Role of INK4a/Arf Locus-Encoded Senescent Checkpoints Activated in Normal and Psoriatic Keratinocytes

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During malignant transformation in skin, epidermal keratinocytes (KCs) frequently acquire the capacity to by-pass cellular senescence, a response that normally limits their unrestricted proliferation. Despite growing interest in the role for senescence during aging of skin and cutaneous carcinogenesis, little is known regarding regulation of three proteins encoded by the INK4a/ARF locus (p12, p14^{ARF}, p16) in KCs. In this study, several molecular pathways are explored using cultured KCs and KCs freshly isolated from psoriatic plaques. p16 and p14^{ARF} are predominantly expressed spontaneously when foreskin-derived early-passage KCs undergo confluency-induced premature senescence. Induction of p14^{ARF} on confluency occurred with low E2F-1 levels. Suspension of KCs in methylcellulose induced p12 expression. Addition of various cytokines (interferon- γ , tumor necrosis factor- α) or a phorbol ester [12-O-tetradecanoylphorbol-13-acetate (TPA)] only induced p16, but not p14^{ARF}. Confluent KCs up-regulated Ras activity and the downstream signaling involving ERK. Addition of MAPK inhibitor blocked cytokine and TPA-induced p16 expression. Confluency and interferon- γ induced premature senescence and p16 expression was linked to induction of the transcription factor Egr-1. KCs derived from chronic psoriatic plaques were characterized by enhanced p16, p14^{ARF}, and p12 expression accompanied by elevated Egr-1 levels. These results demonstrate that multiple and highly divergent stimuli can trigger the senescent checkpoint in human KCs with differential regulation of p16, p14^{ARF}, and p12. Although abnormal mitogenic signaling by oncogenic Ras is generally cited as being responsible for induction of premature senescence, our findings indicate that a broader perspective is warranted, to include confluency and cytokine-/TPA-induced pathways for KCs. (*Am J Pathol* 2003, 162:161–170)

Cellular senescence involving skin-derived cells is under investigation because of links to aging and neoplasia.^{1–3} A role for *INK4a/Arf* in regulating replicative senescence in human keratinocytes (KCs) has been established by several groups,^{4,5} and by-passing onset of senescence by alteration in the *INKa/Arf* locus on chromosome 9p occurs in many cutaneous epithelial neoplasms.^{6–8} The *INK4a/Arf* locus encodes three distinct proteins produced by tissue-specific alternative splicing including p16, p14^{ARF}, and p12.⁹ Although the mechanism by which p16 influences tumor suppressors such as Rb and p14^{ARF} influences p53 are well-established,¹⁰ molecular pathways governing differential expression of p16 and p14^{ARF} by normal KCs remain relatively unexplored. Depending on growth conditions (feeder layers, defined medium,¹¹ or overexpression of Id-1,^{12,13}) the replicative capacity of human KCs can be enhanced with reduction in p16 levels, accompanied by a delay in the onset of senescence.

Previously, we observed that even early passage neonatal foreskin-derived KCs strongly up-regulated p16 expression and underwent irreversible growth arrest, hallmarks of premature senescence, on reaching confluency.¹⁴ To further investigate the molecular pathway contributing to p16 expression in human KCs, a number of experimental conditions were used. Several key mediators were selected based on results using other cell types, particularly skin-derived fibroblasts. Premature senescence in fibroblasts was observed after oncogenic Ras activation.^{15,16} Besides Ras itself, we also explored potential modulators of Ras by exposing KCs to either cytokines such as interferon (IFN)- γ , transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α , or phorbol ester [12-O-tetradecanoylphorbol-13-acetate (TPA)].^{17–20} Because the use of confluent cultures, or KCs acutely exposed to various cytokines or phorbol esters, can rapidly change the phenotype of KCs, the mechanisms involved are independent of telomere shortening that require many population doublings.¹¹

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Hence, these studies provide insight into the behavior of cells that simulate replicative aging, but under more physiological conditions. Moreover, given a previous suggestion that KCs contained within psoriatic plaques resemble cells undergoing a senescent switch,²¹ experiments were extended from *in vitro* conditions to include *in vivo* samples. Psoriasis is a common and complex skin disease in which numerous signaling pathways are activated. Psoriasis was also examined because it arises within a defined cytokine network including IFN- γ and TNF- α ,²² and is accompanied by altered protein kinase C signaling.²³ Because Egr-1 can influence epithelial cell proliferation and protect cells from apoptosis, a role for Egr-1 in the onset of KC senescence was also explored.²⁴

