Tumor Necrosis Factor Receptor 1/c-Jun-NH_2-Kinase Signaling Promotes Human Neoplasia

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Abstract

The tumor necrosis factor α receptor (TNFR1) activates downstream effectors that include the mitogen-activated protein kinase kinase 7 (MKK7)/c-Jun-NH_2-kinase (JNK)/activator protein 1 (AP1) cascade. Here, we report that JNK is activated in a majority of spontaneous human squamous cell carcinomas (SCC). JNK pathway induction bypassed cell cycle restraints induced by oncogenic Ras and cooperated with Ras to convert normal human epidermis into tumors indistinguishable from SCC, confirming its oncogenic potency in human tissue. Inhibiting MKK7, JNK, and AP1 as well as TNFR1 itself using genetic, pharmacologic, or antibody-mediated approaches abolished invasive human epidermal neoplasia in a tumor cell autonomous fashion. The TNFR1/MKK7/JNK/AP1 cascade thus promotes human neoplasia and represents a potential therapeutic target for human epithelial cancers. [Cancer Res 2007;67(8):3827–34]

Introduction

Inflammation mediated by the cytokine tumor necrosis factor α (TNFα) and its receptor, TNFR1, is important in autoimmunity and has recently been implicated in neoplasia. Both activator protein 1 (AP1) and nuclear factor-κB (NF-κB) family transcription factors are concomitantly activated by signals transmitted through TNFR1, via c-Jun NH_2-terminal kinases (JNK) and IκB kinases (IKK), respectively (1, 2). Like NF-κB, AP1 subunits, which include Jun, Fos, activating transcription factor, and Fra family members, function as heterodimers or homodimers to regulate expression of a diverse array of genes. JNKs are encoded by three different genes, *Jnk1, Jnk2*, and *Jnk3*, which produce as many as 10 isoforms due to differential splicing (3). JNK3 expression is limited to brain and testis, whereas JNK1 and JNK2 are ubiquitously expressed in multiple tissues, including skin. The immediate upstream activators of JNK are the mitogen-activated protein kinase (MAPK) kinases MKK4 and MKK7, with the latter displaying higher JNK specificity (4).

The role of JNK/AP1 in human tumorigenesis has been controversial due to the complexity of subunit redundancy and their tissue- and species-specific effects (5). Recent murine genetic loss-of-function studies have shown that targets of the JNK signaling cascade, particularly c-Jun and c-fos, may contribute to chemical or UV-induced murine epidermal neoplasia (4, 6–15). Moreover, our recent studies have shown that uncontrolled epidermal proliferation occurs with impairment of NF-κB function and that this process is dependent on TNFR1-JNK-AP1 signaling (16, 17). These findings together suggest that JNK/AP1 action may play a role in inducing abnormal cell growth and proliferation associated with human epidermal carcinogenesis.

Epidermal neoplasias include basal cell carcinoma and squamous cell carcinoma (SCC). SCC is among the most common invasive cancers in the world, with a yearly incidence of 250,000 in the United States (18). Although the molecular mechanisms leading to SCC are likely to be heterogeneous, recent studies have shown that concurrent activation of Ras, a GTPase that is activated frequently in SCC by a variety of mechanisms, along with NF-κB blockade transforms primary human epidermal cells into a malignancy with features indistinguishable from spontaneous SCC (Supplementary Fig. S1; ref. 19). To study the role of the TNFR1/MKK7/JNK/AP1 cascade in human oncogenesis, we examined the effects of gain and loss-of-function of specific cascade components in experimentally induced human SCC. Our findings show that the TNFR1/MKK7/JNK/AP1 cascade promotes human neoplasia in a tumor cell–intrinsic manner and that its blockade at multiple levels abrogates tumorigenesis, indicating that targeted inhibition of this pathway represents a possible therapeutic approach for SCC.

Materials and Methods

Plasmids and gene transfer. To facilitate efficient gene transduction of human primary keratinocytes, the coding regions for active MKK7 (GFP-MKK7/3E) and dominant-negative c-Jun (TAM67) were subcloned into the LZRS retroviral vector through gateway cloning (Life Technologies) for viral packaging in 293T phoenix cells as described in previous studies (20–22). The puromycin-resistant pSM2 retroviral vectors targeting TNFR1 or control were purchased from Open Biosystems. pshMKK7 was cloned by inserting putative MKK7 small interfering RNA (siRNA) oligonucleotide (TAAGCTACTTGAACACAGCGC) into pSM2. Short hairpin RNA retroviruses are packaged in LINX cells (Open Biosystems). For gene transduction, primary human keratinocytes were cultured in KSF (Life Technologies) at a density of 3×10^5 to 4×10^5 cells on 100 mm or 3×10^4 to 4×10^4 on 35-mm dishes for infection with retroviruses encoding LacZ, mutant IκBα (23), active MKK7, or Ras (H-Ras^{G12V}). For multiple gene transductions, keratinocytes were infected sequentially at 12-h intervals with each retrovirus. Protein expression was confirmed by immunoblotting.

