RSK2 as a key regulator in human skin cancer

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**Our previous report demonstrated that RSK2 plays an important
role in cell proliferation and transformation induced by tumor
promoters such as epidermal growth factor mediated through the
N-terminal kinase domain of RSK2 in JB6 Cl41 mouse skin epider-
mal cells *in vitro*. However, no direct evidence has been reported
regarding the relationship of RSK2 activity and human skin can-
cer. To elucidate the relationship of RSK2 activity and human skin
cancer, we examined the effect of knocking down RSK2 expres-
sion on epidermal growth factor-induced anchorage-independent
transformation in the premalignant HaCaT human skin keratino-
cyte cell line and on soft agar colony growth of SK-MEL-28 malig-
nant melanoma cells. We found that the phosphorylated protein
levels of RSK2 were enhanced in cancer tissues compared with
normal tissues in a human skin cancer tissue array. We found that
UVB stimulation induced increased in not only the total and phos-
phorylated protein levels of ERKs and RSK2 but also the nuclear
localization and gene expression of RSK2. RSK2 knockdown
inhibited proliferation and anchorage-independent transforma-
tion of HaCaT cells and soft agar colony growth of malignant
melanoma cells. Moreover, RSK2$^{-/-}$ mouse embryonic fibroblast
(MEF) showed enhanced sub-G$_1$ accumulation induced by UVB
stimulation compared with RSK2$^{+/+}$ MEFs, indicating that RSK2
might play an important role in tolerance against stress associ-
ated with ultraviolet. Importantly, activated RSK2 protein levels
were highly abundant in human skin cancer tissues compared with
matched skin normal tissues. Taken together, our results demon-
strated that RSK2 plays a key role in neoplastic transformation of
human skin cells and in skin cancer growth.**

Introduction

The mitogen-activated protein (MAP) kinases are key regulators of
cell proliferation and oncogenesis (1,2). The MAP kinase extracel-
lular signal-regulated protein kinases (ERKs) mediate their activation
signals through the phosphorylation of 90-kDa ribosomal S6 kinases
(p90^{RSKs}) (3,4), which are a family of serine/threonine kinases that
respond to many growth factors, peptide hormones and neurotrans-
mitters (5,6). RSK2 is a member of the p90^{RSK} family and is activated
by ERKs and phosphoinositide-dependent kinase 1 (PDK1)(3,4),
but not p38 kinases (7). When activated, RSK2 is translocated to the
nucleus and phosphorylates various nuclear proteins, including c-Fos,
Elk-1, histones, cyclic AMP responsive element binding (CREB) pro-
tein (8–13), activating transcription factor 4 (ATF4) (14), p53 (15),
NFAT3 (16) and ATF1 (17). Therefore, the RSK2 protein is likely to
mediate a variety of cellular processes, including proliferation and

Abbreviations: BCC, basal cell carcinoma; DAPI, 4′,6-diam
idino-2-phenylindole; EGF, epidermal growth factor; ERK, extra-
cellular signal-regulated protein kinase; FBS, fetal bovine serum;
MAP, mitogen-activated protein; MM, malignant melanoma; PBS,
phosphate-buffered saline; RSK2, ribosomal S6 kinase 2; SCC, squamous cell
carcinoma; UV, ultraviolet; UVB, ultraviolet B.

transformation as well as cell cycle. However, direct evidence link-
ing RSK2 to human skin cancer development has not yet been fully
obtained.

The Ras-ERKs-RSK2 pathway regulates cell proliferation, sur-
vival, growth and motility (18,19) and tumorigenesis (13). RSK2 is
a direct substrate kinase of ERKs and is located between ERKs and
its own target transcription factors (7,17). Furthermore, many human
solid cancers exhibit an activated *Ras* mutation (Ras^{G12V}; constitutively
active Ras) with the highest incidence observed in colon cancer (45%),
pancreatic cancer (90%), non-small-cell lung cancer (35%) and mela-
nomas (15%) (20). Furthermore, our previous study demonstrated that
the total RSK2 protein level was significantly higher in human skin
cancer tissues and cancer cells compared with normal skin tissues and
premalignant cell lines (7). We also demonstrated that the N-terminal
kinase domain of RSK2 plays an important role in transducing the
RSK2 activation signal to its substrates (7). RSK2 requires activation of
its C-terminal domain by its upstream kinases, ERKs, in order to phos-
phorylate and activate its substrates (7,17). For example, a recombinant
RSK2 protein purified from *Escherichia coli* cannot phosphorylate the
RSK2 substrate, NFAT3, but phosphorylation of RSK2 by ERKs at
the linker region of RSK2 gives RSK2 the ability to phosphorylate
its substrates through its N-terminal kinase domain. This suggests that
ERKs/RSK2 signaling plays an important role in RSK2-mediated cell
proliferation and cell transformation (7,17). Importantly, our study
demonstrated that coexpression of Ras^{G12V} and RSK2 in NIH3T3 cells
increased foci formation compared with Ras^{G12V} alone and si-RNA
knockdown of RSK2 in Ras^{G12V}- or Ras^{G12V}/RSK2-coexpressing
NIH3T3 cells completely blocked foci formation (13). This indicated
that the Ras/ERKs/RSK2 signaling pathway plays a key role in cell
transformation. However, the role of RSK2 activation and signaling in
human skin cancer has not yet been fully elucidated.

Ultraviolet (UV) light is a well-known environmental carcinogen
and is highly associated with skin carcinogenesis (21). Squamous
cell carcinomas (SCCs) and basal cell carcinomas (BCCs), account
for ~80% and 16% of all skin cancers, respectively. They occur pre-
dominantly on UV-exposed areas of the body and have been linked
with chronic exposure to UV (22). Studies in various skin cell lines
have demonstrated that epidermal growth factor (EGF) receptors (23),
MAP kinases (24) and phosphatidylinositol 3-kinase (PI3-K) (25) are
specific signaling molecules in ultraviolet B (UVB)-induced skin
carcinogenesis. UVB treatment markedly enhances ERKs and p38
kinase signaling (26) and inhibition of ERKs and p38 kinase abro-
gated UVB-induced c-*fos* gene transcription and protein expression
as well as AP-1 transactivation activity in human keratinocytes (21).
Taken together, these results indicated that the Ras-ERKs-RSK2 sign-
aling pathway might play an important role in human skin carcino-
genesis. However, direct evidence linking RSK2 activity with human
skin cancer development has not yet been reported. In this study, we
found that UVB induced increased levels of total and phosphorylated
ERKs, RSK2 and c-Fos in HaCaT cells and also caused nuclear accu-
mulation of activated RSK2. Importantly, the activated RSK2 protein
level was higher in human skin cancer tissues compared with normal
tissues, demonstrating that activated RSK2 plays an important role in
human skin cancer development and progression.