Herein, we demonstrate that there are several pathways that can lead to the differential expression of either p16, p14^{ARF}, or p12 in KCs depending on the stimuli. Moreover, in psoriatic plaques not only is p16 overexpressed, but it is accompanied by p14^{ARF} and p12 expression. These results support the notion that cellular senescence involves complex patterns of p16, p14^{ARF}, and p12 expression by human KCs that can be regulated independently. Although the current experimental results encompass three different approaches (comparing KCs after various times in culture, the effects of cytokines and phorbol ester, and tissue-based studies of normal and diseased skin samples) they are all linked by their shared overexpression of proteins such as p16 encoded by the INK4a/Arf locus. Taken together the current data provides the basis for future studies aimed at preventing or reversing age-related cutaneous pathologies, including premature senescence, psoriasis, and skin cancer.

Materials and Methods

Cell Culture and Treatments

Primary KCs were isolated from freshly excised neonatal foreskin as described previously.¹³ KCs were maintained in a low calcium (0.15 mmol/L), serum-free growth medium (KGM; Clonetics, San Diego, CA) for optimal proliferation. When KCs began to become juxtaposed to each other, these cultures were deemed to be at an early stage of confluency. When parallel dishes were continually fed 3 days after the early stage of confluency, they were designated as late confluent cultures. In late confluent cultures >90% of the KCs were tightly crowded in the dish. Treatments to induce p16 included addition of: recombinant IFN- γ , as well as recombinant TNF- α (10³ units/ml; Genentech Inc., San Francisco, CA), TPA (5 nmol/L; Sigma Chemical Co., St. Louis, MO), and TGF- β (10 ng/ml; Upstate Biotechnology, Inc., Lake Placid, NY), in KGM for 24 to 72 hours. The PKC inhibitor GF 109203X (GF; bisindolymaleimide I), which is competitive for ATP and inhibits PKC activity, was purchased from Alexis Biochemicals (San Diego, CA) and used at 5 μ mol/L. The MEK inhibitor PD98059 was purchased from Sigma Chemical Co. and used at 50 μ g/ml.

Extraction of Proteins from Tissues

Tissues from normal human skin ($n = 5$) and untreated chronic psoriatic plaques ($n = 10$) were removed after obtaining informed consent and institution review board approval, suspended in CHAPS buffer [20 mmol/L HEPES, 140 mmol/L NaCl, 10 mmol/L CHAPS, 2 mmol/L ethylenediaminetetraacetic acid (EDTA)] supplemented with complete protease inhibitor cocktail (Roche Diagnostic Corp., Indianapolis, IN) and sonicated to homogeneity. The homogenate was microfuged for 10 minutes at 4°C and supernatants were frozen at -80°C until use.

Determination of Ras Activity

Ras activity was measured by a GST pull-down assay in which GST-Raf was used to pull down activated Ras (Upstate Biotechnology). In brief, cells were washed with phosphate-buffered saline (PBS) and lysed in 1 ml of lysis buffer (25 mmol/L Hepes, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mmol/L MgCl₂, 1 mmol/L EDTA), and a protease inhibitor cocktail (Boehringer, Ingelheim, Germany). Total protein (~500 μ g) was incubated overnight (at 4°C) with 10 μ g of the GST-Raf1 agarose conjugate. After washing the beads three times with the lysis buffer, bound GTP-Ras was released by boiling in 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer and the released proteins were analyzed by immunoblotting using monoclonal antibody to Ras (clone RAS10, Upstate Biotechnology).

Immunoblotting and DNA Binding

For immunoblotting, 30 μ g of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 6% and 10% polyacrylamide gels. Proteins were transferred onto Immobilon-P membrane (polyvinylidene difluoride), and blocked in 5% nonfat dry milk in TBST (40 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.01% Tween 20). Nuclear cell lysate or total cell lysate were prepared to detect different proteins as previously described.¹⁴ In brief, for nuclear lysates cells were washed with PBS, pelleted in buffer A (20 mmol/L Hepes, pH 7.9, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5 mmol/L dithiothreitol with 0.1% Nonidet P-40), incubated in ice for 15 minutes, and microcentrifuged, and the supernatant was discarded. The pellet was resuspended in buffer C (20 mmol/L Hepes, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride) for 15 minutes on ice. Lysates were vortexed and microcentrifuged, and the supernatants were saved (frozen at -80°C). For the total cell lysates, KCs were washed with PBS and were incubated in ice for 15 minutes in CHAPS buffer.¹⁴ Lysates were microcentrifuged, and supernatants were saved (frozen at -80°C). The protein concentration of each sample was determined by Lowry assays. Unless otherwise indicated,

equivalent protein loading was confirmed by Ponceau S staining of the gels as previously described.¹⁴

