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Survivin expression in normal human bronchial epithelial cells: an early and critical step in tumorigenesis induced by tobacco exposure

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The inhibitor of apoptosis protein survivin is selectively expressed in tumor cells. The tobacco component nicotine increases the transcription of the survivin gene in non-small cell lung cancer cells. However, the role of survivin expression induced by tobacco component is not clear during lung carcinogenesis. We investigated the effects of the tobacco components nicotine and its related carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on survivin expression in normal human bronchial epithelial (NHBE) cells and examined the role of survivin in the malignant transformation of normal human bronchial epithelial (HBE) cells induced by these components. We found that survivin messenger RNA (mRNA) expression was detected in 41% (7 of 17) of bronchial brush specimens from heavy smokers. Nicotine and NNK increased survivin mRNA and protein expression levels in primary cultured NHBE cells and immortalized HBE cells. Bronchial epithelium in mice administered NNK also showed increased staining for survivin. Nicotine and NNK stimulated the Akt–mammalian target of rapamycin (mTOR) pathway in NHBE cells, leading to increased *de novo* synthesis of survivin protein. Induced survivin expression increased the survival potential of the cells, which was blocked by transfection with survivin-specific small interfering RNA (siRNA). siRNA-induced down-regulation of survivin expression also suppressed the tumorigenic potential of premalignant and malignant HBE cells exposed to the tobacco components. These findings suggest that NNK and nicotine induce survivin protein synthesis in NHBE cells by activating the Akt–mTOR pathway and thus blockade of the pathway effectively inhibits the tobacco-induced malignant transformation of HBE cells.

Introduction

Lung cancer is the leading cause of cancer death in the US and worldwide. More than one million new cases are diagnosed globally every year (1). Exposure to tobacco smoke is the most significant risk factor for lung cancer. Genotoxic metabolites of tobacco components, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polyaromatic hydrocarbons, cause mutations in vital genes that lead to neoplastic transformation (2,3). Cells containing mutated DNA are normally eradicated via apoptosis; however, activation of cell survival mechanisms can disrupt the cell death process, resulting in a loss of control over cell growth and ultimately leading to tumor initiation and progression (4). Nicotine and its related carcinogens have been shown to activate Akt, whose phosphorylation of several key cell survival- and cell cycle-related molecules is critical to tumor progression (5,6).

Abbreviations: BaP, benzo[a]pyrene; EGF, epidermal growth factor; HBE, human bronchial epithelial; IAP, inhibitor of apoptosis protein; KSFM, keratinocyte serum-free medium; MEF, mouse embryo fibroblast; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; mut, mutant; NHBE, normal human bronchial epithelial; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NSCLC, non-small cell lung cancer; pAkt, phosphorylated Akt; PI3K, phosphoinositol-3 kinase; siRNA, small interfering RNA; TSC2, tuberous sclerosis complex 2; WT, wild-type.

Therefore, identifying molecules that are induced by tobacco components and have a key role in the survival of premalignant human bronchial epithelial (HBE) cells with genetic or epigenetic alterations would be an important step in developing effective strategies for lung cancer chemoprevention and treatment.

One such molecular marker may be survivin, a member of the inhibitor of apoptosis protein (IAP) family, which is involved in cell division and inhibits apoptosis by directly binding to caspases-3, -7 and -9 and promotes cell survival against a variety of apoptotic stimuli, including conventional chemotherapeutic drugs (7–9). Survivin is strongly expressed in embryonic organs but rarely expressed in most normal adult tissues (8,10,11). However, survivin is frequently over-expressed in human cancers, including lung cancer, and it is associated with tumor progression. Survivin expression is regulated at the transcriptional and post-translational levels. Recent findings have shown that nicotine activates survivin transcription in lung cancer cells via increased recruitment of the transcription factor E2F1 and concomitant dissociation of the retinoblastoma tumor suppressor protein (Rb) from the survivin promoter and that the Akt pathway is involved in the nicotine-mediated induction of survivin gene transcription (7). Akt also mediates growth factor-induced survivin expression (12). Previous studies of normal human bronchial epithelial (NHBE) cells *in vitro* and *in vivo* have revealed that Akt activation is an early event in tobacco-induced lung carcinogenesis: the expression of phosphorylated Akt (pAkt) increases on treatment with tobacco components such as nicotine and NNK (6,13,14). In this light, we hypothesized that survivin expression is induced and plays a role during tobacco-induced lung carcinogenesis.

This study was designed to investigate whether the tobacco components nicotine and NNK can induce survivin expression in NHBE cells and whether survivin plays a role in the malignant transformation of these cells. In addition, we investigated the mechanism by which survivin expression is regulated by nicotine and NNK in NHBE cells.