Materials and methods

Reagents and antibodies
Chemical reagents, including Tris, NaCl and sodium dodecyl sulfate for
molecular biology and buffer preparation were purchased from Sigma–Aldrich
(St Louis, MO). Cell culture medium and other supplements were purchased
from Life Science Technologies (Rockville, MD). Antibodies for west-
ern blot analysis were purchased from Cell Signaling Technology (Beverly,
MA), Santa Cruz Biotechnology (Santa Cruz, CA) or Upstate Biotechnology
(Charlottesville, VA).

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Cell culture and transfection

HaCaT, a premalignant human skin keratinocyte cell line, and SK-MEL-28, a malignant melanoma (MM) cell line, were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% CO_2 incubator. N/TERT1, a normal skin keratinocyte cell line, and SCC-13, a human skin squamous cell carcinoma cell line, were cultured in keratinocyte–serum-free medium (Life Science Technologies) supplemented with human recombinant epidermal growth factor and bovine pituitary extract as described in the manufacturer's suggested protocols.

MTS assay

To estimate proliferation, HaCaT, N/TERT1, SCC-13 and SK-MEL-28 cells (1×10^3 cells/well) stably expressing *sh-mock* or *sh-RSK2* were seeded into 96-well plates. After culturing for 2 h, 20 µl of the CellTiter 96® Aqueous One Solution (Promega, Madison, WI) were added to each well and cells were then incubated for 1 h at 37°C and 5% CO_2. To stop the reaction, 25 µl of a 10% sodium dodecyl sulfate solution were added and absorbance was measured at 492 and 690 nm. The MTS assay was conducted at 24 h intervals for 96 h.

Western blotting

Samples containing equal amounts of protein were resolved by the appropriate percentage sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated in blocking buffer and then probed with specific primary antibodies against appropriate target proteins. The western blots were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences Corp., Piscataway, NJ).

Anchorage-independent soft agar assay

EGF-induced cell transformation was investigated in *pKLO-sh-mock-* or *pKLO-sh-RSK2*-infected HaCaT normal human skin keratinocytes. Colony growth of cancer cells was assessed using *pKLO.1-sh-GFP* scramble (*psh-mock*) or *pKLO.1-sh-RSK2* (*psh-RSK2*) infected SK-MEL-28 MM cells. In brief, HaCaT cells (8×10^3) were exposed to EGF (10 ng/ml) in 1 ml of 0.3% Basal Medium Eagle top agar containing 10% FBS. SK-MEL-28 cells (8×10^3) were cultured in 1 ml of 0.3% Dulbecco's modified Eagle's medium top agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO_2 incubator for 14 days and the cell colonies were scored using a microscope and the Image-Pro PLUS (v.6.2) computer software program (Media Cybernetics, Silver Spring, MD) as described by Colburn *et al.* (27).

Nuclear localization of RSK2

To separate the nuclear and cytosolic fractions of HaCaT cells after UVB stimulation, cells (4×10^6) were plated into 100 mm dishes, cultured, starved overnight, stimulated with UVB (4 kJ/m^2) and then harvested at the indicated time points. The cytosolic and nuclear fractions were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagent (PIERCE, Rockford, IL) following the manufacturer's suggested protocol.

Quantitative real-time PCR

HaCaT cells (4×10^6) were plated into 100 mm dishes, cultured, starved overnight, stimulated with UVB (4 kJ/m^2) and then harvested at the indicated time points. Total RNAs were extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) and RSK2 gene expression was analyzed with 50 ng of total RNA, an RSK2-specific real-time primer set (RPS6KA3 Cat no.: HS00177936_m1) and a glyceraldehyde 3-phosphate dehydrogenase-specific real-time primer set (Hs99999905_m1), by quantitative one-step real-time PCR using the TaqMan RNA-to-C_T 1-step kit (Applied Biosystems, Foster City, CA) following the manufacturer's suggested protocols. The C_T values of RSK2 gene expression were normalized with the C_T values of glyceraldehyde 3-phosphate dehydrogenase as an internal control to monitor equal RNA utilization.

Immunocytofluorescence assay

HaCaT cells (2×10^4 cells/well) were seeded in 2-chamber slides and cultured for 24 h, starved overnight and then stimulated with UVB (4 kJ/m^2). The cells were fixed with 4% formalin at the indicated time point after UVB stimulation. The cells were permeabilized with 0.5% triton X-100 in 1× phosphate-buffered saline (PBS) for 5 min at room temperature, blocked with 3% bovine serum albumin in 1× PBS for 1 h at 37°C and hybridized with phospho-RSK2 (T577) primary and Alex 488-conjugated secondary antibodies. The cells were mounted with Fluoro-Gel II with 4′,6-diamidino-2-phenylindole (DAPI) (Electron Microscopy Science, Hatfield, PA) and nuclear localization of activated RSK2 was observed under a laser scanning confocal microscope (NIKON C1^{si} Confocal Spectral Image System, NIKON Instruments Co., Melville, NY; ×200). Fluorescence intensity was measured using the Image J computer software program (v.1.45) and normalized with nuclear DAPI intensity as described previously (7).

Tissue array

A human skin tissue array (SK801) was purchased from US Biomax (Rockville, MD) and tissue array analysis was conducted according to the manufacturer's suggested protocols. The tissue array includes matched normal tissues (H1–H7), which were biopsied from the adjacent tissue of each cancer tissue (A1–A7) from seven individual patients. Briefly, the slide was baked at 60°C for 2 h, deparaffinized and rehydrated. Antigens were then unmasked by submerging the slide into boiling sodium citrate buffer (10 mM, pH 6.0) for 10 min. The slide was blocked with 3% BSA in 1× PBS/0.03% Triton X-100 in a humidified chamber for 1 h at room temperature, and then hybridized with a phospho-RSK2 (T577) antibody (1:200 dilution in 1× PBS/0.03% Triton X-100) at 4°C in a humidified chamber overnight. The slide was washed, hybridized with the secondary antibody-conjugated with Alex 488 (1:1000) for 1.5 h at room temperature in the dark. Optical sections containing the activated RSK2 protein levels were captured by laser scanning confocal microscopy (NIKON Instruments Co., Melville, NY). Each image was converted and incorporated into a TIF file. The fluorescence intensity from a whole frame of each photo was individually measured using the Image J computer software program (v.1.45) and normalized with nuclear DAPI intensity as described previously (7).