The following antibodies were used in this study: p16 (SC-468; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody recognizes both p16 and p12. We also used p12 antibodies generated to peptide (Ac-CNHRP-PPGDALGAWETKE). p53 (DO-1 (Oncogene Science, San Diego, CA), p14^{ARF} (SC-1063), Egr-1 (SC-110, Santa Cruz Biotechnology), anti-Ras (Upstate Biotechnology), and ERK were purchased from Cell Signaling (Beverly, MA), and anti-actin antibody was obtained from ICN Bio-medicals Inc. (Irvine, CA).

For DNA binding, nuclear extracts were prepared and used for electrophoretic mobility shift assay as previously described.²⁵ Double-stranded oligonucleotides were radioactively labeled by T4-kinase and purified on native 10% gels.²⁶ Binding reactions were performed for 30 minutes at room temperature in a buffer containing 10 mmol/L HEPES, 2.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 5% glycerol with a proteinase inhibitor cocktail (Boehringer). The sequence used for electrophoretic mobility shift assay was: Egr-1 5'-AACGGGGCGGGGCGGATTT-3'.

Reporter Assays

For the p16 reporter gene assay, KCs were cultured in six-well plates. At ~50 to 60% confluency, cells were transfected with 0.8 μ g of p16-luc reporter vector (Clontech Laboratories, Palo Alto, CA) and 0.2 μ g of pRL-TK plasmid DNA that contains *Reneilla* luciferase gene to normalize the transfection efficiency. The same amount of control vector in which the k enhancer was removed from p16-LUC was also used to co-transfect the cells.

DNA was transfected into KCs or Id-1-infected cells using Fugene-6 Transfection Reagent (Boehringer Mannheim) according to the manufacturer's protocol. After 24 hours of transfection, cells were pretreated with inhibitor for 2 hours and then TPA (100 nmol/L) for 24 hours. The preparation of cell lysate and luciferase activity measurements were made with Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. The sample was placed in a TD-20/20 luminometer (Clontech Laboratories) for detection of light intensity.

RNase Protection Assays

Total cellular RNA was extracted using Trizol Reagent (Life Technologies, Inc., Grand Island, NY). The RNase protection assay was performed according to the supplier's instructions (PharMingen, San Diego, CA). Briefly, human cell-cycle template set HCC-2 was labeled with α -³²P uridine triphosphate. RNA (10 μ g) and 8×10^5 cpm of labeled probes were used for hybridization, and after RNase treatment, protected probes were resolved on a 5% urea sequencing gel.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as previously described.⁹ Briefly, RNA was isolated from cultured KCs, normal skin, and psoriatic plaques with the following primer sequences used to detect p16 and p12: 5'-AAC GCA CCG AAT AGT TAC G-3' and 5'-TTC CCG AGG TTT CTC AGA G-3' (common for both p12 and p14^{ARF}; p14^{ARF}: 5'-CAT GGT GCG CAG GTT CTT-3'; actin: 5'-GAA ACT ACC TTC AAC TCC ATC-3' and 5'-CGA GGC CAG GAT GGA GCC ACC-3'.

Retroviral Vectors and Infection of Human KCs

The Egr-1 cDNA (kindly provided by Dr. Barbara Hoffman, Temple University, Philadelphia, PA) was subcloned into *Bam*HI and *Not*I sites of pMSVC vector. The pMSVC vector was kindly provided by Dr. A.G. Balliet (Temple University). The Phoenix-ampho retroviral packaging cells were obtained from American Type Culture Collection (Manassas, VA) with permission from Dr. Gary P. Nolan (Stanford University School of Medicine, Stanford, CA). The packaging cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and transfected with Egr-1/pMSVC or pMSVC+ vector using CaCl₂ and 2 $\times$ Hanks' balanced salt solution. After overnight incubation, the cells were fed with fresh medium and incubated at 32°C for an additional 24 to 48 hours. The supernatants containing the recombinant virus were collected for infection of KCs, which were seeded into six-well plates and infected with 300 μ l of viral supernatant in the presence of 4 μ g/ml of hexadimethrine bromide for 1 hour at 32°C, then the supernatant was removed and replaced with fresh medium, and incubated at 37°C in 5% CO₂ overnight as previously described.¹³ After being washed with PBS, the infected cells were treated with G418 for neomycin selection (purchased from Life Technologies, Inc.), propagated and harvested to detect p16 levels. Overexpression of Egr-1 was detected by Western blot analysis.