Animal studies. Animal experiments included five independently grafted or injected animals per experimental group, with consistent data in all cases for each group studied. Findings representative of at least two independent experiments are shown. For surface skin regeneration, primary human keratinocytes were serially transduced to express genes of interest. Cells were then seeded on devitalized human dermis followed by surface grafting onto immunodeficient severe combined immunodeficient (SCID) mice as described (22). Biopsies were taken from the regenerated skin at 4 to 8 weeks after grafting. JNK inhibition *in vivo* was achieved through daily topical application of SP600125 (1 mg/200 μL DMSO) onto 1-week-old

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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skin grafts for 5 weeks with DMSO solvent as control. For antibody administration, mice bearing 1- or 4-week-old skin grafts expressing Ras and IκBα were administered by weekly i.p. injection of monoclonal antibodies against human IgG control (Jackson ImmunoResearch) or human TNFR1 (R&D Systems, Mab 225; 1 mg for first injection, 500 μg in 200 μL PBS thereafter) for 3 or 4 weeks, respectively. For s.c. tumor growth kinetic analysis, 10^6 cells were suspended in 150 μL culture medium plus 50 μL EMC Matrigel (BD Bioscience) for each injection. For siRNA knockdown, SCC25, A431, or primary keratinocytes expressing Ras and IκBα were transduced with retrovirus targeting TNFR1 or MKK7 followed by selection with puromycin (2 μg/mL) for 2 to 5 days before injection. S.c. tumors were measured weekly for up to 6 weeks. Antibody-mediated TNFR1 blockade in nude mice was achieved as described above for SCID mice. For visceral tumor growth, primary human keratinocytes expressing LacZ or Ras and MKK7 were introduced into SCID mice via tail veil injection (10^6 cells in 100 μL KSF medium). Pulmonary tissues from these mice were harvested 6 weeks postinjection for histologic analysis and cytokeratin expression. Animal studies were conducted in accordance with protocols approved by the Stanford Animal Care and Use Committee.

Immunohistochemistry. For immunoperoxidase staining, 5-μm paraffin sections of SCC tissue microarray (Biomax), regenerated grafts, and pulmonary tissues were deparaffinized, rehydrated, and antigen unmasked by boiling in 50 mmol/L Tris-HCl (pH 9.5) for 15 min. Sections were then incubated with primary antibodies, including rabbit against pJNK (Promega), pc-Jun (Cell Signaling Technology), or cytokeratin 14 (DAKO) followed by biotin-conjugated secondary and 3,3′-diaminobenzidine detection (LabVision). For immunofluorescence staining, 5-μm cryosections were incubated with primary antibodies against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR; Santa Cruz Biotechnology, Santa Cruz, CA), human type VII collagen (Chemicon), Ki-67 (LabVision), or desmoglein 3 (Chemicon) followed by Cy2/3-conjugated secondary antibodies (Jackson ImmunoResearch). Tissue sections were then counterstained with Hoechst 3342 (100 μg/mL in PBS). Images were taken under a Zeiss Axiovert microscope, and mitotic indices were calculated from the number of Ki-67(+) cells/100 μm basement membrane zone (BMZ). pJNK staining was graded by two independent observers according to the percentage of number of tumor cells positive with nuclei pJNK staining: >75% (strong), 25% to 75% (moderate), and <25% (negative).

Gene expression. Total RNA (5 μg) isolated from primary keratinocytes expressing genes of interest was used to generate biotin-labeled cRNA. Fragmented cRNA was then hybridized on Affymetrix human Genechip (U133A2.0 plus) and analyzed by Gene Spring software (accession no. GSE 65959). For immunoblotting, protein lysates were immunoblotted with antibodies against cyclinA, cyclinB1, cyclinB2, cdc2, TFDP1, actin and total Rb (Santa Cruz Biotechnology), cdc6 (Abcam), p-Rbs (Cell Signaling Technology), and CDK4 (Upstate Technology). For *AP1* gene reporter analysis, 293T cells were cotransfected in triplicates with retroviral vectors encoding LacZ, active MKK7 or dominant-negative c-Jun (DNc-Jun) along with AP1-driven firefly luciferase, and cytomegalovirus (CMV)–driven renilla-luciferase constructs using FuGene-6 transfection reagents (Roche). Luminometer dual-luciferase readings (Promega) were taken at 48-h posttransfection, and relative luciferase reading units were obtained by normalizing the readings of interest to that of LacZ control cells. For *AP1* gene reporter analysis in primary human keratinocytes, isolated keratinocytes were transduced with retroviruses for expression of Ras, MKK7, Ras, and MKK7 or LacZ. Cells were then trypsinized and transfected in triplicates by nucleofection (Amaxa) with AP1-driven firefly-luciferase and CMV-driven renilla-luciferase constructs followed by dual-luciferase assay at 48-h posttransfection.

Results

JNK activation occurs in spontaneous human SCC. To study the frequency of JNK induction in human SCC, we probed tissue microarrays of spontaneous human SCC samples (n = 52) with antibodies directed against the phosphorylated active forms of JNK. Eighty-one percent of SCCs examined exhibited either strong or moderately strong induction of phosphorylated JNK (pJNK) over normal tissues (Fig. 1*A*). JNK activation was also detected in genetically defined experimental epidermal neoplasia generated from primary human keratinocytes expressing Ras and IκBα (Fig. 1*B*). Furthermore, high levels of pJNK were found in s.c. tumors produced with cell lines derived from spontaneous human SCCs, including SCC25 (24), A431 (25), and CAL27 (ref. 26; Supplementary Fig. S2). Thus, JNK is activated in both spontaneous and experimentally induced human SCC.