Materials and methods

Cells and reagents

For our study, we used SV40 large tumor antigen-immortalized BEAS2B HBE cells, premalignant (1799 and 1198) HBE cells and malignant (1170-I) HBE cells (gifts of Dr A.Klein-Szanto, Fox Chase Cancer Center, Philadelphia, PA) (15). We also used the non-small cell lung cancer (NSCLC) cell lines H1299, H460, A549, H596, H661, H322, H226B and H226Br (all from American Type Culture Collection, Rockville, MD), NHBE cells (Cambrex Bio Science Walkersville, Walkersville, MD) and $TSC2^{-/-}/p53^{-/-}$ and $TSC2^{+/+}/p53^{-/-}$ mouse embryo fibroblasts (MEFs) (gifts of Dr D.J.Kwiatkowski, Brigham and Women's Hospital, Boston, MA) (16). pSilencer 2.1-U6 neo small interfering RNA (siRNA) expression vectors (Ambion, Austin, TX) containing wild-type (WT) or mismatched mutant (mut) survivin siRNA were gifts of Dr L.Yang, Emory University, School of Medicine, Atlanta, GA. The following reagents were used: NNK (Midwest Research Institute, Kansas City, MO); nicotine, benzo[*a*]pyrene (BaP), rapamycin and corn oil (Sigma Chemical Co., St Louis, MO) and LY294002 (Cell Signaling Technology, Beverly, MA).

Western blot analysis

Total protein was isolated and subjected to western blot analysis as described previously (17). The following antibodies were used for the western blots: mouse monoclonal antibodies detecting survivin; rabbit polyclonal antibodies detecting pAkt (Ser473), pAkt (Thr308), pmTOR (Ser2448), $pp70^{S6K}$ (Thr389), p4E-BP1 (Thr70) or pS6 (Ser235/236) (Cell Signaling Technology); rabbit polyclonal antibodies detecting unphosphorylated Akt, mammalian target of rapamycin (mTOR), $p70^{S6K}$, 4E-BP1, S6 and XIAP (Cell Signaling Technology); a rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit anti-mouse IgG–horseradish peroxidase conjugate, a donkey anti-rabbit IgG–horseradish peroxidase conjugate and a rabbit anti-goat IgG–horseradish peroxidase conjugate (Santa Cruz Biotechnology).

The membranes were washed extensively, the proteins were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and the proteins were quantified using the National Institutes of Health Image software program (version 1.61; Bethesda, MA). Representative results from two separate experiments were reported.

Immunohistochemical analysis

Bronchial tissue specimens from A/J mice were fixed in 10% formaldehyde, embedded in paraffin, sectioned into 5 μm-thick slices and processed for immunohistochemical analysis according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA) as described previously (17). The primary antibody against survivin (Santa Cruz Biotechnology) was used.

Staining intensity was rated as 0 (none), 1 (low), 2 (moderate) or 3 (strong). The percentage of cells at each level of staining intensity was determined by scanning a 200 μm-wide specimen of bronchial epithelium. Survivin levels were expressed in terms of cytosolic and nuclear scores. Immunostaining scores were derived by multiplying the percentage of cells at each intensity by the intensity value (0–3) and then adding the scores. All findings were evaluated and scored independently by a pathologist blinded to the experimental mouse treatment conditions and to all patient information.

Reverse transcriptase–polymerase chain reaction

Using TRIzol reagent (Invitrogen, Carlsbad, CA), we isolated total RNA from HBE cells and human bronchial brush specimens from asymptomatic heavy smokers (at least 20 packs/year). Heavy smokers were categorized as current smokers and former smokers; the latter had stopped smoking for at least 12 months at the time of sample collection.

cDNA was synthesized as described previously (17). The primer sequences were 5′-GCATGGGTGCCCCGACGTTG-3′ (sense) and 5′-GCTCCGGCCAGAGGCCTCAA-3′ (anti-sense) for survivin and 5′-GGTGAAGGTCGGTGTGAACGGATTT-3′ (sense) and 5′-AATGCCAAAGTTGTCATGGATGACC-3′ (anti-sense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (control). The thermocycling conditions were 2 min at 94°C (one cycle), 30 s (hot start) at 94°C, 1 min at 62°C, 1 min at 72°C (25–35 cycles) and 10 min at 72°C (one cycle). Amplification products were resolved in a 1.2% agarose gel, stained with ethidium bromide, visualized by transillumination and photographed.