Results

UVB activates ERKs-RSK2 signaling

The MAP kinase signaling pathway not only promotes cell proliferation but also mediates cell survival and is upregulated in various cancer cells (20). Our previous study demonstrated that EGF, a tumor promoter, induced cell transformation through Ras-ERKs-RSK2 signaling (13). UV irradiation is the most common environmental carcinogen. It acts as a tumor initiator by inducing mutations in tumor suppressor proteins and functions as a tumor promoter by enhancing cellular signaling through the MAP kinase signaling pathways including ERKs (26). However, ERKs-RSK2 signaling in human skin cancer induced by UVB is not yet fully understood. To determine whether ERKs-RSK2 signaling is activated by UVB, we conducted immunofluorescence assessment of total RSK2 proteins in normal human skin tissues using a specific antibody. We found that RSK2 was expressed in epidermis and dermis of normal human skin tissues (Figure 1A). Notably, we found that phosphorylation of ERKs, RSK2 and c-Fos was increased by exposure to UVB (4 kJ/m^2) in HaCaT human skin keratinocytes (Figure 1B). Unexpectedly, the total protein levels of ERKs, RSK2 and c-Fos were also enhanced by UVB stimulation in HaCaT human skin keratinocytes (Figure 1B). We used real-time PCR to confirm that the elevated RSK2 protein level resulted from increased *rsk2* gene expression (Figure 1C). Importantly, we found that UVB treatment induced nuclear localization of activated RSK2 [i.e. phosphorylated RSK2 (Thr577)] in a time-dependent manner (Figure 1D). These results demonstrated that ERKs-RSK2 signaling has an important role in UV-induced human skin carcinogenesis.

RSK2 enhances tolerance against UVB-induced stress

Our previous studies indicated that UV-activated RSK2 phosphorylates proapoptotic Bcl-2-associated death promoter (BAD) at Ser112 resulting in BAD inactivation and increased cell survival (28). At the same time, RSK2 also upregulates the transcription of antiapoptotic Bcl-2associated death promoter through CREB phosphorylation and activation (29). Because apoptosis is presumed to have a protective function against carcinogenesis, these effects suggest that RSK2 plays a role in enhancing tumorigenesis. To examine this hypothesis, we treated cells with UVB (4 kJ/m^2) and found that the sub-G_1 population was increased in RSK2-deficient (RSK2$^{-/-}$) MEFs compared with RSK2 wild-type (RSK2$^{+/+}$) MEFs (Figure 2A). Notably, nuclear localization of activated RSK2 and the total RSK2 protein level was increased by UVB treatment (Figure 2B). Moreover, we found that UVB enhanced the activated and total RSK2 protein levels in various human skin cells, including HaCaT, N/TERT-1, SCC-13 and SK-MEL-28 (Figure 2C). In addition, the activated and total RSK2 proteins were highly abundant in the nucleus following UVB treatment of HaCaT, N/TERT-1, SCC-13 and SK-MEL-28 cells (Figure 2D). These results demonstrated that RSK2 plays an important role for protection of cells from stress such as UV.
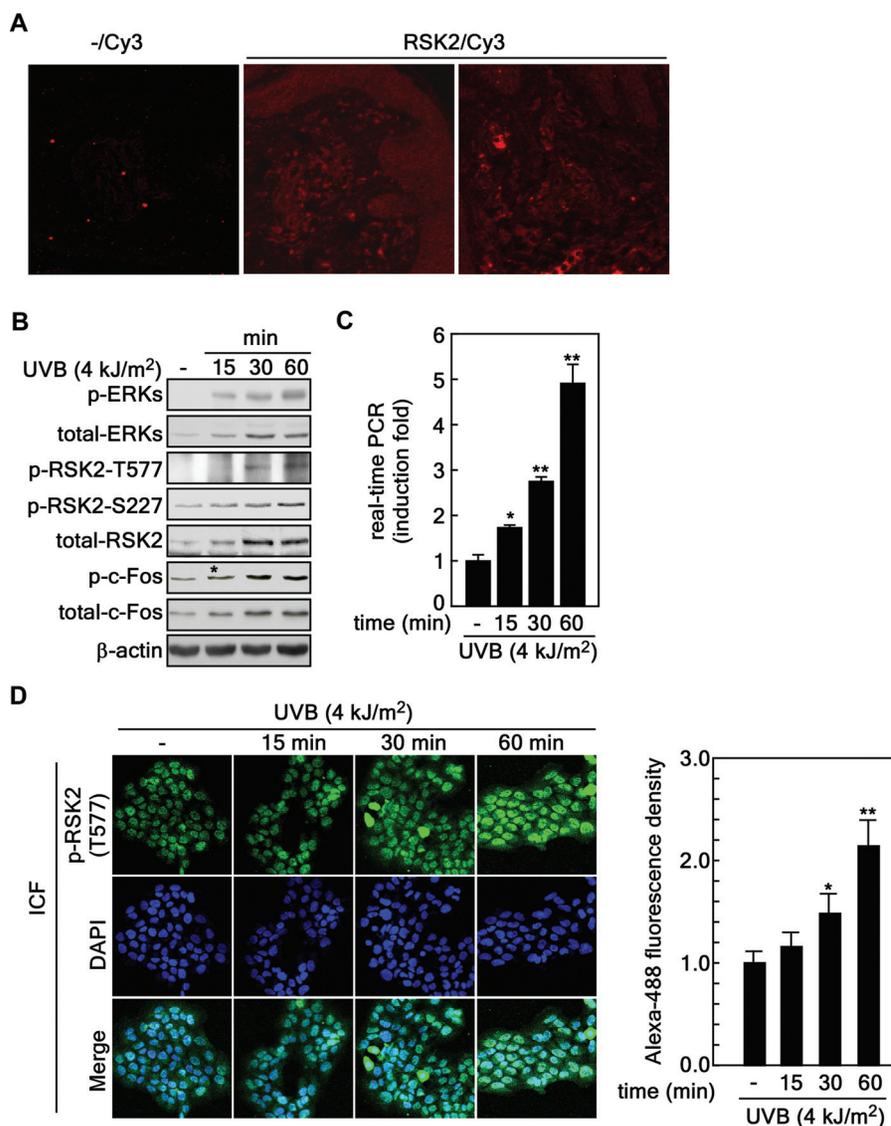
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Fig. 1. UVB activates ERKs-RSK2 signaling. (**A**) Immunofluorescence of total RSK2 proteins in normal human skin tissues. Normal human skin tissues were used to visualize total RSK2 proteins using an RSK2-specific primary antibody and an Alexa568-conjugated secondary antibody. Optical sections showing total RSK2 protein levels were captured by laser scanning confocal microscopy as described in Materials and methods. (**B**) Activation profile of ERKs-RSK2 signaling induced by UVB exposure. HaCaT human skin keratinocytes (4×10^6) were seeded in 100 mm culture dishes, incubated for 24 h, starved overnight, and then stimulated with UVB (4 kJ/m^2). The cells were harvested and proteins extracted. Activation of ERKs-RSK2 signaling was analyzed by western blotting using specific antibodies as indicated. β-Actin was used as an internal control to monitor equal protein loading. (**C**) UVB stimulates increased *rsk2* mRNA expression. HaCaT human skin keratinocytes (4×10^6) were seeded, cultured for 24 h, starved overnight and stimulated with UVB (4 kJ/m^2). Total RNA was extracted at the indicated time point and *rsk2* mRNA levels were analyzed by real-time RT–PCR as described in Materials and methods. Data are represented as means ± SD of values obtained from two independent triplicate experiments and significant differences were calculated using the Student's *t*-test (*$P < 0.05$, **$P < 0.001$). (**D**) UVB induces nuclear localization of RSK2. HaCaT human skin keratinocytes (2×10^4) were seeded into 2-chamber slides, cultured for 24 h, and then serum-starved overnight. Cells were stimulated with UVB (4 kJ/m^2), fixed with 4% formalin for 15 min and permeabilized with 0.5% triton X-100 in 1× PBS. Nuclear localization of RSK2 was observed by fluorescence microscope (×200) using a phospho-RSK2 (T577) primary antibody and an Alexa 488-conjugated secondary antibody. The fluorescence intensity was measured by the Image J computer program (v.1.45) and normalized by fluorescence of DAPI intensity. Data are represented as means ± SD of values obtained from 20 randomly selected cells and significant differences were calculated using the Student's *t*-test (*$P < 0.05$, **$P < 0.005$).