Inhibition of JNK/AP1 cascade elements inhibits human epidermal tumorigenesis. Although JNK activation is seen in SCC as well as in other diseases, such as glioma, osteosarcoma, prostate carcinoma, and psoriasis (26–29), a functional requirement for this cascade in human tumorigenesis has not been clearly defined. To address this, we first studied the effect of a JNK-specific inhibitor, SP600125 (30), which has been shown to inhibit JNK function and epidermal hyperplasia in epidermis expressing IκBα or deficient in *relA* (16). Inhibition of the JNK cascade in epidermal tissue by

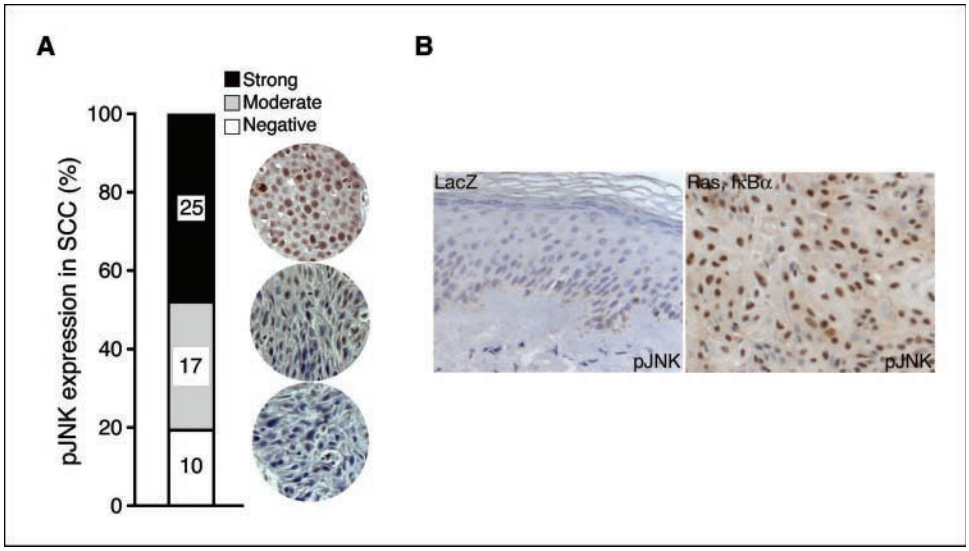

Figure 1. JNK cascade activation occurs in spontaneous human SCC. *A,* immunoperoxidase staining of tissue microarrays of patient SCCs (n = 52). Samples were graded as strong, moderately strong, or negative, with representative histology shown: pJNK (*brown*) and nuclei (*blue*). Magnification, ×100. *B,* immunoperoxidase staining of 6-week-old skin grafts regenerated with keratinocytes expressing LacZ or Ras and IκBα for pJNK. Magnification, ×100.

