The c-Jun NH$_2$-Terminal Kinase 2 Plays a Dominant Role in Human Epidermal Neoplasia

Hengning Ke$^1$, Rebecca Harris$^1$, Jonathan L. Coloff$^2$, Jane Y. Jin$^1$, Benjamin Leshin$^1$, Paula Miliani de Marval$^1$, Shiyiing Tao$^3$, Jeffrey C. Rathmell$^2$, Russell P. Hall$^1$, and Jennifer Y. Zhang$^1$

Abstract

The c-Jun NH$_2$-terminal kinase (JNK) signaling cascade has been implicated in a wide range of diseases, including cancer. It is unclear how different JNK proteins contribute to human cancer. Here, we report that JNK2 is activated in more than 70% of human squamous cell carcinoma (SCC) cells and that inhibition of JNK2 pharmacologically or genetically impairs tumorigenesis of human SCC cells. Most importantly, JNK2, but not JNK1, is sufficient to couple with oncogenic Ras to transform primary human epidermal cells into malignancy with features of SCC. JNK2 prevents Ras-induced cell senescence and growth arrest by reducing the expression levels of the cell cycle inhibitor p16 and the activation of NF-$\kappa$B. On the other hand, JNK, along with phosphoinositide 3-kinase, is essential for Ras-induced glycolysis, an energy-producing process known to benefit cancer growth. These data indicate that JNK2 collaborates with other oncogenes, such as Ras, at multiple molecular levels to promote tumorigenesis and hence represents a promising therapeutic target for cancer. Cancer Res; 70(8): 3080–8. ©2010 AACR.

Introduction

The c-Jun NH$_2$-terminal kinases (JNK) make up one family of the mitogen-activated protein kinases (MAPK), along with extracellular signal–regulated kinase (Erk) and p38 (1). The JNK proteins, also known as stress-activated protein kinases (stress activated protein kinases), include JNK1, JNK2, and JNK3, which are encoded by three different genes. JNK1 and JNK2 are ubiquitously expressed and each has four splice isoforms whose expression levels vary among different cell types and environmental conditions. In contrast, JNK3 is abundant in a limited number of tissues, including brain, testis, and heart, although wider expression of JNK3 has been implicated in recent studies. JNK is highly responsive to a variety of extracellular stimuli, including a wide range of inflammatory cytokines (2). Signals transmitted from membrane receptors activate MKK4 and MKK7, which then activate JNK via dual phosphorylation on the ThrProTyr (TPY) motif. MKK7 is highly specific for JNK, whereas MKK4 also activates p38 (3). The major downstream targets of the JNK cascade are members of the activator protein 1 (AP-1) family transcription factors, including Jun and Fos proteins that function as hetero- or homo-dimers (2).

The JNK signaling pathway is not only involved in cellular stress responses but also plays key roles in tissue homeostasis by regulating cell proliferation, apoptosis, and differentiation in a cell type– and tissue-specific manner. Abnormal JNK signaling has been associated with a number of pathologic conditions in animal models, including neurodegenerative disorders, diabetes, arthritis, atherosclerosis, and cancer (4–7). In line with these animal data, increased JNK activation has been detected in an array of human cancers, including glioma, prostate carcinoma, osteosarcoma, and squamous cell carcinoma (SCC; refs. 8–11). SCC is the most common and invasive type of nonmelanoma skin cancer, with an annual incidence of more than 250,000 cases in the United States alone. It has been recently recognized as one of the most costly types of cancers that entail a huge financial burden to the health care system (12). Therapeutic strategies targeting the JNK signal transduction pathway has already been under active development for a broad spectrum of other diseases (13). It is imperative to explore their use in human SCC. Our previous studies by immunostaining have shown that the JNK is highly activated in more than 75% of patient SCC samples examined. Moreover, JNK activation by expression of a constitutively active mutant of MKK7 is sufficient to couple with oncogenic Ras to promote invasive epidermal malignancy with features of SCC (11). These findings underscore a dominant role of the JNK signaling pathway in human SCC. However, the functional significance and molecular mechanisms of different JNK proteins in SCC are still unclear.