β -Galactosidase (β -Gal) Staining

Detection of senescence-associated β -Gal (SA- β -Gal) was performed by staining cultured KCs maintained in Lab-Tek chamber slides as previously described.²⁷

Results

KC Confluency-Induced Up-Regulation of p16 and p14^{ARF} Correlates with Activation of the Ras-ERK Pathway

Primary human KCs in culture exhibit limited proliferation potential and cease proliferation on confluence. Based on their morphology, these cells appear to have undergone senescence as previously described.¹⁴ By comparing proliferating KCs to cells undergoing early- and late-

Figure 1. Activation of cell-cycle regulators and Ras/Raf/ERK pathway in proliferating *versus* confluent KCs. Immunoblot showing the total levels of indicated cell-cycle regulators (**left**), as well as Ras and Ras activity (**right**), in proliferating as well as early and late confluent KCs.

stage confluency, the confluent KCs were characterized biochemically by the appearance of active (hypophosphorylated) Rb and decreased E2F-1 levels, with increases in cyclin D1, p27, p21, p16, and p14^{ARF} (Figure 1, left). No p12 was detected in either proliferating or confluent KCs (data not shown). The results portrayed in all figures are representative of one of at least three independent experiments using different KC cultures from separate donor specimens. Moreover, both protein and mRNA levels were obtained by harvesting parallel cultures at the same time for their respective Western blot and RPA analysis.

To elucidate the molecular mechanisms by which these cells activate a senescence program after a limited number of doublings in confluent culture, we initially determined whether Ras plays any role.^{28–30} As shown in Figure 1, right, protein levels and activity of Ras increased markedly after KCs reached confluence and stopped dividing as compared to proliferating cells. Downstream pathways related to Ras signaling were explored and only phosphorylated forms of Raf and ERK were increased on confluency, without detection of activated forms of either JNK or p38 (UV-light induces phosphorylation of proteins that can be detected under identical assay conditions; data not shown). These results suggest that the Ras/Raf/ERK pathway might be involved in confluency-induced up-regulation of p16 and p14^{ARF}. Because Ras is shown to positively regulate expression of p16 and p14^{ARF} in other cell systems, these observations raised the possibility that the activated Ras in confluent KCs also regulates the expression of p16 and p14^{ARF}.

To further elucidate the signaling pathways activated during confluency-induced senescence in human KCs, we compared the steady-state levels of RNAs, known to encode growth/senescence-inducing proteins, among proliferating, early-confluent, and late-confluent KCs. As shown in Figure 2, left, the steady-state levels of various transcripts encoding the growth-inhibitory and senescence-inducing proteins (for example, p130, pRb, p14^{ARF}, and p53) were higher in confluent cells than

proliferating cells. Interestingly, the levels of p16 RNA did not increase significantly, suggesting that the increase in the levels of p16 in confluent KCs involve posttranscriptional mechanisms. Moreover, consistent with the RNA data, the protein levels of p27, p21, and p14^{ARF} were also higher in confluent cells than proliferating cells with late confluent KCs expressing higher levels than early confluent KCs.

Further evidence to confirm that confluency induces a senescent state in human KCs was revealed by SA- β -Gal staining in which the majority of confluent, but not proliferating, KCs were positive (Figure 2, right).

Modulation of p16 Levels by Cytokines, TPA, and Inhibitors of Signaling Pathways

To elucidate the signaling pathways regulating the levels of p16 in KCs, we treated proliferating KCs with the indicated modulators of cell growth (for example, TNF- α , IFN- γ , TGF- β , and TPA), and inhibitors of signaling pathways (for example, PD98059 and GF). Figure 3A reveals the kinetic analysis by which the indicated cytokines and TPA enhanced levels of p16 in KCs. For all cytokine and TPA treatments, the appearance of elevated p16 levels were delayed, generally requiring 48 to 72 hours after initial exposure for clear-cut enhancement in levels compared to untreated KCs.