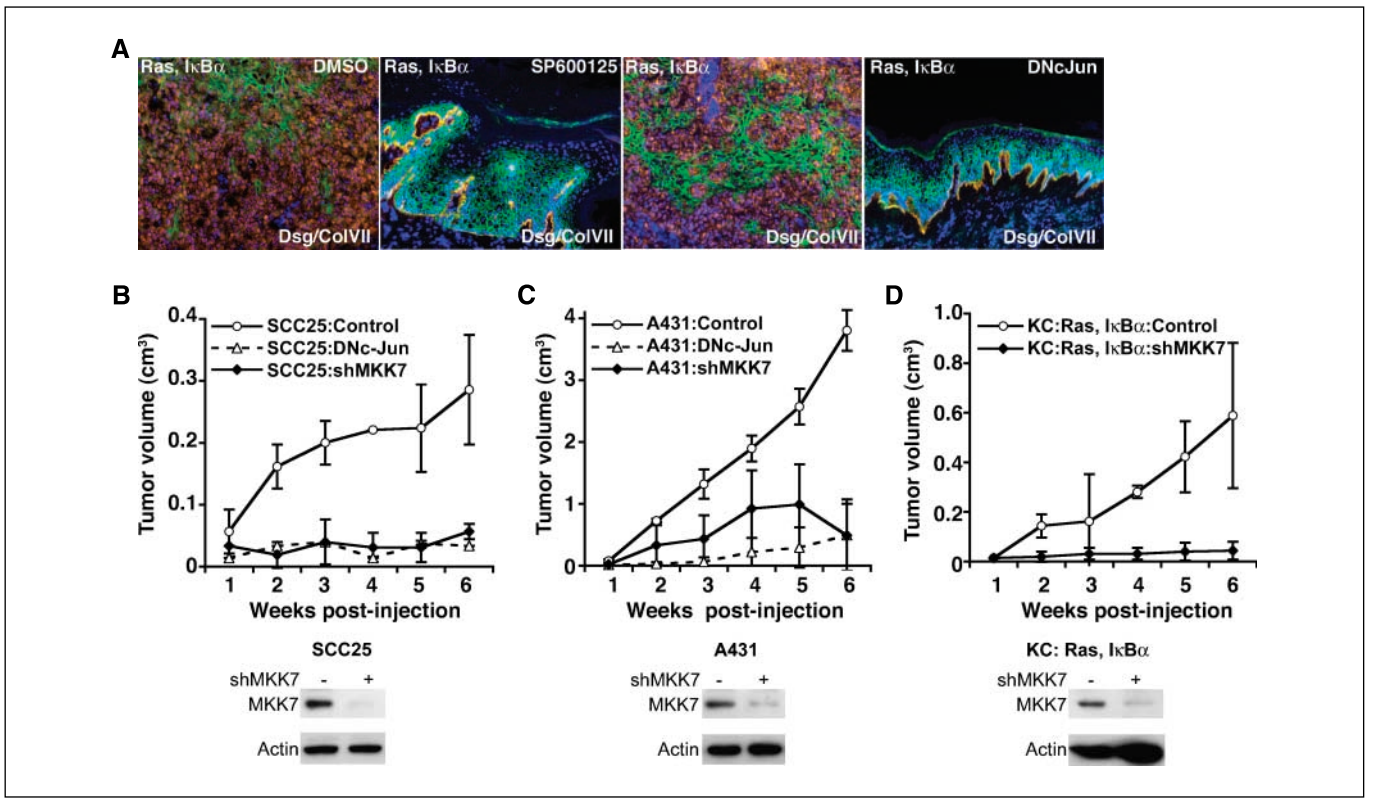

Figure 2. JNK/AP1 activity is required for epidermal neoplasia. *A,* blockade of JNK-AP1 function prevents Ras-driven tumorigenesis on surface skin grafts. Immunostaining of 6-week-old skin grafts expressing Ras and IκBα treated with topical DMSO vehicle or JNK inhibitor SP600125 in DMSO or grafts expressing Ras and IκBα with or without DNc-Jun [type VII collagen (*ColVII, orange*; BMZ marker), desmoglein-3 (*Dsg, green*; cell-cell junctional protein, serves as a human epithelial marker), and nuclei (*blue*, Hoechst 3342)]. Note the intact linear BMZ in tissues treated with SP600125 or expressing DNc-Jun. Magnification, ×40. *B* to *D,* DNc-Jun or MKK7 knockdown inhibits s.c. tumor growth of SCC25 (*B*), A431 (*C*), and primary keratinocytes (*D*) expressing Ras and IκBα in immunodeficient nude mice. Reduced expression of MKK7 is confirmed by Western blot analysis with actin serving as a loading control, as shown below each graph.

SP600125 was confirmed by immunostaining for the activated target molecule phospho-c-Jun (Supplementary Fig. S3). Ras-driven epidermal neoplasias generated on the surface of immunodeficient SCID mice were treated topically with DMSO vehicle or SP600125 daily for 5 weeks. DMSO and untreated control tissues displayed typical findings of neoplasia, including invasion and disruption of the BMZ, as visualized by type VII collagen staining (Fig. 2*A*). In contrast, tissues treated with SP600125 lacked invasive neoplasia and retained sharp delineation of the BMZ, with a human-specific desmoglein-3 antibody confirming the human origin of these tissues (Fig. 2*A*). To confirm this pharmacologic finding, we next studied the effects of inhibiting JNK cascade action through expression of the dominant-negative c-Jun TAM67 (DNc-Jun) that inhibits the function of AP1, the major target of JNK (7). DNc-Jun completely inhibited Ras-driven human epidermal tumorigenesis (Fig. 2*A*), suggesting that tumor cell–intrinsic AP1 function is required for this process. Thus, both pharmacologic and genetic approaches suggest that JNK/AP1 function is required for Ras-driven human epidermal tumorigenesis.

To investigate whether JNK/AP1 dependency is a general process common to other SCC cell types, we expressed DNc-Jun in spontaneous human SCC cell lines, A431 and SCC25, and found that DNc-Jun significantly inhibited their s.c. tumor growth as well (Fig. 2*B–C*). Furthermore, retroviral-mediated RNA interference knockdown of MKK7, the upstream activator of JNK, also prevented s.c. tumor growth of A431, SCC25, and primary keratinocytes transformed by Ras and IκBα (Fig. 2*B–D*). MKK7/JNK/AP1 function is thus necessary for human epidermal neoplasia and operates in a tumor cell–intrinsic fashion.

JNK cascade induction promotes Ras-driven epidermal neoplasia. We next asked whether components of the JNK cascade could directly promote human epidermal neoplasia. We expressed either active MKK7 or c-Jun in primary keratinocytes and examined their capacity to promote tumorigenesis in combination with Ras in the absence of IκBα. First, we confirmed induction of AP1-dependent gene reporter activity by MKK7 *in vitro* (Supplementary Fig. S4). Surface skin grafts were then regenerated with primary keratinocytes expressing MKK7 and Ras; these developed into clinical tumors resembling SCC (Fig. 3*A*). Dermal invasion, one of the hallmarks of spontaneous human SCC, is apparent in tumors induced by MKK7 and Ras as revealed by both histologic appearance and BMZ disruption (Fig. 3*B–C*). MKK7 and Ras-induced neoplasia depends on intact AP1 function as concurrent expression of DNc-Jun prevents tumorigenesis (Fig. 3*A–C*). Thus, active MKK7 acts in conjunction with Ras to directly convert human epidermal tissue into malignancy in an AP1-dependent manner.

Capacity for growth in nonphysiologic environments is a feature of malignant tumors. We therefore next tested the capacity of active MKK7 and Ras to produce s.c. tumors. These cells were able to form tumors and displayed growth kinetics similar to those of cells transduced with Ras and IκBα (Fig. 3*D*). In contrast, cells

expressing LacZ, MKK7, or Ras alone do not produce measurable tumors. Cells expressing MKK7 and Ras are also capable of pulmonary dissemination when introduced i.v. Mice display a wasting process and an increase in mortality at 4 weeks postinjection due to the expanding tumor burden within the pulmonary parenchyma (Fig. 3*E*). Tumor nodules display SCC morphology and keratin 14 expression, confirming their epidermal cell origin (Fig. 3*E*). Of interest, although Ras by itself activates AP1, coexpression of Ras with MKK7 induces significantly higher levels of AP1 activity in primary human keratinocytes than either one alone (Supplementary Fig. S5), indicating that Ras and MKK7 act in synergy to induce downstream signaling. Overall, these findings indicate that activation of JNK cascade components is sufficient to promote malignant cell transformation of primary human keratinocytes in synergy with oncogenic Ras.

JNK cascade induction rescues Ras-induced epidermal cell growth arrest. Oncogenic Ras alone induces growth arrest in primary human keratinocytes in the G_1 phase of the cell cycle (19, 31). We therefore predicted that one of the necessary roles of the JNK cascade in Ras-induced tumorigenesis might be to promote escape from G_1 growth restraints. To examine the effects of JNK cascade induction on epidermal homeostasis, we regenerated human skin grafts with epidermal expression of active MKK7 alone on SCID mice. Epidermal tissues expressing MKK7 display hyperplasia and an increased mitotic index, indicating a positive effect on epidermal cell cycle progression, whereas tissue expressing MKK7 alone show some irregular areas of downgrowth into the epidermis, the tissue remains contiguously connected above the basement membrane and was noninvasive (Fig. 4*A*). MKK7 bypassed cell cycle G_1 arrest induced by Ras, as shown by flow cytometry (Fig. 4*B*). Thus, MKK7 promotes epidermal cell proliferation and rescues Ras-induced cell cycle arrest.