RSK2 knockdown inhibits proliferation of normal human skin cells and skin cancer cells

To examine the direct effect of RSK2 on proliferation of human skin cells, we chose HaCaT cells, a premalignant human skin keratinocyte cell line, N/TERT-1 cells, a human skin keratinocyte cell line immortalized by telomerase (30), SCC-13 cells, a human skin epidermal squamous cell carcinoma cell line (31) and SK-MEL-28 MM cells. We first examined the RSK2 protein level in these cells. We found that N/TERT-1 expressed a very low level of RSK2. RSK2 was weakly expressed in HaCaT cells, moderately expressed in SCC-13 cells, but highly expressed in SK-MEL-28 cells (Figure 3A). We infected each cell type with *sh-mock* or *sh-RSK2* for stable expression, and confirmed that *sh-RSK2* almost totally blocked RSK2 expression in these cells (Figure 3B). To explore the effect of knocking down RSK2 expression on proliferation, we used the MTS assay and found that RSK2 knockdown inhibited proliferation of HaCaT, SCC-13 and SK-MEL-28 cells (Figure 3C). Interestingly, N/TERT-1 human skin keratinocytes were not significantly affected by RSK2 knockdown (Figure 3C). Importantly, the efficiency of proliferation inhibition induced by RSK2 knockdown corresponded with the endogenous
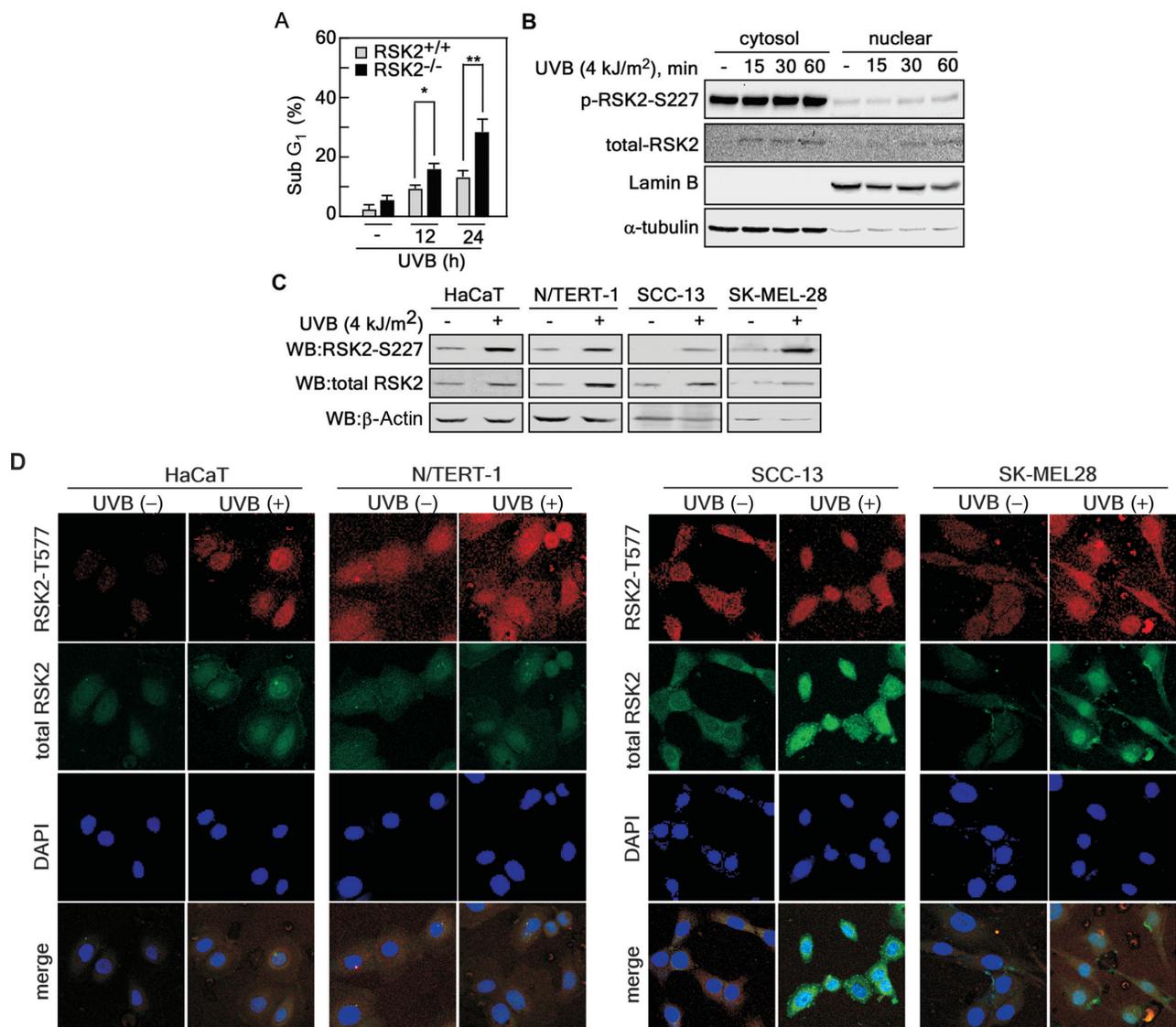
Fig. 2. RSK2 enhances tolerance against UVB-induced stress. (**A**) RSK2 wild-type (RSK2$^{+/+}$) and deficient (RSK2$^{-/-}$) MEFs were treated with UVB (4 kJ/m^2) and the sub-G$_1$ population was analyzed by flow cytometry at the indicated time point. Data are represented as means ± SD of values obtained from an experiment with triplicate samples and significant differences were calculated using the Student's *t*-test (*$P < 0.05$, **$P < 0.005$). (**B**) HaCaT human skin keratinocytes (4×10^6) were seeded in a 100 mm dish, cultured for 24 h, starved, stimulated with UVB (4 kJ/m^2), and then harvested at the indicated time point. The cytosolic and nuclear proteins were extracted using NE/PER nuclear and cytoplasmic extraction reagents. Activated and total RSK2 protein levels were visualized using specific antibodies as indicated. Lamin B and α-tubulin were used as nuclear and cytoplasmic marker proteins, respectively. (**C**) UVB stimulation induces increased RSK2 protein levels in various skin cell lines. HaCaT, N/TERT-1, SCC-13 and SK-MEL-28 cells were seeded, starved, stimulated with UVB (4 kJ/m^2) and cultured for 60 min in a 37°C/5% CO$_2$ incubator. The proteins were extracted and then activated and total RSK2 protein levels were visualized by western blotting using specific antibodies as indicated. β-Actin was used as an internal control to monitor equal protein loading. (**D**) HaCaT, N/TERT-1, SCC-13 and SK-MEL-28 cells (2×10^4) were seeded in 4-chamber slides, starved for 24 h, stimulated with UVB and cultured for 60 min in a 37°C/5% CO$_2$ incubator. The cells were fixed, permeabilized, triple-stained with Alexa-488 to detect total RSK2, Alexa-568 to detect phosphor-RSK2 (T577) and DAPI for nuclear staining. Activated and total RSK2 protein levels were visualized under a laser scanning confocal microscope using specific antibodies as indicated.

