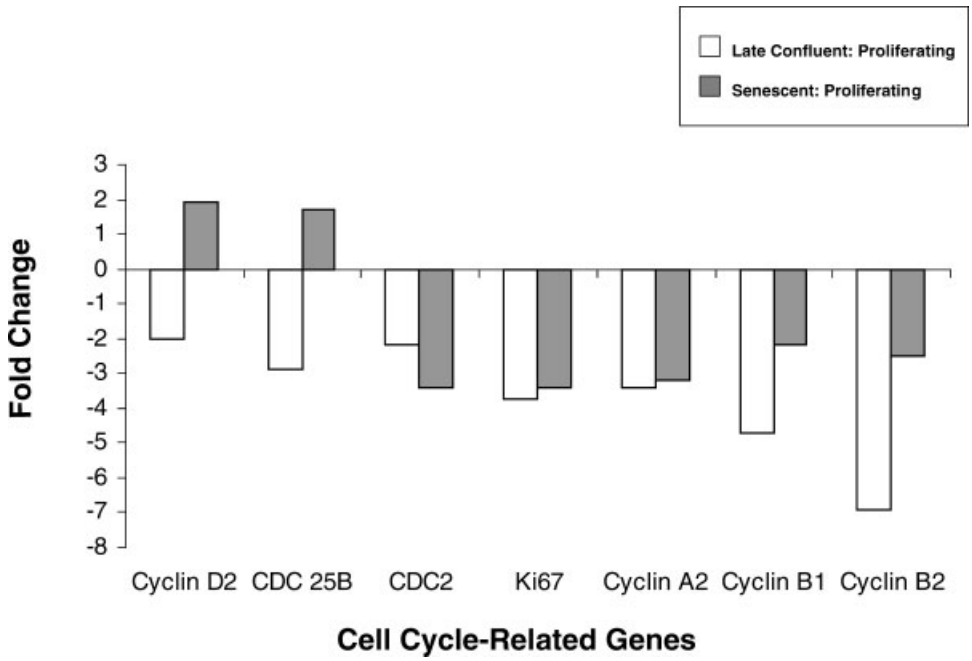

Fig. 6. Relative levels of genes regulating the cell cycle and proliferation of KCs. For each gene of interest the ratio of the level of expression in PR KC cultures is compared to either LC KC cultures, or between PR KCs and KCs undergoing replicative senescence. Note that five of seven cell cycle-related genes are characterized by similar reduction in mRNA levels when comparing PR KC cultures against either LC or SN KC cultures.

in LC KC cultures by twofold relative to PR KCs, whereas this same gene was increased twofold in the SN KC cultures relative to PR KCs. Similarly, a phosphatase that activates cell cycle kinases (e.g., cdc25B) was also decreased in LC, but increased in SN KC cultures relative to PR KC cultures. Taken together, these results provide insight into molecular pathways that are activated when PR KCs undergo either late confluency or senescence, which can impact cell-cycle progression.

Differential Expression of KC Differentiation-Related Genes

Figure 7 portrays some of the markers of early and late KC differentiation with the fold change values of the associated genes. As can be seen, there emerges a general pattern in the gene expression levels when growth arrested LC and SN KC cultures are compared to the proliferative stage. Many differentiation-related genes are highly upregulated in LC stage, with lesser increases observed for SN KC cultures. These differentiation-related genes include: SPRR1A and SPRR1B, envoplakin, involucrin, keratin 15, profillagrin, transglutaminase, and keratin 1. However, other genes that may regulate differentiation were reduced in both LC as well as SN KC cultures relative to PR KCs including LAMC2, WNT7A, DKK1, LAMC2, and KRTHA4. Taken together, these results indicate that even under low calcium conditions, the onset of late confluency, as well as the replicatively SN state triggers many genes that can induce differentiation in epidermal KCs.