Cell viability and colony-forming assays

To evaluate NNK's role in HBE cell viability, cells were plated in 96-well plates at a density of 5×10^3 cells per well or in six-well plates at a density of 3×10^5 cells per well. The cells were treated with 0, 1 or 10 μM NNK for 3 days in keratinocyte serum-free medium (KSFM; Invitrogen) in the presence or absence of epidermal growth factor (EGF). To investigate the role of survivin in cell survival, we left the cells untransfected or transiently transfected them with survivin or scrambled siRNA (Dharmacon, Lafayette, CO) via oligofectamine (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, the cells were left untreated or were treated with NNK (10 μM) for a further 1 or 3 days in KSFM in the absence of EGF. Then the cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For the analysis of anchorage-dependent colony formation, cells were re-plated in six-well plates at a density of 5×10^2 cells per well and cultured for 7 days. Colonies were stained and counted as described previously (18). For the analysis of anchorage-independent colony formation, cells were suspended in 0.3% agar in KSFM at a density of 1×10^3 cells/ml, re-plated in six-well plates pre-coated with 1 ml of 0.6% agar and cultured for 20 days. Colonies >0.2 mm in diameter were counted.

Cell cycle and apoptosis assays

BEAS2B and $BEAS2B_{N90d}$ cells were left untransfected or were transfected with survivin siRNA for 1 day and incubated in the absence of EGF for a further 1 day. Floating and attached cells were collected to determine cell cycle distribution using flow cytometry analysis or cell apoptosis using an APO–BrdU kit (Phoenix Flow Systems, San Diego, CA) as described previously (13,19).

Animal models

All experiments using animals were approved by The University of Texas M. D. Anderson Cancer Center's Institutional Animal Care and Use Committee. A/J mice (Jackson Laboratories, Bar Harbor, ME) were treated with NNK and BaP (3 μmol each in 0.1 ml of corn oil) for 10 weeks, as previously reported (6). At 10 weeks, the mice were humanely euthanized by CO_2 asphyxiation. Lung tissues were removed, embedded in paraffin and subjected to immunohistochemical analysis of survivin.

Human tumor xenograft models were created using 5- to 6-week-old female nude mice (Harlan Sprague Dawley, Indianapolis, IN) as described previously (19). In brief, 1170-I cells were transfected with WT or mut survivin siRNA and selected by G418 (500 μg/ml). Cell colonies lacking survivin were identified by western blot analysis. Then, an exponentially growing single colony of control (untransfected), WT or mut 1170-I cells (1×10^7 cells suspended in 100 μl of 1× phosphate-buffered saline) was injected subcutaneously into the right flank of each mouse (eight mice per group).

Immunofluorescence analysis

Cells were plated on laminin-coated cover slips in 24-well plates at a density of 5×10^4 cells per well. The next day, the cells were incubated with or without 10 μM NNK or 10 μM nicotine for 3 days in KSFM in the absence of EGF. A modified immunofluorescence analysis was performed as described previously (20). The primary rabbit polyclonal anti-pmTOR, anti-pAkt and anti-survivin antibodies (1:200; Cell Signaling Technology) were used. The primary antibodies were recognized by Alexa 488 goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Nuclei were stained with 500 nM 4′,6-diamidino-2-phenylindole dilactate (Molecular Probes), and cytoplasmic actin was stained with 1 U/ml Alexa 594–phalloidin (Molecular Probes). Slides were analyzed by epifluorescence microscopy (Olympus America, Lake Success, NY) and data were acquired using digital image analysis (Scanalytics, Fairfax, VA).

^{35}S metabolic labeling

Metabolic labeling of survivin was performed as described previously (19). In brief, NHBE cells reaching 60% confluence in 100 mm diameter dishes were pre-treated with or without 10 μM NNK for 1 day. Next, the cells were incubated in a methionine- and cysteine-free medium for 2 h and then incubated with or without the phosphoinositol-3 kinase (PI3K) inhibitor LY294002 (10 μM) or the mTOR inhibitor rapamycin (10 nM) in medium containing [^{35}S]methionine–cysteine for 6, 12 or 18 h. Metabolically labeled survivin proteins were immunoprecipitated and analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, autoradiographed and quantified by densitometric analysis. Two independent experiments were performed and the results recorded.

Statistical analysis

Data from cell viability and colony-forming assays were analyzed using simple *t*-tests or two-sided log-rank tests. $P < 0.05$ was considered statistically significant.