RSK2 protein level (Figure 3C). These results demonstrated that RSK2 has an important role in the proliferation of human skin cells.

Knockdown of RSK2 suppresses EGF-induced anchorage-independent transformation and colony growth of skin cells

To examine whether RSK2 knockdown affects anchorage-independent cell transformation induced by EGF or cancer cell colony growth under anchorage-independent conditions, we conducted a soft agar assay with HaCaT (32) and SK-MEL-28 cells. We found that HaCaT cells expressing *sh-mock* formed colonies when stimulated by EGF (Figure 4A). However, colony formation was dramatically reduced by expression of *sh-RSK2* (Figure 4A). Furthermore, RSK2 knockdown suppressed growth of SK-MEL-28 MM cells in soft agar compared

with *sh-mock* control cells (Figure 4B). In addition, soft agar assay results indicated that *sh-RSK2* inhibited the number of colonies formed as well as colony size (Figure 4). These results demonstrated that RSK2 also has an important role in cell transformation and cancer cell colony growth.

Tissue array analysis of RSK2 in solid human skin cancer and normal skin tissues

To examine the physiological relevance of RSK2 in human skin cancer, we analyzed a human skin cancer tissue array using 80 human skin core tissues, which included 70 human skin cancer tissues and 10 normal skin tissues. Our previous study demonstrated that human skin cancer tissues contain higher RSK2 protein levels compared with
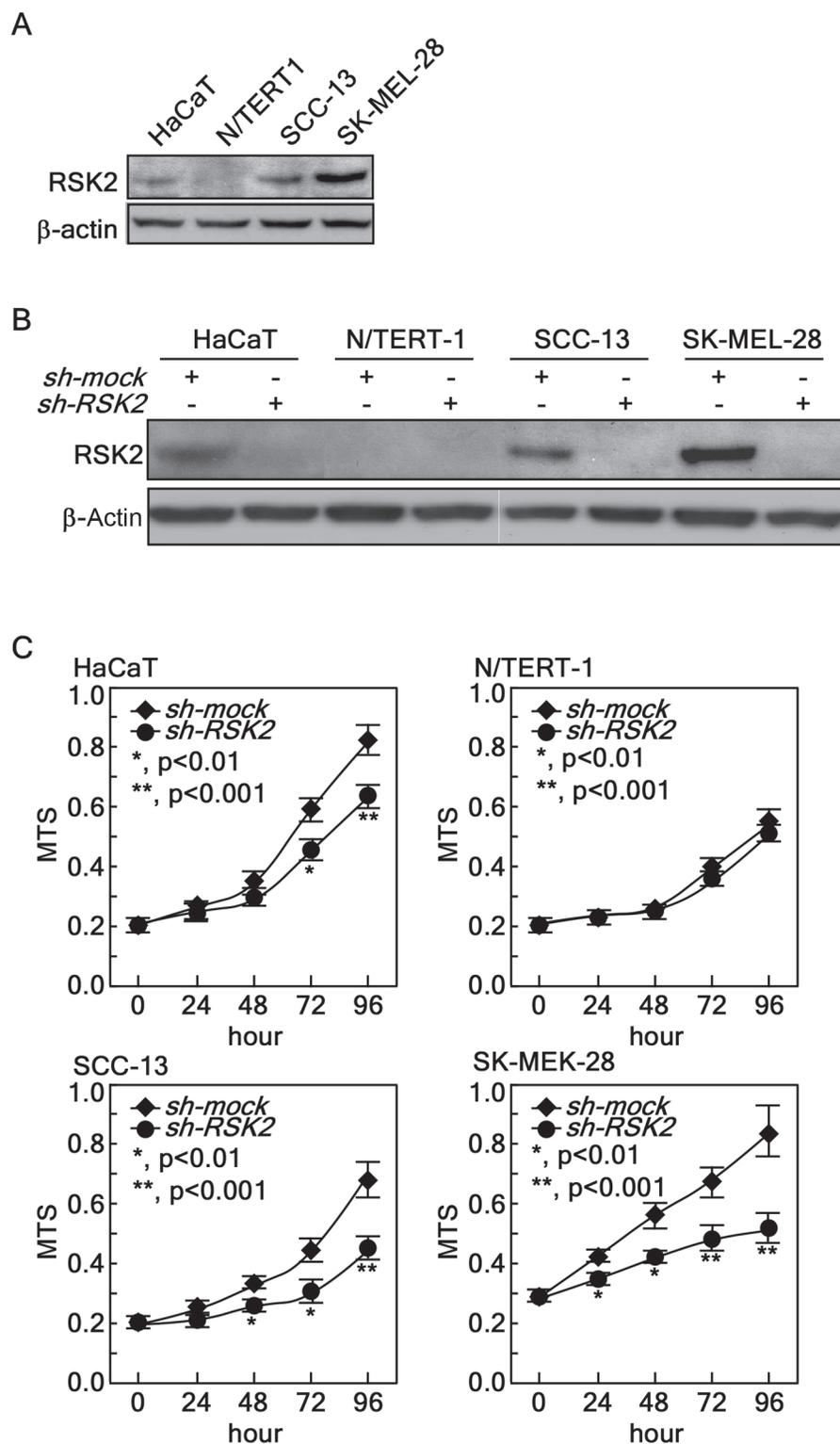
Fig. 3. RSK2 knockdown inhibits normal human skin and cancer cell proliferation. (**A**) Endogenous RSK2 protein levels in normal human skin HaCaT, N/TERT-1 keratinocytes, human skin SCC-13 and SK-MEL-28 cancer cells. Cells were cultured with complete cell culture media and proteins were extracted. The RSK2 protein levels were analyzed by western blotting using RSK2-specific primary antibody and a horseradish peroxidase-conjugated secondary antibody. β-Actin was used as an internal control to monitor equal protein loading. (**B**) Endogenous RSK2 expression is silenced by infection with *sh-RSK2* in HaCaT, N/TERT-1, SCC-13 and SK-MEL-28 cells. Knockdown efficiency was analyzed by western blotting using an RSK2-specific primary antibody and a horseradish peroxidase-conjugated secondary antibody. β-Actin was used as an internal control to monitor equal protein loading. (**C**) The cells from (**B**) were used for a proliferation assay. Cells (1×10^3) were seeded into 96-well plates and proliferation was measured by MTS assay at 24 h intervals up to 96 h. Data are represented as means ± SD of values obtained from six experiments and significant differences were calculated using the Student's *t*-test (*$P < 0.05$, ** $P < 0.005$).
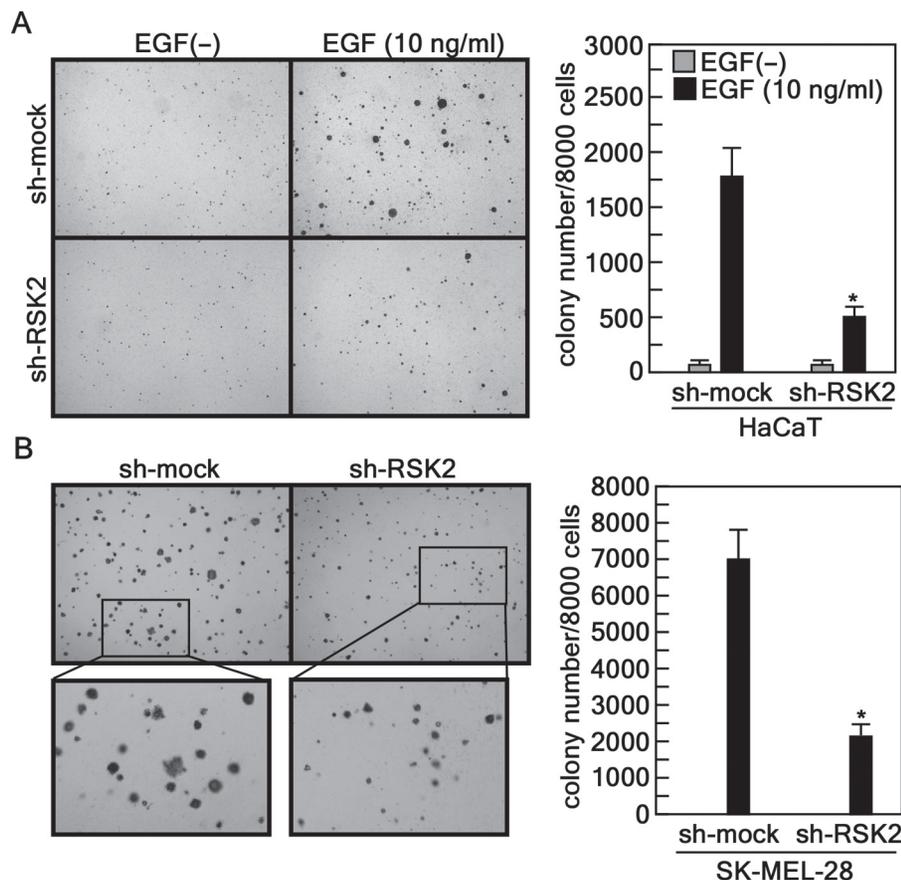
Fig. 4. Knockdown of RSK2 suppresses EGF-induced anchorage-independent cell transformation and colony growth in soft agar. (**A**) Suppression of anchorage-independent transformation of HaCaT cells by knocking down endogenous RSK2. Cells stably expressing *sh-mock* or *sh-RSK2* were cultured and subjected to an EGF-induced anchorage-independent cell transformation assay. Cells (8×10^3) were exposed to EGF (10 ng/ml) in 1 ml of 0.3% Basal Medium Eagle agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO_2 incubator for 14 days and then colonies were counted using a microscope and the Image-Pro PLUS (v.6.2) computer software program. Data are represented as means ± SD of values from triplicate experiments and statistical significance was determined using the Student's *t*-test (*$P < 0.001$). (**B**) Colony growth in soft agar is inhibited by knocking down endogenous RSK2. SK-MEL-28 cells stably expressing *sh-mock* or *sh-RSK2* were tested for colony growth under anchorage-independent conditions. Cells (8×10^3) were mixed with 0.3% top agar in complete cell culture medium. The cultures were maintained in a 37°C, 5% CO_2 incubator for 10 days and then colonies were counted using a microscope and the Image-Pro PLUS (v.6.2) computer software program. Data are represented as means ± SD of triplicate values and statistical significance was determined using the Student's *t*-test (*$P < 0.001$).