Because IFN- γ and TPA produce the greatest increase in p16 levels in KCs, further studies were performed on these two different stimuli. Figure 3B reveals the influence of IFN- γ or TPA on Ras signaling in proliferating KCs. Although total Ras protein levels did not change consistently, IFN- γ or TPA could both produce increased Ras activity (within 2 to 4 hours).

Pretreatment of KCs with TNF- α , IFN- γ , TGF- β , or TPA followed by incubation with a specific inhibitor of MEK (PD98059) prevented induction of p16, suggesting that in proliferating KCs induction of p16 is regulated by the signaling pathway involving MEK for all of these stimuli (Figure 3C).

When KCs are placed in suspension, marked induction of p12 is observed with a lesser increase in p16 (Figure 3D). Addition of the PKC inhibitor GF blocks this p12 and p16 induction, implying a role for PKC signaling in a suspension-induced appearance of p16 and p12.

To confirm and extend these protein measurements, functional studies using a luciferase reporter assay were performed. Figure 4 reveals that exposure of proliferating KCs to IFN- γ , TPA, or TNF- α activated the p16 promoter activity (Figure 4, left). Relative to untreated proliferating KCs, exposure for 6 hours to IFN- γ , TPA, or TNF- α increased p16 promoter activity by several fold depending on the stimulus based on luminometry. No significant promoter activity was detected using vector-only controls (data not shown). Furthermore, the presence of the MEK inhibitor (PD98059) or the PKC inhibitor (GF) inhibited the TPA-mediated p16 reporter activity (Figure 4, right). Thus not only are p16 protein levels induced by these stimuli, but such increases are associated with enhanced acti-

Figure 2. Effect of confluency on mRNA levels for cell-cycle regulators. RNase protection assays for proliferating, early confluent, and late confluent KCs (**left**). Staining to detect SA-β-Gal expression in proliferating and confluent KCs. Note the enhanced expression of the senescent biomarker in the confluent KCs.

vation of the p16 promoter as reflected by these functional results.

Role for Egr-1 in Confluency and IFN-γ-Induced Senescence

To explore possible transcription factors that could influence p16 promoter activity, several different studies were performed focusing on Egr-1. Although proliferating KCs have low to undetectable nuclear levels of Egr-1, when KCs achieve either early or late confluency, or 24 hours after addition of IFN-γ, Egr-1 levels are increased (Figure 5A, left). DNA-binding assays confirm increased Egr-1 binding in KCs at confluency or after IFN-γ treatment (Figure 5A, right).

When proliferating KCs were infected with a retrovirus containing Egr-1, there was abrupt growth arrest (data not shown), accompanied by elevated p16 levels and increased p16 promoter activity (twofold; Figure 5B). Moreover, the morphology changed dramatically with small cuboidal rapidly proliferating KCs infected with the empty (control) retrovirus being converted to enlarged

and flattened cells with vacuoles in KCs with forced over-expression of Egr-1 (Figure 5C).

Dissection of Senescent-Related Signaling Pathway in Vivo: Psoriatic Plaques

To move beyond the *in vitro*-based results, tissue samples from psoriatic plaques were examined for the same molecular mediators described in the earlier sections. Compared to all five normal healthy skin samples examined, several psoriatic plaques contained KCs with elevated levels of p16, p14^{ARF}, and p12 (Figure 6A). Of the 10 psoriatic samples examined, 8 were found to contain elevated levels of p12, 6 contained elevated p14^{ARF}, and 4 contained elevated p16 levels. In addition, Ras signaling mediated by phospho-ERK was also identified in psoriatic plaques (Figure 6A). By examining RNA derived from these skin samples, using RT-PCR the levels of various transcripts were elevated in psoriatic plaques compared to normal skin including p12, p14^{ARF}, and p16 (Figure 6, B and C). Although no transcripts were detected in any of the RNA extracted from five normal skin samples examined, positive RT-PCR signals were identi-

Figure 3. Composite profile of Western blot analysis for p16 before and after exposure of proliferating KCs to anti-proliferative reagents (TNF- α , IFN- γ , TGF- β , and TPA) for the indicated time intervals (**A**) and after 48 hours including pretreatment with the MEK inhibitor (PD98059) (**C**). **B:** Total Ras protein level and activity in KCs before and after exposure to TPA or IFN- γ . **D:** Western blot analysis of KCs before and after suspension in methylcellulose to detect p12 and p16 levels in the absence and presence of the PKC inhibitor, GF.

fied for p12 in 33% of psoriatic samples, with 88% of psoriatic samples positive for p14^{ARF} and 33% of psoriatic samples positive for p16.