To further characterize molecular targets of MKK7 action that facilitate escape from G_1 restraints triggered by oncogenic Ras, we did gene expression profiling using mRNA isolated from primary keratinocytes expressing either Ras or MKK7 alone versus Ras and MKK7, with and without DNc-Jun. Oncogenic Ras changed the expression of ~300 genes (>3-fold), whereas MKK7 opposed

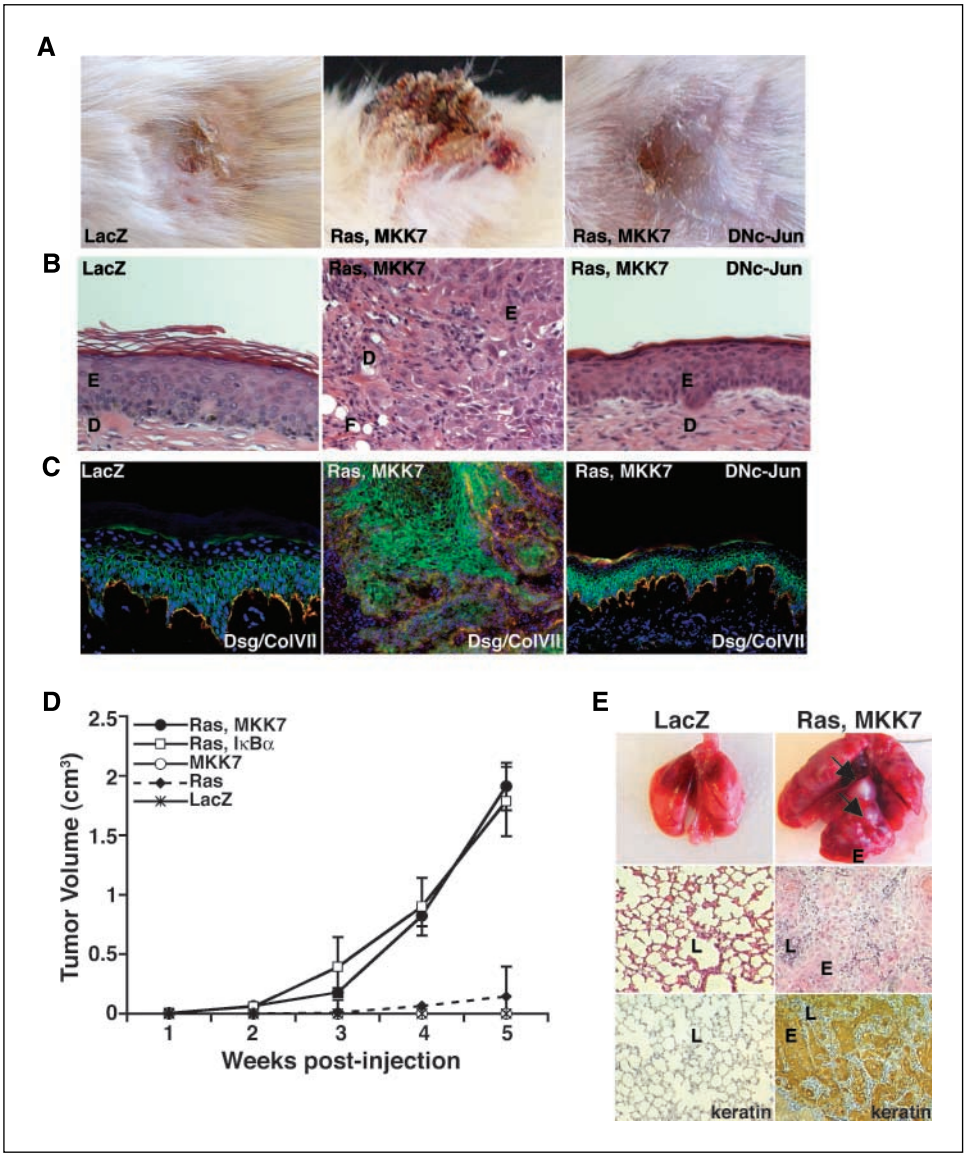

Figure 3. MKK7/JNK/AP1 cascade promotes Ras-driven epidermal neoplasia. *A,* clinical appearance of regenerated skin grafts. *B,* histologic appearance of grafted tissues (*E,* epidermal tissue; *D,* dermis; *F,* fat). Magnification, ×40. *C,* abnormal tissue polarity of neoplasia induced by Ras and MKK7 [type VII collagen (*orange*), desmoglein-3 (*green*), and nuclei (*blue*, Hoechst 3342)]. Magnification, ×40. *D,* s.c. tumor growth kinetics of primary human keratinocytes expressing LacZ, MKK7, Ras, Ras and MKK7, or Ras and IκBα. *Points,* tumor volume, from three independent experiments; *bars,* SD. *E,* visceral dissemination of primary keratinocytes expressing Ras and MKK7 to pulmonary tissue. Appearance of lungs from mice injected i.v. with cells transduced with LacZ or Ras and MKK7. Cross-sections of the lung tissue exhibit epithelial tumors expressing epidermal cytokeratins (*brown*; *L,* lung tissue). Magnification, ×20.