JNK proteins exhibit both redundant and differential roles in tissue homeostasis, as shown by recent mouse genetic
studies (14). Deletion of either Jnk1 or Jnk2, together with or without Jnk3, produces viable mice with defects in T-helper cell differentiation. Compound deletion of Jnk1 and Jnk2 leads to early embryonic lethality (E10.5) due to aberrant cell death in the forebrain and hindbrain (15, 16), which indicates that Jnk1 and Jnk2 are functionally redundant. On the other hand, studies using isolated mouse fibroblasts or erythroblasts have shown opposite roles for Jnk1 and Jnk2 in cell proliferation, with Jnk2 being recognized as a negative growth regulator (17). Similar observations have been reported for epidermal cells such that Jnk2−/− and Jnk1−/− epidermis is hyperplastic and hypoplastic, respectively (18). On the contrary to the growth phenotype, Jnk2−/− mice are resistant whereas Jnk1−/− mice are sensitive to chemically induced skin carcinogenesis (7, 19). Of further interest, only Jnk1 is involved in UV-induced signal transduction in epidermis (20). Taken together, these findings underscore important functions for each Jnk in murine cell growth and neoplasia. It is yet to be determined whether human cells respond to Jnk in the same manner as mouse cells do, as well as the molecular mechanisms that govern the Jnk effects on pathogenesis. To address these questions, we studied the gain-of-function and loss-of-function effects of Jnk1 and Jnk2 on epidermal neoplasia by using the recently developed human epidermal tissue models regenerated on immunodeficient mice (11, 21, 22).

We found that activation of Jnk2, but not Jnk1, was able to act in concert with oncogenic Ras to transform normal epidermal cells to malignancy with features of spontaneous SCC. Jnk2 reduced protein levels of p16 cell cycle inhibitor, blocked Ras-induced NF-κB activation, and prevented Ras-induced epidermal cell senescence and growth arrest (21). On the other hand, Jnk activity is essential for Ras-induced glycolysis, a metabolic feature known to confer survival advantage to cancer (23). Moreover, Jnk2 not only is required for Ras-driven tumorigenesis but also is essential for tumorigenesis of spontaneous SCC cells. Our data indicate that Jnk2 plays a dominant role in human epidermal neoplasia and implicate that selective Jnk2 inhibition promises therapeutic values.

**Materials and Methods**

**Cell culture and gene transfer.** cDNAs encoding the dominant negative c-Jun (DNc-Jun; ref. 24), the constitutively active fusions of MKK7-JNKA1 (Jnk1) and MKK7-JNKA2 (Jnk2; refs. 25, 26), and the kinase-deficient mutants of Jnk1 (Jnk1 [APF]) and Jnk2 (Jnk2 [APF]; ref. 26) were subcloned into the LZR retroviral vector. The shRNA retroviral vectors targeting Jnk1 or Jnk2 and the control vector were obtained from Open Biosystems. Viral production and infection of human keratinocytes were done as described (27). A431 cells (American Type Culture Collection) were cultured in 10% fetal bovine serum/DMEM and transduced in the same manner as above. For gene silencing, transduced cells were selected with 1 μg/mL puromycin for 5 d before cell growth and soft-agar colony assays. Dual luciferase gene reporter assays were done in 293T cells as described (28). Relative luciferase unit was obtained by normalizing the firefly luciferase reading unit to that of the internal renilla-luciferase control and then to the normalized numbers of LacZ control cells.

**Animal studies.** Animal studies were conducted in accordance with protocols approved by the Duke Animal Care and Use Committee. Human skin regeneration was done using CB.17 severe combined immunodeficient (SCID) mice as described (11, 21). Skin biopsies were taken at 8 wk after grafting. Data shown represent two or more sets of independently grafted animals with three to five animals per group.

**β-Galactosidase staining.** Human keratinocytes were fixed in methanol at 48 h after transduction and then stained for the senescence-associated acidic β-galactosidase (Cell Signaling). Blue cells and non-blue cells were counted on photographed microscopic images (n = 10) taken under an Olympus BX41 microscope.

