Histological and proteomic analysis of reversible H-RasV12G expression in transgenic mouse skin

Won-Jun Oh, Vikas Rishi, Steven Pelech1,2 and Charles Vinson

Laboratory of Metabolism, National Cancer Institute, Centre for Cancer Research, National Institutes of Health, Building 37, Room 3128, Bethesda, MD 20892, USA; 1Kinexus Bioinformatics Corporation and the 2Department of Medicine, University of British Columbia, Vancouver, British Columbia V6T1Z3, Canada

To whom correspondence should be addressed. Tel: +301 496 8753; Fax: +301 496 8419; Email: vinsonc@de37a.nci.nih.gov

We have used a two-transgene tetracycline system to reversibly express oncogenic H-RasV12G in mouse skin and primary keratinocytes culture using the bovine keratin 5 promoter. Induction of H-RasV12G expression in skin at 30 days after birth causes epidermal basal cell hyperplasia, an eruption of keratinous cysts and loss of hair follicles by 3 weeks. Subsequent H-RasV12G de-induction for 3 days results in massive apoptosis in the non-H-RasV12G-expressing stroma as well as in the suprabasal cells of the epidermis. Several procaspases such as CASP3, 1a, 5 and 12 disappeared, whereas the pro-apoptotic proteins AIF, Bax and Fas ligand were induced in H-RasV12G-de-induction skin. This process is followed by a wave of cell division at 14 days as hair follicles regrew, returning to near normal histology and skin appearance by 30 days. Using KinetworksTM multi-immunoblotting screens, the phosphorylation status of 37 proteins and expression levels of 75 protein kinases in the skin were determined in three samples: (i) wild-type skin, (ii) hyperplastic H-RasV12G-expressing skin and (iii) skin where H-RasV12G expression was suppressed for 7 days. Following H-RasV12G induction, 16 kinases were increased over 2-fold, and 2 kinases were reduced over 50%. This included increased phosphorylation of both known downstream H-RasV12G targets and unknown H-RasV12G targets. After H-RasV12G suppression, many but not all protein changes were reversed. These results from skin and primary keratinocytes are organized to reflect the molecular events that cause the histological changes observed. These proteomic changes identify markers that may mediate the oncogenic addiction paradigm.

Introduction

Expression in transgenic mice of either oncogenic H-Ras or K-Ras results in both cellular proliferation and tumorigenesis (1) producing a variety of mouse models for human disease including follicular adenoma (2), melanoma (3) and Costello syndrome (4). Several investigators have constitutively expressed Ras or its oncogenic forms in the skin and observed both hyperproliferation of the epidermis and subsequent formation of squamous cell carcinomas (5), demonstrating that activated Ras is sufficient to produce a malignant phenotype. Expression of oncogenic Ras in different compartments of the skin produces different results. Suprabasal epithelial cell expression using the keratin 10 promoter causes hyperplasia and papillomas that slowly progress into carcinomas (6), whereas expression in the hair follicle compartment that contains skin stem cells using the keratin 5 promoter caused papillomas that readily convert into carcinomas (5).

Mutations in each Ras gene have been detected at high frequency in many human cancers, with frequencies ranging from 10 to 90% (7), with different cancers types having reproducible mutations in specific Ras genes. Mouse models of skin chemical carcinogenesis indicate that oncogenic mutations in Ras play a central role in skin tumor development (8), suggesting that the oncogenic properties of Ras are similar in mice and humans.

There are three Ras proto-oncogenes in mammals (1) that have GTPase activity and function to activate kinases (9) whose downstream targets are transcription factors culminating in changes in gene expression (10). Oncogenic Ras is able to transform cells in culture by both promoting cell growth and inhibiting anokis (11), a term for cell death induced in tissue culture when cells fail to make proper cell–cell and cell–stromal interactions (12). The inhibition of anokis is critical for the ability of Ras-transformed cells to grow in soft agar (13).

Ras-signaling pathways underlie cellular transformation as demonstrated by transfection of either Ras or its downstream targets (14). However, in epidermal keratinocytes, conflicting data have been presented, indicating that Ras promotes proliferation and suppress differentiation (15) or does the opposite, such as cell cycle arrest and induction of apoptosis (16).