Results

NNK increases survivin expression, which plays a key role in the survival and tumorigenic potential of HBE cells

Nicotine activates survivin gene transcription in A549 NSCLC cells by increasing the recruitment of E2F1 and the concomitant dissociation of Rb from the survivin promoter (7). On the basis of that information, we examined survivin expression in bronchial brush specimens from 17 heavy smokers. Forty-one percent (7 of 17) of the specimens showed survivin messenger RNA (mRNA) expression (Figure 1A and supplementary Table 1 is available at *Carcinogenesis* Online). This result led us to hypothesize that tobacco components induce survivin gene expression in not only lung cancer cells but also HBE cells. We then treated NHBE and immortalized (BEAS2B, HB56B) HBE cells with nicotine or its metabolite NNK for 1–90 days. As shown by reverse transcriptase–polymerase chain reaction analysis, treatment with nicotine or NNK up-regulated survivin mRNA levels in NHBE and BEAS2B cells (Figure 1B). The survivin mRNA level was higher in BEAS2B cells than in NHBE cells, probably because of the presence of the SV40 oncoprotein in BEAS2B cells, which can increase survivin gene transcription by functionally inactivating Rb proteins (pRB, p107 and p130) (21) and p53 (22). Survivin protein expression was also markedly increased in NHBE, BEAS2B and HB56B cells after short-term (12 h to 3 days) or long-term (90 days) treatment with nicotine or NNK (Figure 1C). We have shown that 1198 premalignant and 1170-I malignant HBE cells have higher level of survivin expression compared with BEAS2B immortalized and 1799 premalignant HBE cells (23). 1198 and 1170-I HBE cell lines had been established by treating immortalized BEAS2B HBE cells with beeswax pellets containing cigarette smoke condensate, whereas premalignant 1799 HBE cells had been established by treating BEAS2B cells with beeswax pellets alone (15). These findings suggested that induced survivin expression is a generic response of HBE cells to tobacco components. We also assessed whether tobacco carcinogens induce survivin expression *in vivo* in the bronchial epitheilium of A/J mice administered orally with NNK and BaP for 10 weeks. Since the expression of survivin at the nuclear or cytoplasmic levels have a different biologic significance (24), we evaluated