Based on the *in vitro* results in which IFN- γ could induce Egr-1 and the important role IFN- γ plays in the

pathophysiology of psoriasis,²² we explored the potential role for Egr-1-mediated p16 expression in psoriatic plaques. Compared to normal human skin in which Egr-1 levels were undetectable, psoriatic plaques had increased Egr-1 levels (Figure 6A). These results indicate

Figure 4. Left: Luciferase-based reporter activity for p16 in cytokine- or TPA-treated KCs. Right: Influence of pretreatment using MEK inhibitor PD98059 or PKC inhibitor GF on TPA-induced p16 reporter activity. Proliferating KCs were examined 6 hours after treatment with either medium alone, or after exposure to the indicated stimuli.

Figure 5. **A, left:** Western blot analysis of Egr-1 expression in proliferating and confluent KCs, as well as proliferating KCs treated for 24 hours with IFN- γ . **A, right:** DNA binding assay reveals enhanced Egr-1 binding in late confluent and IFN- γ -treated KCs compared to proliferating KCs (cold oligo control, **first lane**). Western blot analysis of KCs infected with empty retroviral construct (PMSVC) and KCs infected with retroviral construct containing Egr-1. Note enhanced expression of Egr-1 and p16 in KCs infected with retrovirus PMSVC and Egr-1. **B, left:** Actin expression confirms equal loading. **B, right:** Reporter assay confirms enhanced p16 activity in KCs infected with retrovirus to force overexpression of Egr-1. **C:** Phase-contrast microscopic appearance of proliferating KCs infected with empty retrovirus *versus* retrovirus containing Egr-1. Note induction of senescent phenotype in KCs overexpressing Egr-1.

Figure 6. A: *In vivo* link to psoriasis. Western blot showing the senescence markers p16, p14^{ARF}, p12, as well as, phospho-ERK from a normal skin sample and two psoriatic tissue samples. Additionally, Egr-1 levels were overexpressed in psoriatic samples compared to normal skin. Actin levels confirm equal loading. **B** and **C:** RT-PCR results demonstrating presence of transcripts encoding: p12 (600 bp), p16 (379 bp), and p14^{ARF} (653 bp), in several different psoriatic plaques. M, Molecular weight markers; **lane 1**, PP skin-1 minus RT; **lane 2** is PP skin-1; **lane 3** is PP skin-2; actin confirms positive reaction conditions.

that a pathophysiological pathway resembling senescence may be activated in some psoriatic plaques.²¹ However, we have been unable to demonstrate consistent SA- β galactosidase positivity in the psoriatic lesions (data not shown). Thus, additional studies are required to more firmly establish the molecular mechanisms regulating senescence-like changes in psoriatic plaques.

Discussion

Understanding the molecular basis underlying the triggering and bypassing of senescence in KCs has implications for both normal aging of the skin, as well as for various pathological states including psoriasis and cutaneous neoplasms. In this study, multiple molecular pathways can be activated in KCs with differential induction of p12, p14^{ARF}, and p16. Thus, depending on the state of confluency, or exposure to cytokines or phorbol ester, KCs can respond by activating the Ras signaling pathway that culminates in onset of the senescent phenotype. Such a link between Ras/Raf and premature senescence is consistent with previous reports using other cell types such as fibroblasts.^{15,31,32}

One of the most striking findings in this report was activation of the Ras/Raf/MEK pathway by multiple stimuli including confluence, which was linked to expression of p16 in KCs. It appears that as soon as KCs begin to achieve confluency in culture, Ras is activated with downstream mediators including Raf and ERK, but not JNK or p38. These results are consistent with the notion that the confluency-induced resistance to UV light induced apoptosis,¹⁴ features a survival response mediated by ERK, rather than by the proapoptotic pathway mediated by p38 MAPK.^{30,33–36} This prominent activation of Ras signaling with the concomitant increase in cell-cycle regulators including p27, p21, p16, p14^{ARF}, as well as, induction of a senescent-like state (with SA- β -Gal) may also account for the irreversible growth arrest and inability to replat and restore proliferation in normal confluent KC cultures despite addition of fresh growth medium. Thus, even though neonatal foreskin-derived KCs have a relatively impressive replicative potential and long life span when passaged before achieving confluency (several weeks to months; greater than 20 population doublings), even passage 1 or passage 2 KCs undergo premature senescence by allowing them to become confluent. Such a relatively simple *in vitro* model may facilitate

progress understanding a telomere length-independent mechanism for premature aging and accelerated senescence related to skin biology and behavior of epidermal-derived KCs.^{11,37,38}