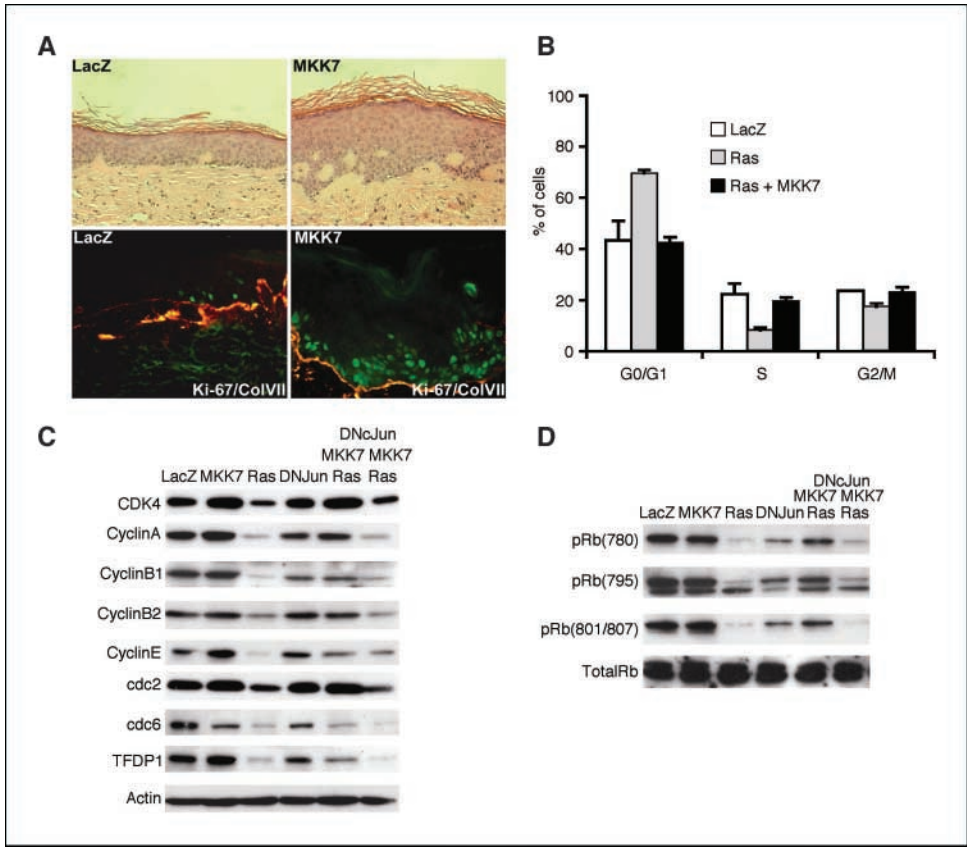

Figure 4. JNK cascade induction rescues Ras-induced cell growth arrest. *A,* skin grafts regenerated on SCID mice with human keratinocytes expressing MKK7 display hyperplasia and hyperproliferation [*top,* H&E; *bottom,* Ki-67 (*green*); type VII collagen (*orange*)]. Magnification, ×40. *B,* MKK7 bypasses Ras-induced G_1 arrest. Cell cycle distribution analysis by flow cytometry of primary human keratinocytes expressing LacZ, Ras, or Ras and MKK7. *C* and *D,* confirmation for cell cycle regulator expression using Western blotting of protein extracts from keratinocytes expressing LacZ, MKK7, Ras, MKK7, and Ras with or without DNc-Jun.

Ras-induced changes in half of those genes. Among these are a group of cell cycle regulators that are significantly down-regulated by Ras and restored by MKK7, including cyclinA, cyclinB1, cyclinB2, cdc2, cdc6, and TFDP1 (GEO accession no. GSE6559); these mRNA changes were confirmed at the protein level by immunoblotting (Fig. 4*C*). CDK4 is a cyclin-dependent kinase promoting G_1 progression previously shown to be posttranscriptionally down-regulated by Ras, a finding whose functional importance has been confirmed because CDK4 can bypass Ras-induced growth arrest (31). CDK4 protein was also restored to near-normal levels by MKK7. Phosphorylated Rb, which reflects active cell cycle progression, was also decreased by Ras, a change that was also reversed by MKK7 (Fig. 4*D*). DNc-Jun prevents the restoration of those progrowth cell cycle regulators by MKK7, indicating that MKK7 action depends on intact AP1 function.

Consistent with the uncontrolled proliferation seen in cancer, human epidermal neoplasia induced by Ras and IκBα, as well as by Ras and MKK7, displays >10-fold increases in mitotic index, compared with LacZ control tissues (Supplementary Fig. S6*A* and S6*B*). Genetic and pharmacologic blockade of AP1 function in these settings reduced cell proliferation toward normal tissue levels (Supplementary Fig. S6*C* and S6*D*). AP1 inhibition abolished hyperproliferation driven by combined MKK7 and Ras action but did not cause graft failure as observed previously with expression of Ras alone (31), indicating that AP1 blockade does not completely abolish MKK7 effects. In support of this, cyclin E expression was minimally responsive to DNc-Jun (Fig. 4*C*). Of interest, JNK-AP1 inhibition does not induce apoptosis, as revealed by histologic analysis and terminal deoxyribonucleotide transferase–mediated nick-end labeling staining (data not shown). Taken together, these findings indicate that MKK7-JNK-AP1 signaling promotes Ras-driven epidermal tumorigenesis by facilitating escape from cell growth restraints.