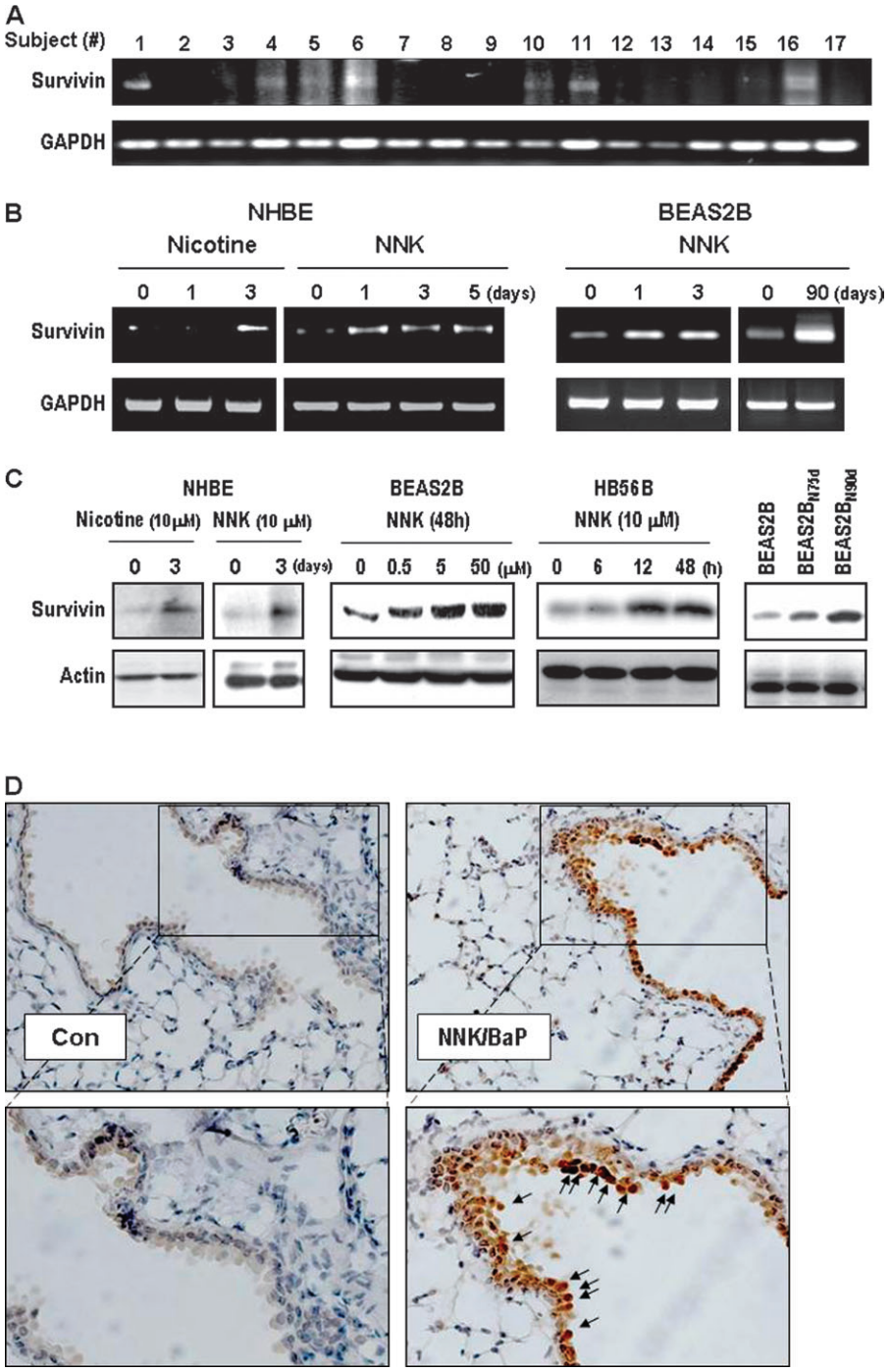
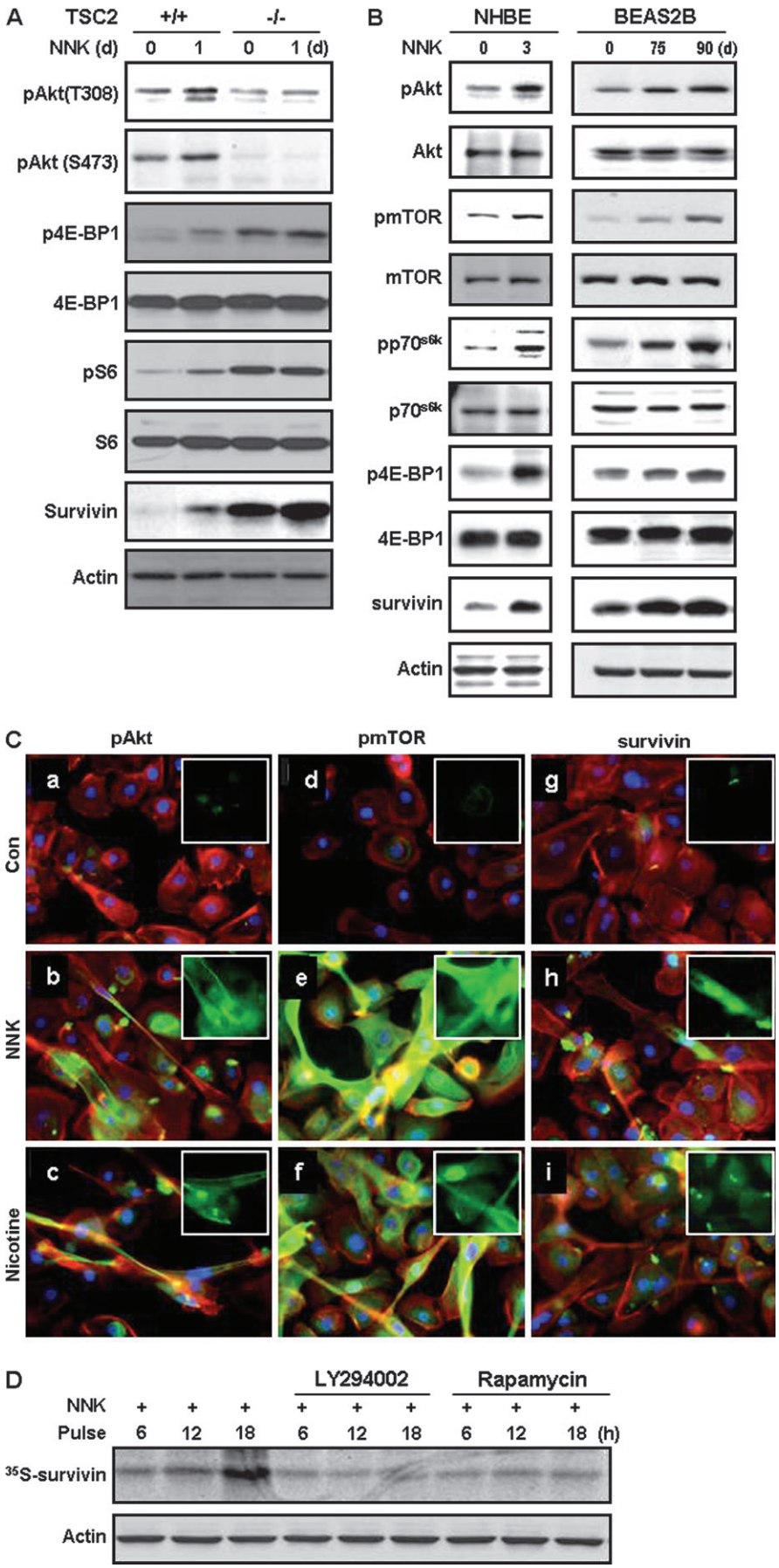