**Immunoblotting and immunohistology.** Protein lysates from surgically discarded human skin and SCC samples (20 μg each) were used for immunoblotting with primary antibodies against phosphorylated Jnk (p-Jnk), Jnk1, Jnk2, and actin (Santa Cruz) followed by IRDye-conjugated secondary antibodies (Odyssey). For immunoprecipitation, 400 μg of each sample were incubated with 2 μg of rabbit antibodies against Jnk1, Jnk2, or granulocyte macrophage colony-stimulating factor (GM-CSF) overnight at 4°C. The precipitated proteins were analyzed by immunoblotting with primary antibodies against p-Jnk (Promega), Jnk1 (Santa Cruz), or Jnk2 (Abcam), followed by a conformation-specific mouse secondary antibody against rabbit (Cell Signaling) and then a horseradish peroxidase–conjugated goat anti-mouse antibody (Chemicon International). For cultured cells, protein lysates (20 μg each) collected 2 d after viral transduction were used for immunoblotting with antibodies against Jnk1, Jnk2, p16, p53 (Genescript), or actin. Nuclei and cytoplasmic fractions were processed as described (29), and 20 μg each were used for immunoblotting with antibodies against RelA, lamin A/C, and actin. Immunohistochemistry and immunofluorescent analyses were done as described (11).

**Real-time reverse transcription-PCR.** Total RNA was isolated from primary human keratinocytes transduced for expression of LacZ, Jnk1, and Jnk2 using Qiagen RNA isolation kit. For gene silencing, cells expressing Jnk2 were transduced with 100 nmol/L siRNA oligonucleotides targeting c-Jun or nonsilencing control (Dharmacon) using RNAi Max reagents (Invitrogen) 24 h before RNA isolation. Standard real-time reverse transcription-PCR (RT-PCR) was done with the following primers: p16, 5′-GGCCGTTTCCAGGTTGTTGGAGTTT-3′ (forward) and 5′-GGCCGTTTCCAGGTTGTTGGAGTTT-3′ (reverse); 18S, 5′-TCTCGGAATCCGAGGCTGAACG-3′ (forward) and 5′-TCTCGGAATCCGAGGCTGAACG-3′ (reverse); and 5′-GGCCGTTTCCAGGTTGTTGGAGTTT-3′ (reverse). Relative mRNA levels of p16 were normalized to that of 18S and presented as fold changes by comparing the normalized data to that of LacZ control cells.

**Glycolysis.** Primary human keratinocytes that have undergone gene transduction were used at 48 h after the final transduction for glycolysis analysis as described (30). The fold changes were obtained by normalizing the number...
of millimoles of glucose per milligram of protein consumed in each sample to that of LacZ control cells. For kinase inhibition, cells transduced for expression of Ras were treated with 10 μmol/L SP600125, LY294002, PD98059, or BI78D3 or 20 μmol/L TAT-JIP peptide for 24 h before glycolysis analysis. All kinase inhibitors were obtained from Sigma.

Results

JNK2 is required for the tumorigenesis of spontaneous SCC cells. Our previous studies have shown that a majority of spontaneous human SCC samples display positive immunostaining for p-JNK (11). In this study, we performed immunoblotting and found that p-JNK was increased in about 70% SCC samples (n = 15) as compared with the normal skin, whereas the total JNK1 and JNK2 were expressed at varying levels in different SCC samples (Fig. 1A; Supplementary Fig. S1A). Antibodies specific for p-JNK1 or p-JNK2 are not available because the JNK activation motif is highly conserved. To determine whether JNK1 and JNK2 are differentially activated in SCC, we performed immunoprecipitation with antibodies targeting JNK1 or JNK2 and then immunoblotting for p-JNK. The validity of this approach was confirmed by the positive detection of an increased level of p-JNK pulled down specifically by the JNK1 antibody from protein lysates of UV-irradiated human keratinocytes (Supplementary Fig. S1B), a finding consistent with a previous report that JNK1 is specifically phosphorylated in response to UV treatment (20, 31). Using this method, we found that the JNK1 antibody pulled down similar levels of p-JNK from protein lysates of normal skin and SCC tissues. In contrast, JNK2 antibody pulled down an average of 2.5-fold higher levels of p-JNK from SCC samples.