Differential Regulation of Genes in KCs Regulating Inflammation/Immune Responses

Figure 8 reveals the relative gene expression of numerous genes that may regulate KC-produced factors capable of influencing local and or systematic inflammatory reactions, as well as immune responses and tissue repair. Compared to the other gene expression profiles in which relatively similar trends were identified for LC KC cultures and SN KC cultures (e.g., genes regulating proliferation—Fig. 6; and genes regulating differentiation–Fig. 7), divergent patterns were apparent for this class of genes. Thus, with the exception of TRAIL, IL-8, Gro-β mRNA levels, which were increased by twofold in both LC as well as SN KCs relative to PR KCs, almost all of the other genes were reduced in the LC KC cultures, but increased in

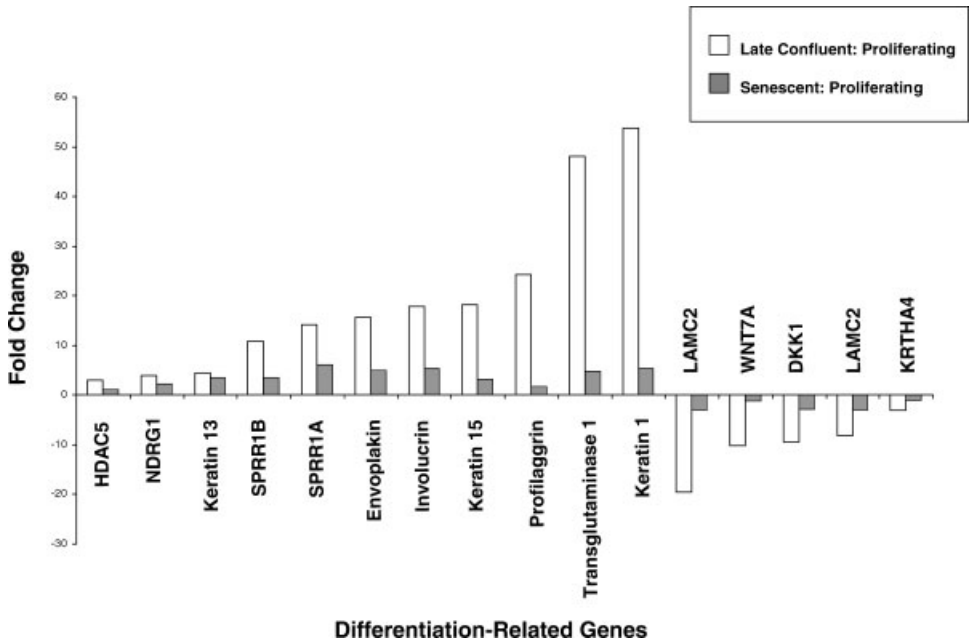

Fig. 7. Relative levels of genes regulating KC differentiation. For each gene of interest the ratio of the level of expression in PR KC cultures is compared to either LC KC cultures, or between PR KCs and KCs undergoing replicative senescence. Note the markedly enhanced levels of differentiation-related mRNA levels for LC KC cultures versus SN KCs when compared to PR KC cultures.