More recently, oncogenic H-RasV12G has been reversibly expressed in melanocytes to show that it is continually needed to maintain the malignant phenotype (17). Similar observations were made for the reversible expression of Myc (18) and a mutant epidermal growth factor (EGF) receptor (19). These observations have been conceptualized to suggest that cancer cells develop a dependence or “addiction” to the continued activity of over-expressed oncoproteins for maintenance of their malignant phenotype (20,21). However, the molecular correlates of this poorly understood phenomenon have not been reported.

To identify such molecular correlates, we have reversibly expressed H-RasV12G in the basal layer of the mouse epidermis using the K5 promoter. Here we show that the expression of H-RasV12G induces hyperplasia in the skin by 3 weeks and subsequent de-induction results in a return to normal skin and histology by 30 days. To help determine the mechanism of tumor regression, we have examined changes in the concentration of over 100 phosphoproteins and proteins that occur in the skin when H-RasV12G expression is activated and subsequently suppressed. The reversibility by H-RasV12G induction and de-induction was also observed in primary keratinocytes. These proteomic changes that occur as H-RasV12G is de-induced may be potential therapeutic targets.

Experimental procedures

Transgenic mice and cell culture

The transgenic mouse containing the tetracycline transactivator under the control of the bovine K5 promoter (K5-tTA) was a kind gift from Dr Adam Glick (22). The tetO-H-RasV12G transgenic mouse (17) was obtained from NCI Mouse Repository (Frederick, MD). Offspring from the cross between K5-tTA and tetO-H-RasV12G mice were genotyped using the polymerase chain reaction primers for H-RasV12G transgene: 5'-GGTCCACTTGGCATATTAGG and 5'-GCCGCGGATCATCCAGGATGTCACAC, and for tTA transgene: 5'-AACAACCCGTAAA-CTCCGCC and 5'-GCAACCTAAGTAAA-GCCCCC.

Primary keratinocytes were isolated from wild-type or K5-tTA: tetO-H-RasV12G (K5-H-RasV12G) transgenic newborn littermates (1 day old) as described (23).

Regulated H-RasV12G expression in mouse skin

To generate K5-H-RasV12G double-transgenic mice, heterozygous K5-tTA mice were crossed to homozygous tetO-H-RasV12G mice in the presence of 200 mg/kg doxycycline (Dox) feed (BioServ, Baltimore, MD). To induce H-RasV12G expression, 30-day-old mice were moved from Dox food. After 3 weeks when K5-H-RasV12G mice showed a phenotype, they were fed food containing 200 mg/kg Dox to suppress expression of the H-RasV12G transgene.

Abbreviations: Dox, doxycycline; VEGF, vascular endothelial growth factor.
Skin tissues were fixed in 10% neutral buffered formalin (NBF) overnight, transferred to 95% EtOH and embedded in paraffin. Six micrometer sections were cut and stained with hematoxylin and eosin. To label dividing cells, mice were sacrificed 60 min after a single intraperitoneal injection of a sterile solution of BrdU (Sigma, Saint Louis, MO; 10 mg/ml in phosphate-buffered saline, 50 mg/kg of body weight). Skin sections were treated for 30 min at 37°C with 2N HCl in phosphate-buffered saline containing 0.5% Triton X-100, rinsed in sodium tetaborate buffer (0.1 M, pH 8.5) and stained using an anti-BrdU antibody (DAKO diagnostics, Angel Drove Ely, UK). Apoptosis was determined in tissue sections using an ApopTag kit (Chemicon, Temecula, CA 92590).

**Western blot analysis**

Protein extracts were made from whole skin or primary keratinocytes using RIPA lysis buffer containing 1% sodium dodecyl sulfate, 1% Nonidet P-40 and 0.5% sodium deoxycholate and cocktail of protease inhibitors. Western blots of lysates were performed on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with α-146 anti-H-Ras monoclonal antibodies (Quality Biotech, Lamden, NJ), anti-Bax polyclonal antibodies and anti-vascular endothelial growth factor (VEGF) polyclonal antibodies (Santa Cruz Biotechnology), differentiation-related antibodies (K1, K10, involucrin and loricrin; Covance, Richmond, CA), anti-Protein kinase C α and anti-phospho-PKCα (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using the enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ).