TNFR1 blockade abrogates invasive neoplasia. The specificity and favorable pharmacokinetics of therapeutic antibodies have contributed to their clinical success (32), with the most accessible targets being extracellular proteins, such as cell surface receptors. TNFR1, the membrane-bound receptor for TNF, acts upstream of the JNK cascade, and is thus a potential target for inhibition of MKK7/JNK/AP1 function. In support of this, TNFR1 has recently been implicated in murine epidermal tumorigenesis. Genetic deletion of *Tnfr1* diminished occurrence of neoplasia in mice induced by both NF-κB blockade and chemical carcinogenesis (33, 34). Moreover, recent findings showed that epidermal NF-κB blockade relieves constitutive repression of JNK activity by the NF-κB RelA subunit and that TNFR1 is required both for the JNK induction as well as the epidermal hyperplasia that occurs in this setting (16, 17). These findings suggest that TNFR1 activity may be functionally important in human epidermal tumorigenesis.

To address this possibility, we treated SCID mice bearing human skin grafts expressing Ras and IκBα with mouse antibodies against TNFR1. Antibodies were delivered via weekly i.p. injection for 3 to 4 weeks starting either before tumor formation (1 week postgrafting) or after tumors were established (4 weeks postgrafting) to distinguish effects on tumor establishment from those on tumor maintenance. Tissues treated with control antibody displayed invasive neoplasia with BMZ disruption and tissue depolarization at both 4- and 8-week time points (Fig. 5*A* and *B*). In contrast, tissues treated with TNFR1 antibody, although hyperplastic, retained BMZ structure and proper desmoglein-3 expression, indicating

intact tissue polarity (Fig. 5*A* and *B*). Blockade of TNFR1 signaling was accompanied by the absence of detectable pJNK in the tissues treated by the TNFR1 antibody (Supplementary Fig. S7). In addition, TNFR1 blockade reduced the mitotic index and limited mitotic cells to the basal layer, whereas proliferating cells were widespread and abundant in the control tumor tissue (Fig. 5*C* and *D*). These findings indicate that antagonizing TNFR1 function prevents Ras-driven tumorigenesis.

Because Ras-MAPK activation and signs of NF-κB loss-of-function occur in a substantial proportion of spontaneous SCCs arising in humans (19), these findings indicate that TNFR1 signaling may be important for SCC in general. In agreement with this, antibody-mediated TNFR1 blockade significantly inhibited s.c. tumor growth of three independent SCC tumor lines, A431, SCC25, and CAL27 (Supplementary Fig. S8*A*). To determine if TNFR1 action in this context is tumor cell intrinsic, we used retroviral-mediated RNA interference to decrease TNFR1 expression. Reduced levels of TNFR1, as confirmed by immunoblotting, significantly inhibited s.c. growth of Ras-driven tumors and SCC lines (Supplementary Fig. S8*B*). This indicates that TNFR1 action is tumor cell autonomous and supports the idea that approaches to inhibit TNFR1 function represent a potential strategy to treat human SCC.

Discussion

This work indicates that the TNFR1/MKK7/JNK/AP1 signaling cascade can functionally promote tumorigenesis in human tissue. Intervention at different levels via multiple approaches, including (*a*) TNFR1 inhibition by antibody blockade and RNA interference, (*b*) MKK7 down-regulation by RNA interference, (*c*) pharmacologic JNK inhibition, and (*d*) dominant-negative interference with AP1 function, inhibits Ras-driven tumorigenesis of primary keratinocytes as well as neoplastic growth of human SCC cell lines.

Therapeutics targeting both NF-κB and JNK-AP1 function are being developed to treat human cancer and other diseases, with the JNK/AP1 cascade recently proposed as a "gold mine" for therapeutic targets (35). Efforts to date are primarily based in murine models (12). The relevance of purely murine studies, however, is sometimes limited by species-intrinsic differences (36). To rationally develop targeted therapeutics, it may thus be helpful to assess effects of each signaling component in human tissue tumor models.

MKK7 and Ras-transformed malignant keratinocytes, like those expressing Ras and IκBα (19), are genetically stable and free of genomic abnormalities commonly observed in many transformed cell lines, as revealed by karyotyping (data not shown), indicating that the malignancy induced by MKK7 and Ras is not triggered by global genomic catastrophe. This suggests that tumorigenesis reflects direct JNK cascade effects. In contrast to many other tissues, epidermal cells display opposite responses to IKK-NF-κB and JNK-AP1 activation by inducing cell growth arrest and hyperproliferation, respectively. Unlike NF-κB, which does not affect differentiation (16, 23), AP1 proteins regulate the epidermal differentiation program. The balance of these two signaling cascades and the combined cellular effects orchestrated by subtle differences in AP1 heterodimerization may ultimately regulate homeostasis of epidermal tissue (16, 17, 37, 38). This possibility is further supported by the differential expression status of AP1 subunits in normal human epidermis as well as psoriatic tissues (37–39). In addition, JNK isoforms have also been shown to induce differential cellular effects (40–42). Consistent with these notions, the role of MKK7/JNK/AP1 in epidermal differentiation appears to be complex and is tissue context dependent, as indicated by the findings revealing that DNc-Jun restores epidermal differentiation of otherwise tumor tissues expressing Ras and IκBα, whereas MKK7 has minimal adverse effect on epidermal differentiation (Supplementary Fig. S9). Therapeutics that discriminate between