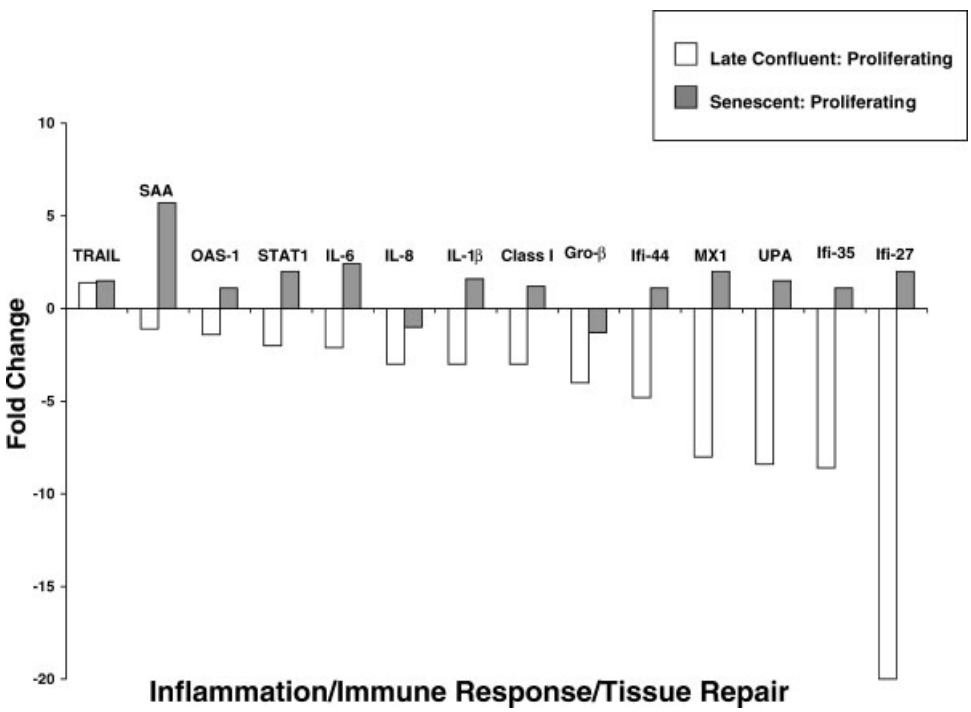

Fig. 8. Relative levels of genes expressed by KCs regulating inflammation and immune responses. For each gene of interest the ratio of the level of expression in PR KC cultures is compared to either LC KC cultures, or between PR KCs and KCs undergoing replicative senescence. Note the markedly decreased inflammation/immune response/tissue repair related mRNA levels for LC KC cultures verses SN KCs when compared to PR KC cultures.

the SN KC cultures. Examples of such divergent genes that were increased in SN KC cultures relative to PR KCs and LC KC cultures included: SAA, OAS-1, STAT1, IL-6, IL-1β, Class I MHC, IFI-44, MX1, UPA, IFI-35, and IFI-27. Taken together, these results suggest that the SN phenotype of KCs is linked to increased interferon-related signaling; whereas in LC KC cultures there may be inhibition of genes that are associated with inflammation and/or immune reactions.

Integration of Signaling Pathways in KC Cultures

In order to accommodate the complex aspects of mammalian functionality (multiple genes, splice variants, isoforms, protein complexes, protein families to name a few), we used a commercial software GeneGo database of novel architecture (see Materials and Methods). Unlike other integration platforms, which have molecular entities (genes, proteins) as principal objects, this system is pathway-centered. The complete set of interactions defines the molecular machinery of human cells: the almost infinite number of multi-step pathways and complexes. Only a fraction of the proteins are expressed and active at any given time, and only a small subset of possible connections is activated. Thus, as detailed below, the complete data set of the gene expression profiles for PR KCs versus LC KCs was subjected to this software-based analysis.

In GeneGo software, metabolic blocks (pathways and subsystems) corresponding to the genetic component of an organism are compiled and connected via intermediates into wire diagrams or reconstruction models [Ekins et al., 2005]. This database and software therefore help us to (1) query the underlying database, (2) generate gene networks using algorithms based on data sets and the manually curated database of human protein–protein and protein–compound interactions, and (3) upload and simultaneously visualize on the gene networks microarray or other high-throughput data. To create molecular pathways, the statistically significant genes from total raw signal intensity values were imported to GeneGo software for further analysis.

We examined pathways and key nodes associated with Drosophila Notch homolog in human skin. As mentioned in the Introduction, Notch receptor mediated signaling has been independently identified by several groups as a key regulatory pathway for KC differentiation [Rangarajan et al., 2001; Nickoloff et al., 2002b; Nicolas et al., 2003; Kolly et al., 2005]. For this purpose we utilized this software to identify closely associated nearest neighbor genes and networks for Notch. One such pathway map was identified and this map was built with already known genes such as HES, CBF-1, NUMB, Jagged-1, Jagged-2, Dsh-1, Dsh-2, and other. By incorporating our own microarray data to this pathway, we were able to create a visual diagram, which is depicted in Figure 9. We found that *Drosophila* Notch homolog is highly expressed and statistically significant in LC KC cultures compared to PR KC cultures. To further validate the microarray-based mRNA levels (Fig. 9A), the relative protein levels for activated Notch-1 (N^{IC}-1) were examined. Compared to PR KC cultures, an increase in N^{IC}-1 protein levels was present in confluent KC culture and N^{IC}-1 protein levels were reduced in SN KC cultures (Fig. 9B). Returning to the pathway analysis panel (Fig. 9C), when a gene is upregulated red color is assigned to a pathway node and blue color is given when a gene is downregulated. Since the initial analysis of differentiation-related gene expression profiles indicated the highest level of such genes was present in LC KC cultures (Fig. 7), the data analysis presented in Figure 9C confirms and extends a key role for Notch receptor pathways in regulating KC differentiation in this model system.