Although others have previously established cell-type-specific roles for p16 and p14^{ARF} in the regulation of cell growth, this report presents novel data focusing on the convergence of various growth inhibitory conditions on KC expression of p12, p14^{ARF}, and p16. To our knowledge, the only report identifying p12 expression in human cells was confined to normal pancreas.⁹ Thus, the ability to induce p12 expression by suspension of cultured KCs in methylcellulose *in vitro*, and the expression of p12 in psoriatic plaques represents additional novel findings.

Even though regulation of mammalian cell division represents a highly complex process involving many different pathways, a growing body of literature points to a key role for proteins encoded by the INK4a/Arf locus. There are several unique aspects of this locus in that it encodes three distinct cell-cycle regulatory proteins using distinct exons and alternate splicing mechanisms. The precise signaling pathway and transcription factors responsible for mediating differential expression of p12, p14^{ARF}, and p16 remain to be elucidated although the current data establish roles for Ras/Raf/MEK and Egr-1 in this response involving KCs. In contrast to a previous study³⁹ in which elevated E2F-1 levels were linked to induction of p14^{ARF}, the current findings involving confluent KCs demonstrate that p14^{ARF} can also be induced in a cellular context in which E2F-1 levels are reduced.

As regards the differential patterns for p12, and p16 in psoriatic plaques, it is possible that such distinct profiles of gene expression may reflect a complex mixture of cytokines and PKC activators known to be present within the psoriatic cytokine network.²² Although quite speculative, because psoriasis represents an autoimmune disease, there may be circulating antibodies that inhibit RNA splicing capable of disrupting the splicing mechanism involved in production of p16.^{40,41} Efforts are currently underway to determine whether psoriatic sera contains such splicing inhibitors.

The current findings confirm and extend an earlier report demonstrating MAPK activation in psoriasis.⁴² We also identified elevated phospho-ERK in psoriatic plaques (Figure 6), but the concomitant overexpression of Egr-1 and the senescence-associated proteins and transcripts for p12, p14^{ARF}, and p16 indicate that KCs within psoriatic plaques may contain not only hyperproliferative cells, but also an additional population of KCs undergoing a senescent switch. Interestingly, another group provided preliminary evidence identifying increased Egr-1 levels in psoriatic epidermis.⁴³ Based on available data, we would propose the following mechanism to explain these observations: Initially, an influx of activated pathogenic T cells into pre-psoriatic skin would expose KCs to IFN- γ and other cytokines producing Ras/Raf/MEK and PKC activation, with induction of Egr-1 (and perhaps other transcription factors) that trigger the senescent phenotype. Egr-1 is a zinc-finger transcription regulator,⁴⁴ that can influence cellular proliferation and apoptosis in many cell types,²⁴ and has been implicated

in cutaneous wound repair.⁴⁵ Because overexpression in proliferating KCs produces premature or accelerated senescence, it is possible that Egr-1 overexpression in psoriatic plaques is contributing to the senescent switch and resistance of psoriatic plaques to apoptosis. Indeed, the induction of a senescent phenotype within the psoriatic plaque provides a biological explanation for the apparent paradox in which KCs within psoriatic plaques are simultaneously resistant to apoptosis, as well as being resistant to cellular transformation.²¹ This notion is supported by previous observations in which loss or dysregulation of Egr-1 expression has been linked to tumor formation in breast and skin carcinogenesis animal models.^{24,46}

In summary, our findings indicate that multiple stimuli can trigger activation of the p16/INK4a locus in human KCs. Despite the diversity of these stimuli ranging from confluency to cytokines and a phorbol ester, they share activation of Ras-mediated signaling with discrete profiles for this differential expression of p12, p14^{ARF}, and p16. Although many mechanistic details remain to be determined to elucidate the molecular basis for differential gene expression, it appears that KCs in culture and *in vivo* (psoriatic plaques) represent excellent models to further our understanding of skin aging.¹

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