**Immunohistochemistry**

Serial 6 μm thick sections were prepared and immunohistochemistry was done using the ABC and DAB kits from Vector Laboratories (Burlingame, CA). Skin was immunostained with goat anti-CD31 (1:200; Santa Cruz Biotechnology) and rabbit anti-cytokeratin antibodies (1:2000; DAKO diagnostics). Histological sections were photographed using an Olympus AX70 microscope (Olympus Optical Center Valley, PA) with analysis software (Soft Imaging System, Center Valley, PA).

**Apoptosis, protein kinase and phosphoprotein screening**

Whole-skin lysate from mice (0.5 mg) was prepared as described previously (24). The Kinoworks™ apoptosis (KAPS-1.0), phosphosite (KPSS-1.3) and protein kinase (KPKS-1.2) screens were performed by Kinexus Bioinformatics (Vancouver, BC) (25).

**Results**

Regulated expression of activated H-RasV12G in the skin basal epidermis was achieved by producing a mouse containing two transgenes. One transgene expresses the tetracycline transactivator under the control of the bovine keratin 5 gene promoter (K5-tTA) that is active in the basal epidermis of the skin both in utero and in the adult. The second transgene contains the oncogenic H-RasV12G gene driven by a minimal promoter containing seven DNA-binding sites for the tet- and tetO-H-Ras V12G mice did not produce any double-transgenic but none of the single-transgenic mice (either K5 or tetO-H-RasV12G mice) developed a wide variety of epithelial alterations, ranging from benign skin hyperplasia to dysplasia, alopecia, keratinous cysts (Figure 1B and 2A) and swelling of the salivary glands (data not shown) that are consistent with other groups findings (5,26). However, we did not observe any macro abnormality of other organs (i.e. oral mucosa, tongue, esophagus, uterine cervix and fore stomach) as reported by Vitale-Cross et al. (5). Massive angiogenesis was also observed in the skin (Figure 1F). Twenty-one days after H-RasV12G expression was induced, expression was suppressed by using food containing 200 mg/kg Dox. This allowed us to examine the dependence of the skin phenotype on continual H-RasV12G expression. The expression of H-RasV12G protein was suppressed in the skin after 3 days (Figure 1A). After 10 days, mouse skin showed intermediate phenotype. Finally, the skin appearance returned to near normal after 30 days (Figure 1C–E). Compared with H-RasV12G-expressing skin (Figure 2A; left panel), dramatic histological changes in the skin were observed at day 3 after suppression of H-RasV12G expression (Figure 2B). Dissected skin expressing H-RasV12G for 3 weeks showed extensive angiogenesis.

**Histological analysis of skin during reversible H-RasV12G expression:**

Three types of histological analyses of the skin revealed cellular events involved in both the H-RasV12G-dependent skin hyperplasia...
and the subsequent regression following H-RasV12G de-induction. These analyses included: (i) hematoxylin- and eosin-stained sections to examine general tissue histology, (ii) BrdU labeling and immunohistological staining to identify cells undergoing cell proliferation and (iii) terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) analysis to identify cells undergoing apoptosis. The dorsal skin of H-RasV12G-expressing (Figure 2C–E) and wild-type (supplementary figure is available at Carcinogenesis Online) mice was examined at eight time points. Four time points were 0, 3, 7 and 21 days of H-Ras V12G expression. Four additional time points examined the consequences of H-Ras V12 suppression for 3, 7, 14 and 30 days after being expressed for 21 days. The quantification of cell growth and apoptosis during this time course is presented for both the K5:H-RasV12G and wild-type mice (Figure 2F and G).

The induction of H-RasV12G expression resulted in hyperproliferation of the epidermis that was evident as early as 3 days. At 7 day, the hyperplasia is more pronounced in both the basal layer of the epidermis and in the hair follicles (Figure 2C), something not observed in wild-type skin (supplementary figure is available at Carcinogenesis Online). At day 21, the epidermis was hyperplastic and developed keratinous cysts. BrdU-positive cells were abundant in basal cells and in cells of the cyst walls (Figure 2D). There were no apoptotic cells observed at either day 3 or 7 although there was a slight increase in TUNEL-positive cells at day 21 (Figure 2E).