DISCUSSION

The precise molecular mechanisms regulating KC proliferation, differentiation, and senescence under physiological conditions is poorly understood [Nickoloff et al., 2002a]. Moreover, as the skin is constantly subjected to environmental insults over several decades by stress-inducing stimuli including solar irradiation, temperature fluxes, infectious agents, etc., this outermost protective coat is being studied to gain insight into age-related pathologies and senescence pathways [Campisi, 1998; Yaar and Gilchrest, 1998; Steinert, 2000]. Despite the ability to easily visualize the skin with the naked eye, and the immense cosmeceutical interest in reversing chronological and photo-ageing, defining the phenotype, and function of SN KCs (either replicative senescence or

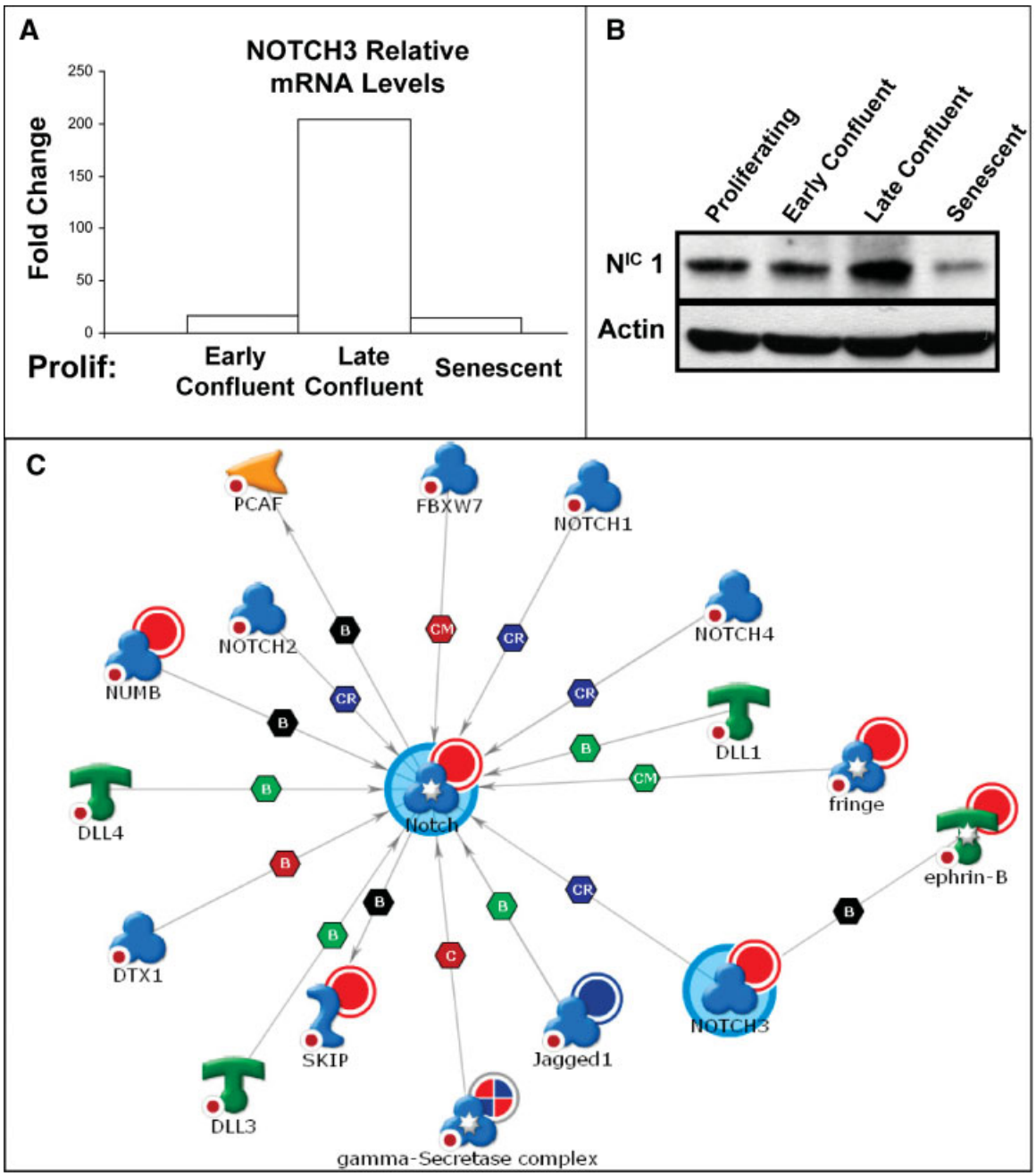

Fig. 9. Integration of gene expression profiling in KCs. **A**: Relative mRNA levels for Notch 3 are determined using PR KC cultures versus either EC, LC, or SN KC cultures. Note the strongest induction in Notch 3 mRNA levels was present when KCs underwent late stage confluency. **B**: Relative protein levels for activated Notch 1 receptor; N^{IC}-1 (97 kd) in the induced KC cultures. Note the enhanced N^{IC}-1 levels in KCs derived from late stage confluency. **C**: Pathway analysis using mRNA data derived from both PR versus LC KC cultures. Under these conditions many differentiation-induced genes are induced, and the pathway analysis reveals a central role for Notch related signaling.

accelerated senescence) is only in its infancy from a biological perspective [Gilchrest, 2003].

Progress in this field has been hampered by the lack of a transcriptional profile of SN KCs, and in this report we have compared and contrasted gene expression profiles for PR KCs versus KCs undergoing accelerated senescence (LC cultures) and replicative senescence. Several key observations and conclusions can be derived from these investigations. First, excellent concordance was observed amongst distinct population of KCs derived from different

foreskins. Thus, both replicates from cultures derived from two different foreskins displayed tight clustering amongst the PR KC population, as well as for KCs undergoing early confluence, late confluence, or replicative senescence (Fig. 3). Second, while the PR KCs resembled EC KCs in their overall gene expression profile (Figs. 2 and 3), KC cultures of LC KCs expressed a significantly different pattern of gene expression. These results indicate KCs undergoing accelerated senescence activate a gene expression profile that