Figure 1. JNK2 activation is increased in human SCC and is required for tumorigenesis of A431 SCC cells. A, immunoblotting for p-JNK, JNK1, and JNK2 with protein lysates from normal human skin (NS) and SCC samples. JNK1, JNK2, and p-JNK displayed varying intensities at 46- and 54-kDa MW positions among different tissue samples, but the 46-kDa bands were stronger than the 54-kDa bands in most samples. B, immunoprecipitation with antibodies against JNK1, JNK2, or GM-CSF for IgG control followed by immunoblotting for p-JNK, JNK1, and JNK2. Representative images for normal human skin and three SCCs are shown and additional samples are presented in Supplementary Fig. S1. Columns, average relative optical densitometry of the p-JNK band from nine SCC samples examined; bars, SD. C, A431 soft-agar colony formation in response to JNK inhibition by 10 mmol/L SP600125, expression of JNK1(APF) or JNK2(APF), or shRNA-mediated gene silencing of JNK1 or JNK2. Columns, average numbers of colonies from triplicate experiments; bars, SD. JNK kinase inhibition, expression of the JNK mutants, and targeted gene silencing were confirmed by immunoblotting for p-c-Jun, total c-Jun, JNK1, or JNK2 with actin for loading control, as shown below each graph.
than the normal skin (Fig. 1B; Supplementary Fig. S1C). Although cross-reactivity was observed between JNK1 and JNK2 antibodies when used for immunoprecipitation with human tissue samples, the increased detection of p-JNK in SCC samples following immunoprecipitation with JNK2 antibody was highly significant. Thus, these data indicate that JNK2 activation is increased in human SCC.

We next asked whether JNK is directly involved in tumorigenesis. To address this question, we used pharmacologic and genetic approaches to achieve targeted inhibition of JNK1 and/or JNK2 in A431, a spontaneous SCC cell line, and then performed soft-agar colony formation, an assay commonly used to assess anchorage-independent growth of cancer cells. Pharmacologic inhibition of JNK kinase activity with SP600125 inhibited soft-agar colony formation of A431 cells (Fig. 1C). Targeted inhibition of JNK1 or JNK2 was next performed by expression of their corresponding kinase-deficient mutants, in which the JNK signature motifs ThrProTyr (TPY) have been mutated to AlaProPhe (APF; refs. 26, 32). We found that JNK2(APF), but not JNK1(APF), significantly reduced the number of colonies (Fig. 1C). In line with this, retroviral shRNA–mediated gene silencing of JNK2 markedly decreased the number of colonies, whereas gene silencing of JNK1 induced a minimal effect (Fig. 1C). These findings indicate that JNK2 is specifically required for the anchorage-independent growth of A431 cancer cells.

Coactivation of JNK2 and Ras transforms primary human epidermal cells into malignancy. To further examine whether JNK2 plays a dominant role in skin cancer, we expressed constitutively active recombinant proteins of JNK1 or JNK2 along with the oncogenic Ha-Ras (Gly12Val) mutant in primary human keratinocytes through retroviral transduction. Functional activities of the constitutively active JNK proteins have been characterized previously (25, 26) and were further confirmed using AP-1–driven gene reporter assay (Fig. 2A; Supplementary Fig. S2A). Cells transduced for expression of Ras along with JNK1 or JNK2 were seeded onto devitalized human dermis for human skin regeneration on immunodeficient SCID mice. At 6 weeks after grafting, tissues expressing JNK2 and Ras displayed vertically elevated and crusty appearance resembling clinical features of human SCC (Fig. 2B). Tissue invasion is apparent as indicated by the absence of a clear delineation between epidermis and dermis and the aberrant detection of collagen VII, a protein component...
of the basement membrane structure (Fig. 2C and D). In line with the invasive growth phenotype, the epidermal tissue was hyperproliferative as indicated by the increased number of cells positive in Ki-67, a cell proliferation marker (Fig. 2D). In contrast, tissues expressing JNK1 and Ras displayed normal clinical and histologic features of human skin, including the delineated staining of collagen VII and the basal layer–limited cell proliferation (Fig. 2B–D). These data indicate that JNK2, but not JNK1, is sufficient to transform primary human epidermal cells into malignancy when coupled with oncogenic Ras.