The de-induction of H-RasV12G after 21 days of expression resulted in a dramatic shift from cell growth to apoptosis (Figure 2F and G). Three days after H-RasV12G de-induction, BrdU incorporation was not detected, whereas apoptosis, as evident by TUNEL staining, occurred...
primary in the stromal cells that do not express H-RasV12G. In some sections, the entire region of stroma underwent apoptosis. In addition, apoptotic cells also appeared in the suprabasal cells of the epidermis. On day 14 of H-RasV12G de-induction, apoptosis was lower while proliferating cells became detectable in the hair follicles. At this time, many of hair follicles were in the anagen phase as a part of skin recovery.

To identify if the apoptotic cells are epidermal in origin, serial sections of skin were stained using a pan-keratin antibody to detect epidermis cells and a CD31 antibody to detect endothelial cells. With 3 days of H-RasV12G de-induction, apoptotic cells were observed that are pan-keratin negative (Figure 3A and C; left panel). They are near CD31-positive cells adjacent to blood vessels (Figure 3A and B; left panel). In addition, VEGF protein was strongly expressed in H-RasV12G-expressing skin, but completely disappeared within one week of H-RasV12G de-induction (Figure 3D). The significant apoptotic rate of cells lining tumor vessels suggests that continued H-RasV12G expression is required for the critical host–tumor symbiotic interaction that sustains tumor vasculature and is consistent with the reports that K- and H-RasV12G can stimulate VEGF expression (27). Apoptotic cells were also detected in epidermal cells expressing keratin protein (Figure 3A and C; right panel).

**Increase in pro-apoptotic proteins without effecting the expression of differentiation markers in H-RasV12G-suppressing skin**

It has been reported previously that tumor regression upon oncogene de-induction is due to apoptosis and differentiation (28). To identify molecular pathways involved in the induction of apoptosis in skin after H-RasV12G de-induction, the Kinetworks’ Apoptosis Protein Screen (KAPS-1.0) that determines protein levels of 25 candidate apoptosis proteins (Figure 4) was used.

The induction of H-RasV12G for 3 weeks increased concentrations of several proteins including the procaspases (proCASP1, pro-CASP3, CASP5 int and proCASP12) and decreased concentrations of others including CytoC, Fas and CASP5 p20, a matured form of procaspase 5 (Figure 4D). The increase in procaspase level in H-RasV12G-expressing human ovarian cancer model has been described earlier (29).

When H-RasV12G is de-induced, the four procaspases that were most strongly induced by H-RasV12G expression decreased to below wild-type levels. In contrast, several proteins that are pro-apoptotic were induced including AIF, Fas and Fasl. (Figure 4D). Bax protein, which induces the releases of mitochondrial AIF and is a member of Bcl-2 protein family (30), increased over 3-fold in mice after H-RasV12G de-induction (Figure 4E).

To evaluate if the observed apoptosis coincided with cell differentiation as occurs in normal skin (31), we examined the levels of differentiation makers such as keratin 1, keratin 10, involucrin and loricrin. These markers increased with H-RasV12G expression but decreased after H-RasV12G suppression, indicating that the observed apoptosis was not part of the normal differentiation program (Figure 4F).

**Phosphoprotein changes in skin after reversible H-RasV12G expression**

Ras act via downstream effectors cascades to alter cellular growth and apoptosis (32). To identify kinases and protein phosphorylation changes that accompany H-RasV12G activation and subsequent suppression, we used four Kinetworks™ multi-immunoblotting screens to examine the phosphorylation of 37 proteins and expression levels of 75 protein kinases.

We first utilized a Kinetworks™ Phospho-Site Screen (KPSS-1.3) to identify changes in the phosphorylation status of 37 protein phosphorylation sites after H-RasV12G expression in the three skin samples described above (Figure 5A and C). These results are summarized in Figure 5D organized to show the most striking increases in protein phosphorylation at the top and the largest decreases at the bottom of the bar graph. It should be appreciated that the most reliable results are provided when the intensity of the enhanced chemiluminescence signals from the immunoblots are higher. Figure 5D reveals that several known downstream effectors of H-RasV12G including RSK1 (10-fold), STAT3 (9-fold) and MEK1 (3.5-fold) have increased phosphorylation levels following H-RasV12G expression. Other proteins, not previously associated with Ras activation, such as NRI and nucleo-prolin (B23), had over a 2-fold increase in their phosphorylation levels. Decreased phosphorylation is observed with adducin α and γ, CREB1, p38 mitogen activating protein (MAP) kinase and e-jun N-terminal kinase. cAMP response element binding protein (CREB) does not show increased phosphorylation even though it is a direct target of RSK1, which is induced 10-fold (33).