distinguishes them from PR KCs or KCs initially undergoing a confluent state. Third, by comparing LC KCs with KCs undergoing replicative senescence, it became clear that accelerated senescence was also characterized by a distinct transcriptome from the replicative senescence progress in human KCs (Figs. 3 and 4). To validate the relative levels of gene expression as detected by the Affymetrix microarray-based assays, we utilized quantitative RT-PCR for eight different genes that were differentially up or downregulated amongst various KC cultures (Fig. 5). Based on the qualitatively similar relative expression values comparing quantitative RT-PCR with microarray-based data, these validation results led to further exploration of genes that influence KC behavior. By probing into molecular pathways of relevance to the biology of KCs several other noteworthy observations could be added to this list.

Fourth, the growth arrest state induced by late confluency or replicative senescence tended to display a shared pattern of genes regulating cell cycle kinetics in which numerous genes coding for proteins necessary to promote mitosis were downregulated (Fig. 6). Fifth, by contrast to downregulation of cell cycle-related genes, KCs undergoing either accelerated senescence or replicative senescence, displayed increases in a similar set of genes that induce differentiation (Fig. 7). Even though the human KCs were maintained in a submerged state as monolayer cultures using low calcium, serum free medium, when the KCs achieved late confluency or replicative senescence, many differentiation markers were induced. These findings confirm and extend earlier studies and highlight the strong propensity within KCs to activate genes involved in the terminal differentiation process [Eckert et al., 1997b; Chaturvedi et al., 1999; Mehic et al., 2005].

Sixth, the SN KC cultures were associated with an elevation in many genes linked to interferon signaling, and hence could be expected to promote inflammation and/or immune reactions (Fig. 8). It was this set of genes in which the greatest discordance was observed when comparing LC to SN KC cultures reflecting the global gene analysis as summarized in Figure 4. From an immunobiological perspective that data are consistent with the view that the SN state may be linked to enhanced or persistent interferon signaling in KCs maintained under these experimental conditions. It is also tempting to speculate that the increase in autoimmune skin diseases observed with increasing age could reflect this genetic program for KCs undergoing replicative senescence.