normal skin tissues (7). Using the same tissue array, we examined the expression of activated RSK2 using a phospho-RSK2 (T577)-specific antibody and an Alexa 488-conjugated secondary antibody. We assessed the level of activated RSK2 in tissue array specimens by measuring the fluorescence light intensity. We found that activated RSK2 was highly abundant in human skin cancer tissues at about 2-fold above normal tissue expression (Figure 5A, left panel). The light intensity distribution clearly showed that activated RSK2 protein levels are enhanced in human skin tissues compared with normal skin tissues (Figure 5A, right panel). To examine RSK2 (Thr577) more fully in different kinds of skin cancers, the human skin cancer array was divided into SCC ($n = 40$), BCC ($n = 13$) and MM ($n = 12$). We re-analyzed the total RSK2 protein levels from our previous tissue array results (7) and the activated RSK2 protein level. We found that total and activated RSK2 protein levels were most highly abundant in SCC (Figure 5B and 5C). Moreover, although the total protein levels were moderately enhanced in BCC and MM (Figure 5B), the activated RSK2 protein levels were dramatically increased in both BCC and MM (Figure 5C). The average total RSK2 levels were about 200% in SCC and 140% in BCC and MM compared with levels in normal skin tissues (Supplementary Figure 1A, available at *Carcinogenesis* Online). Furthermore, the average protein level of activated RSK2 was about 190% in SCC, 176% in BCC, and 206% in MM compared with the activated RSK2 protein level observed in normal skin tissues

(Supplementary Figure 1B, available at *Carcinogenesis* Online). These results demonstrated that total and activated RSK2 protein levels correspond with human skin cancer development.

Activated RSK2 protein abundance is higher in cancer tissues compared with matched normal tissues

The tissue array panel includes seven matched normal tissues and cancer tissues biopsied from the same patient. Using these tissue samples, we compared the fluorescence intensity (Figure 6A) and found that three SCCs, one chronic inflammation of fibrous tissue and three MM tissues contained 2–3-fold higher activated RSK2 protein levels (Figure 6A and B). The activated RSK2 localization into the nucleus was observed in skin cancer tissue compared with normal skin tissue (Figure 6C). These results corresponded well with the total protein level analyzed from our previous publication (7). These results provide strong evidence showing that RSK2 is an important kinase in human skin cancer development.

Discussion

Our previous studies demonstrated that RSK2, as a downstream kinase of the Ras-ERKs signaling pathway, plays an important role in proliferation and transformation of mouse skin epidermal JB6 Cl41 cells (15). EGF or 12-*O*-tetradecanoylphorbol 13-acetate stimulation enhances
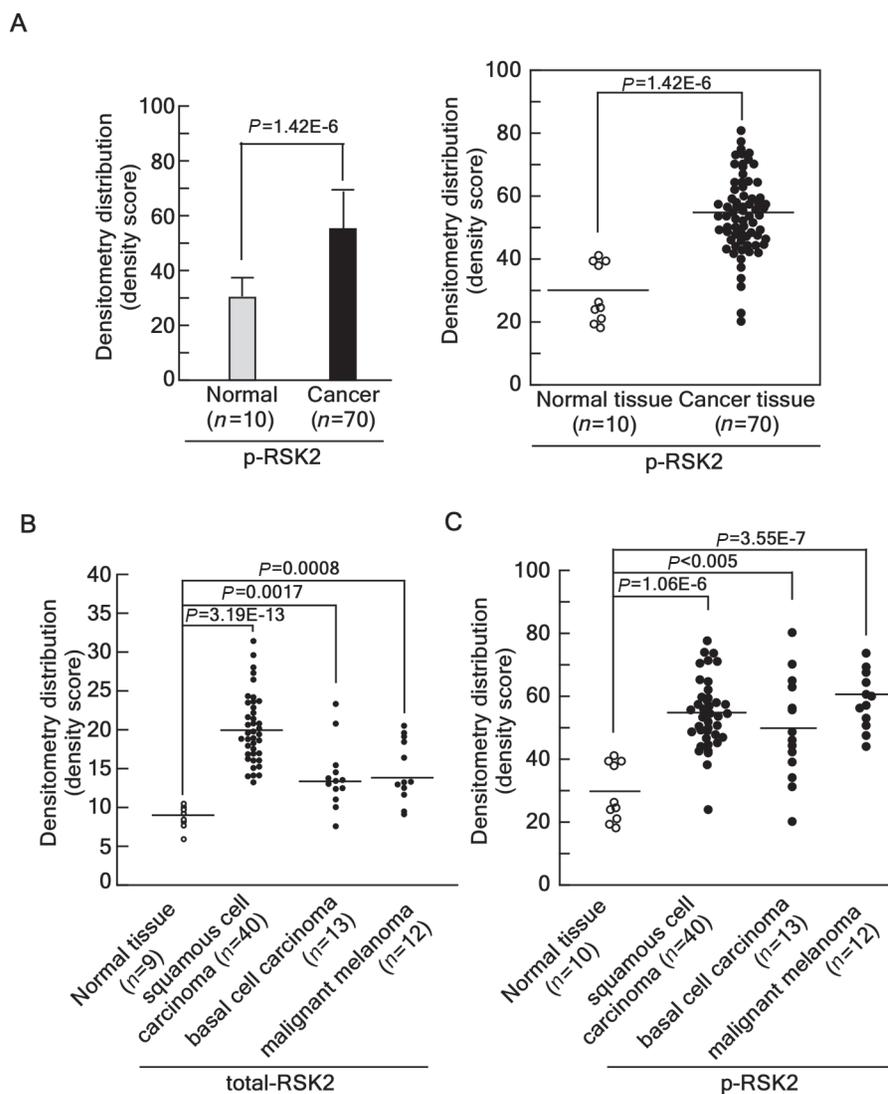
A

B

C

Fig. 5. Tissue array analysis of RSK2 in solid human skin cancer and normal skin tissues. (**A**) Activated RSK2 protein levels are higher in cancer tissues compared with normal tissues. An analysis of activated RSK2 was performed using a human skin tissue array that included matched normal and cancer tissues. Phosphorylated RSK2 was detected using an antibody against phospho-RSK2 (T577) and an Alexa 488-conjugated secondary antibody as described in Materials and methods. The light intensity of each sample was estimated using the Image J computer program (v.1.45). The overall average density of all samples is shown (left panel) along with the density of each individual sample (right panel). The fluorescence intensity of the individual sample was obtained using the Image J computer software program (v.1.45). (**B–C**) The 70 core samples of skin cancer tissues were classified into subclasses of skin cancer categories, including SCC, BCC and MM. The fluorescence light intensity of the activated RSK2 protein and total RSK2 protein levels were re-evaluated. The average density of each subclass is shown in Supplementary Figure 1A (total RSK2) and 1B [phospho-RSK2 [T577]], available at *Carcinogenesis* Online along with the density of each individual sample for total RSK2 (B) and activated RSK2 (C). Significant differences in A–C were evaluated using the Student's *t*-test as described previously (7).

phosphorylation of ERKs and RSKs and the G$_1$/S cell cycle transition in JB6 Cl41 cells (15). Our recent study demonstrated that EGF stimulation induces an interaction between RSK2 and caspase 8, and phosphorylation of caspase 8 at Thr263 results in ubiquitination-mediated caspase 8 degradation (33). The degradation of caspase 8 inhibits apoptosis induced by Fas in HeLa cells (33). Therefore, RSK2 mediates cell survival rather than cell death. These observations correspond well with our finding that RSK2 plays an important role in proliferation and cell transformation induced by tumor promoters such as EGF or 12-*O*-tetradecanoylphorbol 13-acetate in JB6 Cl41 mouse skin epidermal cells (13).