Following H-RasV12G de-induction, the phosphorylation states of many proteins returned to normal (Raf1, MEK1 and B23). However, several proteins had increased phosphorylation (adducinα/γ, S6Kα/2, PKCα/β/ε, GSK3α/β) and may mediate the increased apoptosis observed as the hyperplasic state reverts to a normal.

**Protein kinase changes in skin after reversible H-RasV12G expression**

To evaluate the consequences of H-RasV12G induction and de-induction on protein kinase expression levels, we employed a Kinetworks™
protein kinase screen, which detected 48 out of a possible 75 protein kinases in the mouse skin samples. Following H-RasV12G induction, 16 kinases increased over 2-fold, whereas only two decreased >2-fold. Seven kinases increased 3-fold or more following H-RasV12G expression (PAK3, CDK1/4, BMX, S6Kα/β, Csk, Raf1 and MEK1) (Figure 5E).

When H-RasV12G is de-induced, only three (Pak3, BMX and S6Kα/β) of the seven kinases that increase 3-fold revert to near normal, whereas CDK4 concentration actually increases. Several additional kinases also increase with H-RasV12G de-induction (ZIPK, CaMK4, CaMKK, focal adhesion kinase (FAK) and PKCδ/ε) (Figure 5E).

The protein kinase screen (Figure 5E) and the phosphoprotein screen (Figure 5D) contain signals for 16 common proteins allowing one to determine if the fraction of protein that is phosphorylated changes in the different samples. The stoichiometry of phosphorylation (the ratio of total phospho-site signal divided by the total protein signal) was substantially reduced with H-RasV12G de-induction for Raf1 (by 54%), MEK1 (by 76%), ERK2 (by 55%), GSK3β (by 54%) and PKCδ (by 55%). In contrast, H-RasV12G de-induction increases the stoichiometry of phosphorylation for S6Kα/β T389 (by >1000%) and Rsk 1/3 T359+S363/T356+S360 (by 330%), but there was a compensatory reduction in the total protein levels of these kinases at the same time.

Reversible morphological changes following H-RasV12G suppression in primary keratinocytes

To determine if the reversibility observed in adult mice skin after H-RasV12G suppression was also observed in cell culture, we prepared primary keratinocytes cultures from the K5:H-RasV12G newborn pups. Two days after induction of H-RasV12G expression in primary keratinocytes, distinct morphological changes were observed including swollen cytoplasm with vacuoles. Nine days of H-RasV12G expression causes dramatic morphological changes (Figure 6A; upper panel). These morphological changes were totally reversed within 7 days after suppression of H-RasV12G expression (Figure 6A; lower panel).

We also found that expression of H-RasV12G protein was dramatically decreased within 5 days after addition of Dox (Figure 6B). To confirm the skin-screening results, we examined the levels of PKCa in the keratinocytes. Compared with H-RasV12G-expressing keratinocytes, the phosphorylation of PKCa protein dramatically increased >5-fold on day 5 after H-RasV12G suppression and then decreased on day 7 (Figure 6B). However, compared with H-Ras-expressing keratinocytes, total PKCa protein level was not significantly different in the H-RasV12G-suppressed keratinocytes (Figure 6B).

**Discussion**

We present for the first time a histological and proteomic examination of the reversible expression of oncogenic H-RasV12G in the mouse epidermis under keratin 5 promoter control. This study shows that the H-RasV12G-induced epidermal hyperplasia is reversible after H-RasV12G de-induction, a process that involves apoptosis in both the stroma and the suprabasal cells of the epidermis. The massive induction of apoptosis in the stroma within day 3 of H-RasV12G suppression occurred near blood vessels in an “all or none” type pattern. These results indicate that H-RasV12G expression is needed in a non-cell-autonomous (paracrine) manner to maintain the non-H-RasV12G-expressing cells. Apoptosis also occurred in the suprabasal...
Fig. 5. Kinetworks™ KPSS-1.3 analysis of 37 phospho-sites in cell signaling proteins for three skin samples (A–C) described in Figure 4. The positions of detected target phosphoproteins are indicated with arrows. (D) The percent changes in expression for 17 detected target proteins with H-RasV12G expression (black bars) and H-RasV12G de-induction (white bars) are shown. Error bars indicate the standard error of the means from four separate experiments performed on different samples. (E) The percent change in concentration for 48 detected proteins in Kinetworks™ KPKS-1.2 analysis. Error bars indicate the standard error of the means from two separate experiments performed on different samples.
cells with individual cells being TUNEL positive. To initiate a molec-
ular characterization of this poorly understood phenomenon of cell
death induced by oncogene withdrawal, we have identified changes in
protein expression and phosphorylation status in the skin following
H-RasV12G de-induction. These proteins are potential biomarkers and
molecular mediators of oncogenic addiction.