Seventh, the signaling pathway analysis designed to probe into distinct regulatory components identified a key role for Notch receptors activated when PR KCs underwent late confluency (Fig. 9). During the late confluency stage numerous differentiation-related genes were identified (Fig. 7), and the identification of enhanced Notch 3 at the mRNA level and activated Notch 1 at the protein level reinforce a critical role for Notch signaling and KC differentiation. Since activated Notch-1 receptor signaling has been identified from in vivo and ex vivo organotypic models in regulating KC differentiation, the current results confirm that Notch receptor signaling is also playing a central role in the differentiation process for post confluent KC cultures [Nickoloff et al., 2002b; Nicolas et al., 2003; Kolly et al., 2005]. Future studies are indicated to follow-up on the transcriptome defined for the various human KC cultures by exploring the transcriptome of normal human skin under both physiological and pathological conditions. The coordinated gene expression profile derived from such in vivo studies is likely to shed new insights into the molecular mechanisms regulating tissue homeostasis, as well as identifying novel targets for therapeutic consideration in skin diseases with altered barrier function and/or for aging skin related abnormalities [Sra et al., 2005].

Obviously, much more biological study both in vitro and in vivo are required before any definitive conclusions can be drawn from the data presented herein. However, the differential gene expression profiles that characterize

PR versus LC, and SN KC cultures provide an experimental basis for such studies and offer intriguing insights into the behavior of human KCs that may contribute to advancing our knowledge of many physiological as well as pathological skin disorders [Campisi, 2005].

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REFERENCES

Arany I, Tyring SK, Brysk H, Brysk MM. 1998. Induction by interferon-gamma of its receptor varies with epithelial differentiation and cell type. Arch Dermatol Res 290: 331–334.

Barker JN, Mitra RS, Griffiths CE, Dixit VM, Nickoloff BJ. 1991. Keratinocytes as initiators of inflammation. Lancet 337:211–214.

Brattsand M, Egelrud T. 1999. Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J Biol Chem 274:30033–30040.

Campisi J. 1998. The role of cellular senescence in skin aging. J Investig Dermatol Symp Proc 3:1–5.

Campisi J. 2005. Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors. Cell 120: 513–522.

Candi E, Schmidt R, Melino G. 2005. The cornified envelope: A model of cell death in the skin. Nat Rev Mol Cell Biol 6:328–340.

Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ. 1999. Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem 274: 23358–23367.

Chaturvedi V, Bacon P, Bodner B, Nickoloff BJ. 2004a. Proliferating cultured human keratinocytes are more susceptible to apoptosis compared with mouse keratinocytes. J Invest Dermatol 123:1200–1203.

Chaturvedi V, Qin JZ, Stennett L, Choubey D, Nickoloff BJ. 2004b. Resistance to UV-induced apoptosis in human keratinocytes during accelerated senescence is associated with functional inactivation of p53. J Cell Physiol 198:100–109.

Eckert RL, Crish JF, Banks EB, Welter JF. 1997a. The epidermis: Genes on genes off. J Invest Dermatol 109: 501–509.

Eckert RL, Crish JF, Robinson NA. 1997b. The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol Rev 77:397–424.

Eckert RL, Broome AM, Ruse M, Robinson N, Ryan D, Lee K. 2004. S100 proteins in the epidermis. J Invest Dermatol 123:23–33.

Ekins S, Kirillov E, Rakhmatulin EA, Nikolskaya T. 2005. A novel method for visualizing nuclear hormone receptor networks relevant to drug metabolism. Drug Metab Dispos 33:474–481.

Gandarillas A. 2000. Epidermal differentiation, apoptosis, and senescence: Common pathways? Exp Gerontol 35: 53–62.

Gandarillas A, Goldsmith LA, Gschmeissner S, Leigh IM, Watt FM. 1999. Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. Exp Dermatol 8:71–79.

Gilchrest BA. 2003. Skin aging 2003: Recent advances and current concepts. Cutis 72:5–10.