Increased epidermal growth factor receptor activation is observed within a short time after UV exposure of cultured keratinocytes or skin (34,35), resulting in activation of the ERKs signaling pathway (34). Our results indicated that UVB irradiation induces phosphorylation of ERKs and RSK2 at Thr577, which is a site phosphorylated by ERKs (Figure 1B), and also causes RSK2 nuclear localization (Figures 1D, 2B and D), resulting in phosphorylation of c-Fos (Figure 1B). The

phosphorylation of c-Fos (Ser362) by RSK2 plays an important role in osteosarcoma development (36) through its effects on cell cycle progression (37). Our previous results demonstrated that ectopic expression of RSK2 enhances cell proliferation; and knockout of RSK2 attenuates proliferation and cell cycle progression in JB6 Cl41 mouse skin epidermal cells (13). Based on these results, we found that RSK2 knockdown suppressed proliferation of HaCaT, SCC-13 and SK-MEL-28 cells (Figure 3C). Similar findings in A431 human skin epidermoid carcinoma, SK-MEL-5 and SK-MEL-28 MM and HCT-116 human colon cancer cells were obtained by treatment with the chemical inhibitor kaempferol, which is reported to suppress RSK2 N-terminal kinase activity (7). RSK2 knockdown suppressed Ras^{G12V}-induced foci formation of JB6 Cl41 cells (13). We confirmed that knockdown of RSK2 attenuated anchorage-independent transformation of HaCaT cells induced by EGF (Figure 5A) and soft agar colony growth of SK-MEL-28 MM cells (Figure 5B). Therefore, our results demonstrated that RSK2 plays a key role in human skin cancer.
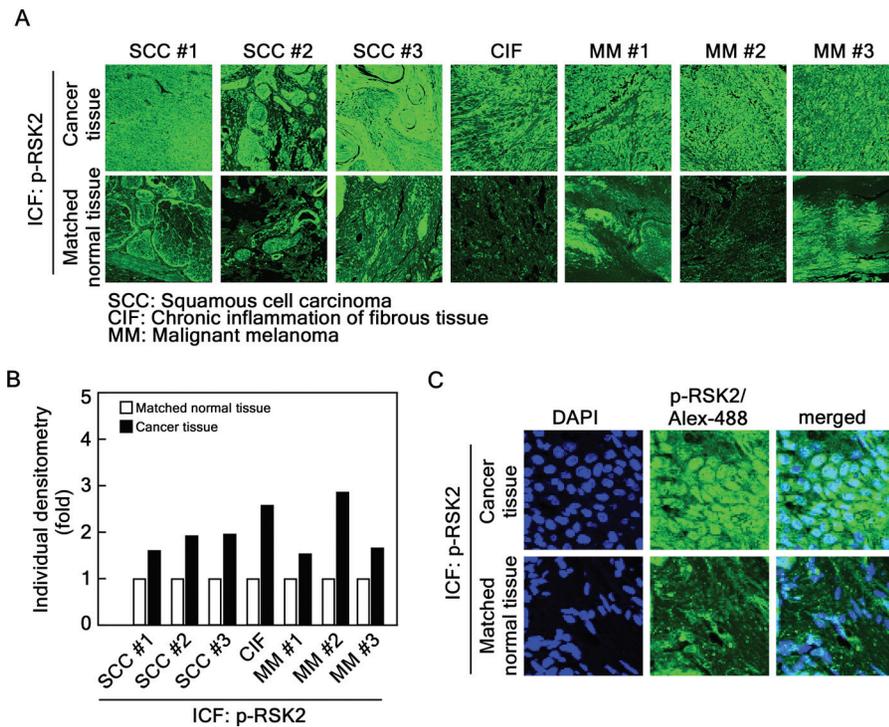
A

SCC: Squamous cell carcinoma
CIF: Chronic inflammation of fibrous tissue
MM: Malignant melanoma

B

C

Fig. 6. The activated RSK2 protein level is higher in cancer tissues compared with matched normal tissues. (**A**) The levels of activated RSK2 proteins were compared with matched human normal and cancer tissues, including SCC, chronic inflammation of fibrous tissue and MM. Representative samples are shown. (**B**) The relative light intensity of individual activated RSK2 in cancer tissues compared with matched each normal tissue sample is shown. (**C**) Elevation of the activated RSK2 protein levels in skin cancer tissue. A matched human skin cancer tissue and normal tissue in (A) were magnified to confirm whether activated RSK2 protein levels were accumulated in nucleus skin cancer tissue. The nucleus was stained with DAPI, phosphorylated RSK2 (T577) was stained with Alexa-488 and nuclear localization of RSK2 was confirmed by merging of DAPI and Alexa-488 as indicated.

Our previous results demonstrated that human skin cancer tissues contain higher total RSK2 protein levels compared with normal skin tissues (7). Using the same tissue array, we found that phospho-RSK2 protein levels were about 2-fold higher in skin cancer tissues compared with normal skin tissues (Figure 5A). Furthermore, we re-evaluated our previous and current tissue array results by dividing the samples into subclasses of skin cancer, SCC, BCC and MM (Figure 5B). We found that total and activated RSK2 levels were most increased in SCC compared with normal skin tissues (Figure 5B and C; Supplementary Figure 1, available at *Carcinogenesis* Online). Although the total protein levels in BCC and MM tissues were moderately enhanced (about 1.5-fold), the activated RSK2 protein levels were increased by about 2-fold in skin cancer tissues (Figure 5B and 5C; Supplementary Figure 1, available at *Carcinogenesis* Online) compared with normal tissues. Furthermore, additional results demonstrated that the activated RSK2 protein level was higher in cancer tissues compared with normal adjacent skin tissues in the same patient (Figure 6). Taken together, our results provide strong evidence of an association of total and activated RSK2 protein levels and human skin cancer development. Moreover, our results further suggest that identifying inhibitors against RSK2 activity might be an effective means to treat skin cancer in humans.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjour-nals.org/

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