AP-1 family transcription factors are the major downstream targets of JNK. To determine whether AP-1 function is required for JNK2-induced tumor growth, we examined the effect of expression of an NH2-terminal deletion mutant of c-Jun (DNc-Jun), which functions as a dominant negative protein to AP-1 function (24). We found that skin tissues generated with primary human keratinocytes expressing DNc-Jun along with JNK2 and Ras displayed normal histologic appearances comparable to those of control tissues (Fig. 2C and D). These data indicate that JNK2, but not JNK1, is sufficient to transform primary human epidermal cells into malignancy when coupled with oncogenic Ras.

**JNK2 prevents Ras-induced cell senescence.** Oncogenes, including Ras, often induce cell senescence and/or cell growth arrest of primary human cells in the absence of other genetic alterations (33, 34). When expressed in human keratinocytes (Supplementary Fig. S2B), Ras induced up to a 3-fold increase in the number of cells positive in acidic β-galactosidase, a marker associated with senescent cells (Fig. 3A). Expression of JNK2 or siRNA-mediated gene silencing of p16, a cell cycle inhibitor commonly associated with cell senescence (35), prevented Ras induction of senescence (Fig. 3A; Supplementary Fig. S3A). In contrast, expression of JNK1 increased the number of β-galactosidase–positive cells by itself and failed to inhibit Ras-induced cell senescence (Fig. 3A). These data indicate that JNK2, but not JNK1, is able to prevent Ras-induced cell senescence. Immunoblotting and quantitative RT-PCR revealed that Ras increased, whereas JNK2 inhibited, Ras induction of p16 at both protein and mRNA levels (Fig. 3B and C). We predicted that JNK2 acted through AP-1 family transcription factors to regulate p16. Consistent with this idea, siRNA-mediated gene silencing of c-Jun, a predominant AP-1 subunit, diminished JNK2-mediated downregulation of p16 (Fig. 3D). Interestingly, albeit less efficient than JNK2, JNK1 also downregulated p16 mRNA level (Fig. 3C). In contrast to JNK2, JNK1 significantly increased p16 protein level (Fig. 3B), which suggests that JNK1 upregulates p16 at the posttranscriptional level. In line with the in vitro data, tissues expressing Ras and JNK2 expressed much lower levels of p16 than those expressing LacZ or Ras and JNK1 (Supplementary Fig. S3B). Taken together, these findings indicate that JNK2 is able to prevent cell senescence by downregulating p16 mRNA and protein levels in an AP-1–dependent manner.

**JNK prevents Ras-induced NF-κB activation.** NF-κB has been recognized as a major mediator in Ras-induced epidermal

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Figure 3. JNK2 prevents Ras-induced cell senescence. A, staining for senescence-associated acidic β-galactosidase (β-Gal(+)) cells; blue. Columns, average percentages of β-Gal(+) cells; bars, SD. B, semiquantitative immunoblotting for p16 with protein lysates from transduced primary human keratinocytes. Relative optical densitometry is shown below each p16 band. C and D, quantitative RT-PCR for p16 with total RNA isolated from cells transduced as in A with or without additional treatment with siRNA oligonucleotides targeting c-Jun (si-c-Jun) or nonsilencing control (siCon). Gene silencing of c-Jun was confirmed by immunoblotting for c-Jun with actin as control, as shown below the graph. Columns, average of triplicate experiments; bars, SD. Expressions of the exogenous proteins in A to D were confirmed by immunoblotting (Supplementary Fig. S2B).
cell growth inhibition and senescence (21). We thus asked whether JNK2 would be able to suppress Ras-induced NF-κB activation. To address this, we examined the subcellular localization of RelA, a predominant NF-κB subunit that is well known to translocate from cytoplasm to nucleus upon activation (36). As expected, RelA was mostly localized in the cytoplasm in control cells as shown by immunofluorescence staining. In contrast, strong nuclear detection of RelA was seen in cells expressing Ras alone or Ras along with JNK2 and Dnc-Jun, but not in those expressing Ras along with JNK2 (Fig. 4A). Quantitative immunoblotting with nuclear protein extracts revealed that Ras induced up to a 4-fold increase of nuclear RelA, whereas expression of JNK2 diminished this increase (Fig. 4B). Of note, neither Ras nor JNK proteins changed the expression levels of total RelA (Supplementary Fig. S4A). Similar to the findings obtained in the in vitro setting, tumor tissues expressing JNK2 and Ras displayed strong cytoplasmic RelA staining (Fig. 4C), whereas LacZ control tissues had strong nuclei staining especially in the super basal layer where NF-κB has been associated with growth arrest during terminal differentiation (37). These findings indicate that JNK2 inhibits Ras-induced NF-κB activation and suggest that JNK2 activation might provide a mechanism for the cytoplasmic localization of RelA in human SCC, as observed in our previous studies (21).