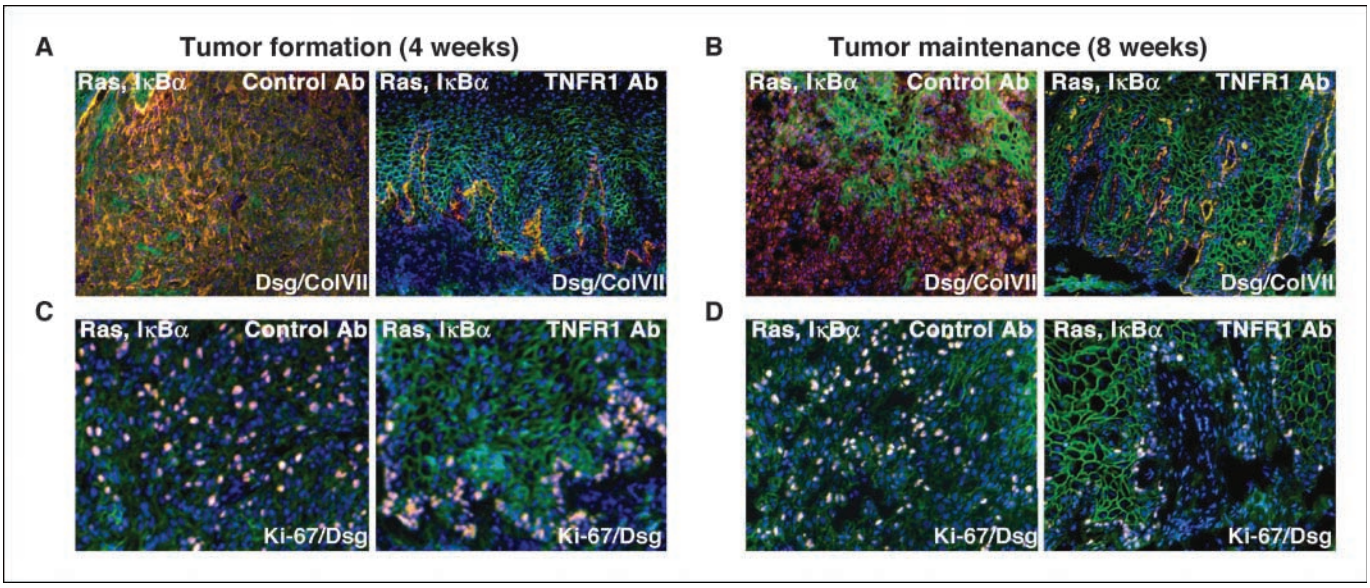

Figure 5. Blockade of TNFR1 with inhibitory antibodies abrogates invasive neoplasia. *A* and *B,* TNFR1 blockade prevents invasive neoplasia. Immunostaining of 4- and 8-week-old skin grafts expressing Ras and IκBα treated with control IgG or inhibitory TNFR1 antibody during weeks 1 to 4 and 4 to 8, respectively [type VII collagen (*orange*), desmoglein-3 (*green*), and nuclei (*blue*, Hoechst 3342)]. Note the tissue polarity and the clear boundary between epidermal and dermal cells in tissues treated with TNFR1 antibody in contrast to the disorganized and invasive neoplasia in control tissue. Magnification, ×40. *C* and *D,* TNFR1 blockade inhibits hyperproliferation [Ki-67 (*orange*), desmoglein-3 (*green*), nuclei (*blue*, Hoechst 3342)]; note the tissue repolarization indicated by Ki-67[+] cells to the BMZ upon anti-TNFR1 treatment. Magnification, ×100.

NF-κB and JNK signaling pathways and different JNK/AP1 proteins therefore represent a potential future therapeutic opportunity.

Activation of MKK7-JNK-AP1 along with Ras induces a collection of molecules that produce hallmark features of cancer (43), including those involved in angiogenesis, tissue invasion, and resistance to apoptosis, unlimited cell proliferation, as well as escape from cellular senescence. For example, both EGFR and VEGF are highly induced in tumor tissues expressing MKK7 and Ras (Supplementary Fig. S10). Critical genetic events that facilitate escape from Ras-induced cellular senescence, as recently observed with the oncogenic activity of cdc6 (44), also seem to operate via MKK7-JNK in epidermal neoplasia. Activating mutations in Ras genes themselves occur in a wide array of tumors but not always frequently enough to account for the majority of sporadic cancers. In contrast, activation of the Ras-MAPK and JNK-AP1 pathways is detected in the majority of epidermal cancers examined, and this may represent a convergent point for protumor growth effects induced by many other genetic or cellular changes. Therefore, inhibiting these two signaling cascades may represent a potential strategy for treating a broad range of epithelial cancers.

Therapeutics targeting TNFα/TNFR1 function have proven beneficial in treating an array of diseases associated with chronic inflammation (32). Two currently approved agents, infliximab and adalimumab, are humanized antibodies designed to deplete human TNFα, the ligand of TNFR1. We observed that both agents, when used at a dosage comparable with and even higher than the dosage used successfully to treat arthritis in human-mouse chimeric models (45, 46), failed to inhibit growth of human tumors in mice. In contrast to blocking antibodies to human TNFR1, however, these antibodies to human TNFα had minimal effects on JNK-AP1 activation in tumor tissues (Supplementary Fig. S11). This may be due to either the action of mouse TNFα from surrounding tissue or the inability of these antibodies to sequester TNFα trimers away from the cell surface, as shown by recent reports (46, 47). In cancer, TNFR1 may therefore be the preferred target rather than TNFα, as recently proposed (48). Of interest, TNFR1-mediated signaling is associated with inflammation, a process directly linked to cancer in other systems. However, the links between inflammation and skin cancer may be more complex than initially envisioned (49). Although it remains unclear whether immunocytes in SCC also supply pathogenically relevant amounts of TNFα, as it seem to occur in inflammatory psoriatic lesions (50), it is evident that epidermal cells can produce TNFα (16). Our findings point to an epidermal cell-autonomous role for TNFR1 in cancer promotion in epithelia because TNFR1 blockade selectively in epidermal tumor cells with siRNAs prevents neoplasia. These findings suggest that blockade of the TNFR1/MKK7/JNK/AP1 signaling cascade is potentially a promising approach for treating human epithelial cancers, such as SCC.

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