Oncogenic dependence or oncogenic addiction is a term used to
decribe the observation that tumors initiated by oncogene expression
are dependent on their continual expression for tumor stability
(20,21). When the oncogene is de-induced, the tumors swiftly regress
mainly by apoptosis or differentiation. For example, H-RasV12G-
induced mouse melanomas regressed via apoptosis when H-Ras V12G
expression was suppressed (17) or partially regress when K-Ras is
suppressed in lung cancers (34). A similar reversal of the tumor phe-
notype via apoptosis is observed when the expression of the c-Myc
oncogene (18) or mutant EGF receptor (19) is de-induced. However,
this phenomenon has never been described in the epidermis.

The induction of apoptosis in the stroma where H-Ras V12G is not
expressed suggests a paracrine function for cells that express
H-RasV12G. One of these proteins could be the secreted VEGF that is
up-regulated by H-RasV12G induction in skin and substantially down-
regulated when H-RasV12G expression is suppressed (17) or partially regress when K-Ras is
suppressed in lung cancers (34). A similar reversal of the tumor phe-
notype via apoptosis is observed when the expression of the c-Myc
oncogene (18) or mutant EGF receptor (19) is de-induced. However,
this phenomenon has never been described in the epidermis.

Using the Kinetworks apoptosis protein screen (KAPS-1.0) (Figure 4),
expression profiles of number of apoptotic proteins were studied.
Several procaspases (1α, 3, 5 and 12) that were induced following
H-RasV12G induction decreased or disappeared in the skin after
H-RasV12G de-induction as the skin switches from cell growth to
apoptosis (Figure 4). Several pro-apoptotic proteins increased includ-
ing Bax and Apoptosis inducing factor (AIF) involved in the intrinsic
apoptosis pathway and FAS and FAS ligand involved in the extrinsic
apoptosis pathway (35). An increase in AIF level suggests that a cas-
pase-independent mechanism may also play a role to induce apoptosis.
However, unlike the previous reports showed that marked differenti-
ation in oncogene inactivation (18,36), H-RasV12G-suppressing skin
did not show any increase in differentiation (Figure 4), suggesting that
differentiation may not be a contributing factor in reversible phenotype.

The molecular changes that accompany tumor regression when the
activating oncogene is de-induced have not previously been de-
scribed. To begin to address this issue, we investigated protein
changes in the skin after H-RasV12G induction and subsequent
de-induction. We examined the phosphorylation status of 37 phospho-
proteins and the expression levels of 75 protein kinases. The proteo-
mic analysis examined the entire skin and describes a global change in
protein concentrations, whereas our histological analysis identified
apoptosis in two distinct cell populations following H-RasV12G sup-
pression, the stroma and the suprabasal epidermis. Future studies will
address where in the skin these proteomic changes occur.

Targets of H-RasV12G activity that induce cell proliferation are
readily identifiable in the hyperplastic skin. These include phosphor-
ylation of several well-known downstream effectors of Ras signaling
via Raf1, including Raf1 itself, MEK1, RSK1 and STAT3. There was
also a decrease in the phosphorylation of MEK6 and its target p38
MAP kinase as well as c-jun N-terminal kinase MAP kinase following