Fig. 1. Tobacco components induce survivin expression in normal bronchial epithelial cells *in vitro* and *in vivo*. (**A**) Total RNA was extracted from bronchial brush specimens obtained from heavy smokers. Reverse transcriptase–polymerase chain reaction was performed to analyze the expression of human survivin and GAPDH. (**B** and **C**) NHBE, BEAS2B and HB56B cells untreated or treated with 10 μM NNK or 10 μM nicotine for the indicated time periods were subjected to reverse transcriptase–polymerase chain reaction (**B**) and western blot (**C**) analyses of survivin expression. GAPDH and actin served as loading controls. (**D**) Survivin expression was immunohistochemically analyzed in the lung tissues of A/J mice treated with or without oral NNK and BaP (3 μmol each in 0.1 ml of cottonseed oil) for 10 weeks.

survivin staining in the two cellular compartments separately. We observed increases in both cytosolic and nuclear survivin staining (Figure 1D). These results suggest that the tobacco components induced survivin expression in NHBE cells *in vitro* and *in vivo*.

Tobacco components induce survivin expression via the PI3K–Akt–mTOR pathway

We investigated the mechanism mediating the tobacco component-induced survivin expression. Because nicotine and NNK activate Akt, which stimulates the mTOR pathway and thus protein synthesis (5,25), we examined the effects of NNK on mTOR activation and survivin protein expression in $TSC2^{-/-}$ and $TSC2^{+/+}$ MEFs. These cells were used because tuberous sclerosis complex 2 (TSC2) inhibits the GTPase Rheb, thereby inactivating mTOR and regulating protein synthesis (25). As measured in terms of 4E-BP1 and S6 phosphorylation, mTOR activity was markedly higher in $TSC2^{-/-}$ MEFs than in $TSC2^{+/+}$ MEFs (Figure 2A). Basal survivin protein expression was also higher in $TSC2^{-/-}$ MEFs than in $TSC2^{+/+}$ MEFs. In the $TSC2^{+/+}$ MEFs, both


A
TSC2
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pS6
S6
Survivin
Actin
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NHBE
BEAS2B
NNK
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pAkt
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pmTOR
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pp70s6k
p70s6k
p4E-BP1
4E-BP1
survivin
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were increased in response to NNK treatment. Knockout of TSC2 blocked this effect of NNK on the expression of survivin, pS6 and p4E-BP1. These findings highlight the importance of TSC2 function in the regulation of survivin expression in response to NNK treatment.

We next studied the effects of NNK on the Akt–mTOR pathway and survivin expression in NHBE and BEAS2B cells. As shown by western blot analysis, NNK induced concomitant increases in the protein levels of pAkt, pmTOR, pp70^{S6K}, p4E-BP1 and survivin (Figure 2B). We also confirmed the NNK- and nicotine-induced increases in the expression of pAkt (Ser473), pmTOR (Ser2448) and survivin in NHBE cells by the use of immunofluorescence analysis. After the treatment with NNK or nicotine (10 μM, 3 days), pAkt (Ser473) localized in the plasma membrane, pmTOR (Ser2448) was increased in the perinuclear region and survivin protein was well detected in the cytoplasm or at one pole of the nucleus in stimulated cells (Figure 2C). To confirm the mTOR pathway's role in inducing survivin protein synthesis in tobacco component-exposed NHBE cells, metabolic labeling of NHBE cells was performed in the absence or presence of the PI3K inhibitor LY294002 (10 μM) or the mTOR inhibitor rapamycin (10 nM). We found that synthesis of ^{35}S-labeled survivin increased in response to NNK but was completely inhibited by treatment with LY294002 or rapamycin (Figure 2D). Together, these findings indicate that NNK effectively increased survivin expression in NHBE cells not only by inducing mRNA transcription but also by increasing *de novo* protein synthesis.