To confirm the functional significance of JNK2-mediated NF-κB blockade, we expressed RelA along with JNK2 and Ras in primary human keratinocytes through sequential retroviral transduction and then used these cells for skin regeneration on immunodeficient mice. Increased expression

Figure 4. JNK2 inhibits Ras-induced NF-κB activation. A, immunofluorescence staining of RelA in human keratinocytes expressing genes noted on top of each image (orange, RelA; blue, nuclei). Magnification, ×120. B, semiquantitative immunoblotting with cytoplasmic and nuclear protein extracts for RelA with actin and nuclear marker lamin A/C as controls. Relative optical densitometry is shown below each RelA band. C, immunostaining of RelA on regenerated human skin tissues expressing LacZ or JNK2 and Ras (brown, RelA; blue, nuclei). D, clinical and histologic appearances (magnification, ×50) of regenerated human skin grafts. Expressions of the exogenous proteins in A to D were confirmed by immunoblotting (Supplementary Fig. S4A).
of RelA inhibited tumorigenesis driven by Ras and JNK2, as shown by the normal appearance of both clinical and histologic features of the regenerated human skin tissue (Fig. 4D). This result indicates that JNK2 inhibition of NF-κB is essential for Ras-driven epidermal tumorigenesis. To test whether the inhibitory effect of NF-κB is limited to Ras-driven tumorigenesis, we expressed RelA in A431, which harbors a genetic deletion of one p53 allele but has no known abnormalities in the Ras signaling pathway (38). We found that exogenous RelA expression reduced the growth rate of A431 cells (Fig. 5A). Moreover, when assessed by soft-agar colony formation, RelA induced >10-fold reduction in the number of A431 colonies (Fig. 5B). These findings indicate that NF-κB tumor suppressor function is relevant not only to Ras-driven epidermal tumorigenesis but also to those induced by other genetic changes, such as p53 haploinsufficiency.

**JNK is essential for Ras-induced glycolysis.** It is intriguing to observe that JNK2, but not JNK1, is able to block oncogenic Ras induction of epidermal growth arrest and senescence. We asked whether JNK1 and JNK2 affected other hallmark processes of cancer. Among these is glycolysis, namely the Warburg effect or the energy-producing process preferentially used by cancer cells in both aerobic and anaerobic conditions (39, 40). Glycolysis confers a cell survival advantage by providing not only energy but also precursors for DNA and lipid synthesis for the rapidly dividing cancer cells (41). Most importantly, increased glycolysis has been recently identified as a consequence of Ras activation in rat fibroblasts (42). Thus, we performed glycolytic analysis in human keratinocytes. We found that Ras induced up to a 4-fold increase in the rate of glycolysis as compared with the LacZ control, whereas JNK1 and JNK2 each induced about a 2-fold increase in the rate of glycolysis as compared with the LacZ control, whereas JNK1 and JNK2 each induced about a 2-fold
increase (Fig. 6A). JNK inhibition with SP600125, BI78D3, or TAT-JIP and phosphoinositide 3-kinase (PI3K) inhibition with LY294002 each abolished Ras induction of glycolysis (Fig. 6B). In contrast, MAP/Erk kinase (MEK) inhibition with PD98059 or AP-1 inhibition with DN-c-Jun did not induce any inhibitory effect on this process (Fig. 6B). These data indicate that JNK, along with PI3K, is essential for Ras-induced glycolysis. Overall, our findings suggest that JNK2 blocks Ras-induced processes that are inhibitory to tumorigenesis while promoting other oncogenic effects of Ras.