Kirkwood TB. 2005. Understanding the odd science of aging. Cell 120:437–447.

Kolly C, Suter MM, Muller EJ. 2005. Proliferation, cell cycle exit, and onset of terminal differentiation in cultured keratinocytes: Pre-programmed pathways in control of C-Myc and Notch1 prevail over extracellular calcium signals. J Invest Dermatol 124:1014–1025.

Koster MI, Huntzinger KA, Roop DR. 2002. Epidermal differentiation: Transgenic/knockout mouse models reveal genes involved in stem cell fate decisions and commitment to differentiation. J Investig Dermatol Symp Proc 7:41–45.

Kulaeva OI, Draghici S, Tang L, Kraniak JM, Land SJ, Tainsky MA. 2003. Epigenetic silencing of multiple interferon pathway genes after cellular immortalization. Oncogene 22:4118–4127.

Laduca JR, Gaspari AA. 2001. Targeting tumor necrosis factor alpha. New drugs used to modulate inflammatory diseases. Dermatol Clin 19:617–635.

LaFleur DW, Nardelli B, Tsareva T, Mather D, Feng P, Semenuk M, Taylor K, Buergin M, Chinchilla D, Roshke V, Chen G, Ruben SM, Pitha PM, Coleman TA, Moore PA. 2001. Interferon-kappa, a novel type I interferon expressed in human keratinocytes. J Biol Chem 276: 39765–39771.

Mehic D, Bakiri L, Ghannadan M, Wagner EF, Tschachler E. 2005. Fos and jun proteins are specifically expressed during differentiation of human keratinocytes. J Invest Dermatol 124:212–220.

Murphy GF, Korngold R. 2004. Significance of selectively targeted apoptotic rete cells in graft-versus-host disease. Biol Blood Marrow Transplant 10:357–365.

Nickoloff BJ, Nestle FO. 2004. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. J Clin Invest 113:1664–1675.

Nickoloff BJ, Qin JZ, Chaturvedi V, Bacon P, Panella J, Denning MF. 2002a. Life and death signaling pathways contributing to skin cancer. J Investig Dermatol Symp Proc 7:27–35.

Nickoloff BJ, Qin JZ, Chaturvedi V, Denning MF, Bonish B, Miele L. 2002b. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ 9:842–855.

Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F. 2003. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 33:416–421.

Norsgaard H, Clark BF, Rattan SI. 1996. Distinction between differentiation and senescence and the absence of increased apoptosis in human keratinocytes undergoing cellular aging in vitro. Exp Gerontol 31:563–570.

Qin JZ, Chaturvedi V, Bonish B, Nickoloff BJ. 2005. Avoiding premature apoptosis of normal epidermal cells. Nat Med 7:385–386.

Quinn AM, Brown K, Bonish BK, Curry J, Gordon KB, Sinacore J, Gamelli R, Nickoloff BJ. 2005.

Uncovering histologic criteria with prognostic significance in toxic epidermal necrolysis. Arch Dermatol 141: 683–687.

Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP. 2001. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 20:3427–3436.

Rosenthal DS, Steinert PM, Chung S, Huff CA, Johnson J, Yuspa SH, Roop DR. 1991. A human epidermal differentiation-specific keratin gene is regulated by calcium but not negative modulators of differentiation in transgenic mouse keratinocytes. Cell Growth Differ 2:107–113.

Sesto A, Navarro M, Burslem F, Jorcano JL. 2002. Analysis of the ultraviolet B response in primary human keratinocytes using oligonucleotide microarrays. Proc Natl Acad Sci USA 99:2965–2970.

Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. 1999. Microarray analysis of replicative senescence. Curr Biol 9:939–945.

Sra KK, Babb-Tarbox M, Aboutalebi S, Rady P, Shipley GL, Dao DD, Tyring SK. 2005. Molecular diagnosis of cutaneous diseases. Arch Dermatol 141:225–241.

Steinert PM. 2000. The complexity and redundancy of epithelial barrier function. J Cell Biol 151:F5–F8.

Yaar M, Gilchrest BA. 1998. Aging versus photoaging: Postulated mechanisms and effectors. J Investig Dermatol Symp Proc 3:47–51.