Survivin induces malignant transformation of tobacco component-exposed HBE cells

We then asked whether induction of survivin expression contributed to the tobacco component-mediated lung carcinogenesis. Because survival in the absence of exogenous growth factors is a hallmark of cell transformation (26), we examined the effect of NNK on the viability of NHBE cells when cultured in the presence or absence of EGF. In the presence of EGF, NNK treatment did not significantly change the viability of these cells; however, in the absence of EGF, NHBE cells treated with 1 μM NNK ($P < 0.001$) or 10 μM NNK ($P = 0.001$) had significantly greater viability than untreated control cells did (Figure 3A). We tested the effects of the transfection

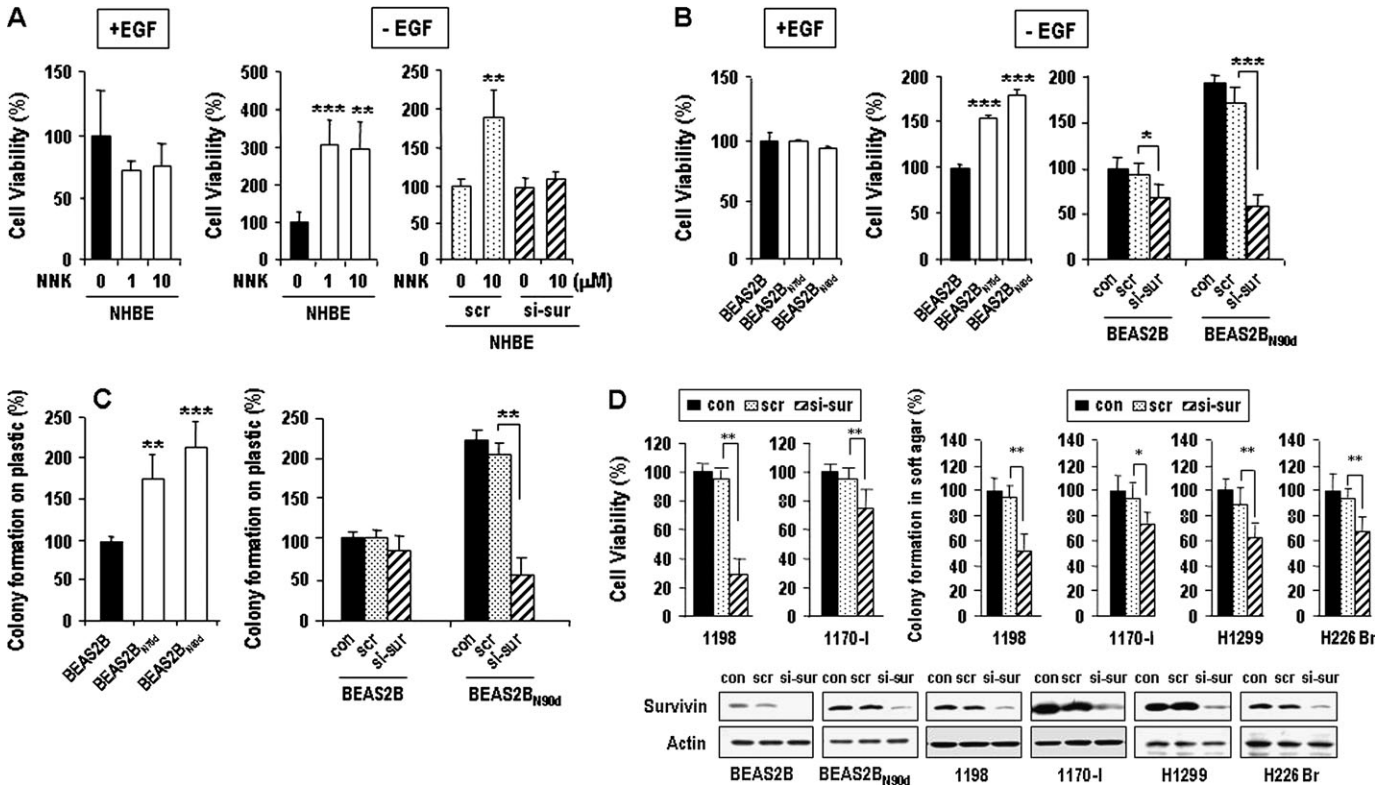

Fig. 3. Inhibition of the NNK-mediated survival potential of HBE cells by knockdown of survivin expression. NHBE, BEAS2B, BEAS2B$_{N75d}$, BEAS2B$_{N90d}$, 1198, 1170-I, H1299 and H226Br cells were untransfected or transiently transfected with scrambled siRNA (scr) or survivin siRNA (si-sur) for 1 day and then were left untreated or were treated with NNK (0, 1 or 10 μM) for 3 days in the presence (+EGF) or absence (−EGF) of EGF. These cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**A**, **B** and **D**), anchorage-dependent colony-forming (**C**), anchorage-independent colony-forming (**D**) and western blot (**D**) assays. The mean values from triplicate experiments are shown. The error bars represent 95% confidence intervals, $^{*}P < 0.01$ and $^{**}P < 0.001$.