Fig. 6. Effect of H-RasV12G expression and suppression on primary keratinocytes morphology and expression profile of PKCα. (A) Primary keratinocytes in
which H-RasV12G protein was expressed for 2 days. After 2 days, H-RasV12G was suppressed or expressed for another 7 days and cell morphologies were monitored
by phase-contrast microscopy. (B) Time-dependent expression levels of H-RasV12G, phospho-PKCα and total PKCα protein.
H-RasV12G induction (Figure 5) consistently repression of the inhibitory roles that these protein kinases normally play in cell proliferation and their pro-apoptotic functions. In contrast, extracellular signal-regulated kinase phosphorylation was not changed much in H-RasV12G-induced skin (Figure 5D). These results have precedence. Mao et al. (37) have shown that phosphorylated extracellular signal-regulated kinase proteins are barely detectable in skin papilloma with the oncogenic H-Ras protein. We speculate that our results may indicate an imbalance of phosphotases and oncogene signaling. We also measured the expression levels of 25 different phosphatases using a Kinetworks® protein phosphatase screen (KPPS-1.2; unpublished data by Oh WJ et al.). H-RasV12G-induced a >2-fold elevation of the catalytic and regulatory A2 subunit levels of protein serine phosphatase 4 and the dual specificity MAP kinase phosphatase 2. With H-RasV12G suppression, there was a complete loss of protein serine phosphatase 4-A'2 expression and a reduction of protein serine phosphatase 4-C levels to even below the wild-type values. In contrast, H-RasV12G suppression also caused even more marked increases in the expressions of MAP kinase phosphatase 2. The finding that the concentrations of diverse protein phosphatases were also markedly altered with the gain of Ras function as well as its subsequent repression adds an extra dimension to the complexity in interpreting the observed changes in protein phosphorylation. The induction of the dual specificity MAP kinase phosphatase may partly account for why the phosphorylation levels of ERK1 and ERK2 were not significantly affected in the skin of H-RasV12G-expressing and repressed animals, despite the increases in expression and activating phosphorylation of the upstream kinase MEK1.

When H-RasV12G is de-induced, signaling pathways upstream of the apoptotic machinery (discussed above) also changes. These changes have previously been shown to have either anti-apoptotic or pro-apoptotic consequences. Anti-apoptotic signals are increased phosphorylation of the α and β subunits of S6K that increase ribosomal translation. An additional anti-apoptotic signal is the increase in FAK protein, a kinase involved in organizing stress fibers in cell culture (38).

Several pro-apoptotic changes are evident. These include decreased phosphorylation of Akt1, a survival factor, which has also been shown to respond to VEGF to promote endothelial cell survival (39), thus contributing to apoptosis in the stroma. In addition, an increase in p38 MAP kinase phosphorylation level (40) and ZIP kinase protein after H-RasV12G de-induction is consistent with previously reported work (41).

Proapoptotic signals that increase include phosphorylation of PKCaβ and PKCe. PKCs promote apoptosis in human gastric cancer cells (42) and mouse keratinocytes (43). Our studies indicate PKCα activation after H-RasV12G suppression could also contribute to cell killing that is often required before the recovery process. The result showing that the phosphorylation of PKCα decreased in H-RasV12G-expressing skin (Figure 5D) was consistent with the previous reports that down-regulation of PKC protein level and activity are seen in cells expressing activated Ras genes (44–48). “Adducin γ/γ that are the targets of phosphorylated PKC also increases, leading to waning of cell-cell interactions and apoptosis 49.” Furthermore, the phenotypic reversibility and an increase in PKCa phosphorylation also occurred in the primary keratinocytes system after H-RasV12G suppression (Figure 6), indicating that it is a cell-autonomous property. More work will be needed to determine if these pathways are active in apoptotic cells. Interestingly, in primary keratinocytes cell culture (Figure 6), the significant apoptotic event did not occur in the reversible manner upon H-Ras suppression (data not shown), suggesting the possibility that the reversibility following Dox addition may reflect a continued requirement for H-Ras in promoting and sustaining cell-autonomous hyperplasia–host interactions that are essential for the skin phenotype and maintenance that are not faithfully mimicked in tissue culture.

In summary, we have identified protein and phosphoprotein changes that accompany H-RasV12G induction and de-induction in mouse skin. These molecular changes may mediate the poorly understood phenomenon of oncogenic addiction.

**Supplementary material**

Supplementary figure can be found at http://carcin.oxfordjournals.org/.

**Acknowledgements**

We thank Stuart Yuspa, Ronald Wolf, Christophe Cataisson and Lyuba Vartiovski for insights into Ras signaling.

**Conflict of Interest Statement:** None declared.

**References**

19. Politi,K. et al. (2006) Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. Genes Dev., 20, 1496–1510.