Discussion

The conversion of normal cells to malignancies typically involves multiple genetic or epigenetic perturbations and requires collaborative actions of multiple signaling pathways leading to cell growth autonomy and evasion of cell apoptosis and senescence. In response to a single mitogenic stimulus, normal human cells often initiate cell growth arrest and programmed cell death, which presumably serve as frontline defenses against cancer. Fitting into this scenario is the response of human epidermal cells to oncogenic Ras, which acts in part through NF-κB to induce cell growth arrest and senescence (21). Thus, the oncogenic activity of JNK2 is manifested by downregulation of cell cycle inhibitor p16 and inhibition of NF-κB activation, while permitting many other Ras-induced molecular changes that support cancer development, including increased levels of epidermal growth factor and vascular endothelial growth factor and glycolysis (11).

p16 loss of function occurs in more than 50% of SCC samples due to either genetic or epigenetic changes or transcriptional deregulations (43). Our findings suggest that JNK2-mediated downregulation of p16 might be clinically relevant in human SCC. Interestingly, it has been recently reported that p16 physically interacts with JNK1 and JNK3 and inhibits their kinase activity toward c-Jun in various cell lines (44). In line with these reports, we found that JNK1 was less efficient than JNK2 in inducing c-Jun activation in human keratinocyte (Supplementary Fig. S4A). Of further interest, p16 mRNA level was decreased whereas its protein level was increased by JNK1, indicating that JNK1 increases p16 at the posttranscriptional level. It will be interesting to examine whether JNK1-p16 interaction enhances p16 protein stability while inhibiting JNK1 kinase activity.

With regard to the cross talk between NF-κB and JNK, it has been well established that NF-κB can suppress the JNK pathway (45); less is clear whether JNK affects NF-κB function. We showed that JNK2 suppressed NF-κB activation in a manner that was presumably dependent on AP-1 target genes. Of note, we found that expression of RelA also increased the expression of p16 at mRNA and protein levels (data not shown), which suggests that Ras acts in part through NF-κB to upregulate p16 and cell senescence. However, sequence analysis of the 5-kb proximal region of the human p16 promoter revealed two responsive elements for AP-1 but none for NF-κB (accession no. NG_007485). It is possible that NF-κB increases p16 via suppression of the JNK/AP-1 or other signaling pathways. Overall, findings to date indicate that JNK2 and NF-κB function antagonistically via cross talks at multiple molecular levels and that their functional balance is a critical determinant to cell growth or senescence. Regarding glycolysis, it has been shown that Ras acts through the PI3K-Akt signaling pathway to increase glucose uptake and to activate glycolytic enzymes in rat fibroblasts (42). Similarly, a recent study has indicated a direct role for JNK1 in activating phosphofructokinase-1 in mouse embryonic fibroblasts (46). It is yet to be determined which glycolytic enzymes are directly regulated by JNK and/or PI3K in human keratinocytes.

The positive role for JNK2 in human epidermal neoplasia is consistent with the mouse data showing that Jnk2−/− mice are resistant to skin carcinogenesis (7, 19). However, we observed species-specific responses at two different levels. First, JNK1 was implicated as a tumor suppressor in mouse (19) and human SCC. JNK1 loss of function induced a minimal effect on the tumorigenesis of A431 cells. We speculate that the insufficiency of JNK1 in promoting Ras-driven human epidermal neoplasia is due to the weaker effects of JNK1 on p16 and NF-κB than those of JNK2. Nevertheless, it is worth noting that cells expressing JNK1 and Ras were able to produce viable skin grafts as opposed to the complete graft failure of cells expressing Ras alone (22). This suggests that JNK1 is able to confer a survival and growth advantage to cells expressing Ras, albeit not sufficient to promote invasive tumor growth. Second, the opposite responses between Jnk1−/− and Jnk2−/− mice were attributed to the lower levels of Erk, Akt, and AP-1 activities in Jnk2−/− skin (19). In contrast, human keratinocytes expressing active JNK1 or JNK2 did not show significant differences in Akt or Erk activation (Supplementary Fig. S4A). These data indicate that JNK proteins induce differential molecular responses between mouse and human cells. Overall, our findings establish that JNK2 is a predominant oncogene in human epidermal neoplasia and may represent a potential therapeutic target for human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Hengning Ke, Rebecca Harris, Jonathan L. Coloff, et al.

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