Fig. 2. Survivin protein translation by tobacco components via the PI3K–Akt–mTOR pathway. (**A**) TSC2$^{-/-}$/p53$^{-/-}$ and TSC2+/+/p53$^{-/-}$ cells were treated with 10 μmol/l NNK for 24 h in serum-free medium. Total protein extracts were subjected to immunoblot analysis on pAkt (T308), pAkt (S473), p4E-BP1 (Thr70) and pS6 (Ser235/236). Expression of the unphosphorylated forms of these proteins, survivin and actin was also analyzed. (**B**) NHBE cells were not treated or treated with 10 μM NNK for 3 days in the absence of EGF. BEAS2B, BEAS2B$_{N75d}$ and BEAS2B$_{N90d}$ cells were incubated in the absence of EGF for 3 days and then harvested and subjected to immunoblot analysis of pAkt (Ser473), pmTOR (Ser2448), pp70^{S6K} (Thr389) and p4E-BP1 (Thr70). Expression of the unphosphorylated forms of these proteins, survivin and actin was also analyzed. (**C**) NHBE cells were incubated with or without 10 μM NNK or nicotine for 3 days in the absence of EGF. After treatment, cells on cover slips were subjected to immunofluorescence analysis of pAkt (Ser473), pmTOR (Ser2448) and survivin (green). Counter staining was performed with 4′,6-diamidino-2-phenylindole dilactate to identify nuclei (blue) and with 1 U/ml Alexa 594–phalloidin to stain cytoplasmic actin (red) as described in Materials and Methods. (**D**) NHBE cells pre-treated with 10 μM NNK for 1 day were treated with or without 10 μM LY294002 or 10 nM rapamycin in a medium containing [^{35}S]methionine–cysteine for the indicated amounts of time. ^{35}S-labeled survivin level was analyzed by immunoprecipitation. Actin expression served as a loading control. ^{35}S-labeled survivin expression was quantified by densitometric analysis of the immunoprecipitated bands.

with survivin siRNA on the viability of NNK-treated NHBE cells. When treated with NNK in the absence of EGF, unlike the cells transfected with scrambled siRNA, the cells transfected with survivin siRNA showed no increase in survivin expression (data not shown) and no stimulation of cell viability (Figure 3A). We further tested the effects of the transfection with survivin siRNA on the viability of BEAS2B cells treated for 75 or 90 days and premalignant 1198 and malignant 1170-I cells to assess the roles of survivin in a late stage of tobacco-induced transformation of HBE cells. Similar to the findings in NHBE cells, BEAS2B cells that had been treated with NNK for 75 days ($BEAS2B_{N75d}$) or 90 days ($BEAS2B_{N90d}$) (15) were significantly more viable than control (BEAS2B) cells ($P < 0.001$ for both comparisons) when incubated for 3 days in the absence of EGF, but not in the presence of EGF (Figure 3B). When incubated in the absence of EGF, $BEAS2B_{N90d}$ cells transfected with survivin siRNA were significantly less viable than BEAS2B cells (BEAS2B: $P = 0.019$; $BEAS2B_{N90d}$: $P < 0.001$) (Figure 3B). Moreover, compared with BEAS2B cells, $BEAS2B_{N75d}$ and $BEAS2B_{N90d}$ cells were better able to form anchorage-dependent colonies ($BEAS2B_{N75d}$ versus BEAS2B, $P = 0.005$; $BEAS2B_{N90d}$ versus BEAS2B, $P < 0.001$) (Figure 3C); however, these cells transfected with survivin siRNA had significantly decreased anchorage-dependent colony-forming abilities (BEAS2B: survivin siRNA versus scrambled siRNA, $P = 0.452$; $BEAS2B_{N90d}$: survivin siRNA versus scrambled siRNA, $P < 0.001$). Decreases in survival potential were greater in $BEAS2B_{N90d}$ cells than in BEAS2B cells. Similarly, when cultured in the serum-free media, 1198 and 1170-I cells transfected with survivin siRNA were significantly less viable compared with the untransfected control cells or cells transfected with scrambled siRNA (Figure 3D), suggesting that the premalignant and malignant HBE cells exposed to tobacco components were dependent on survivin expression for survival. We then examined the effects of the knockdown of survivin expression on the anchorage-independent growth of premalignant and malignant HBE and NSCLC cells. The 1198 and 1170-I HBE cells and the H1299 and H226Br NSCLC cells transfected with survivin siRNA showed significantly reduced anchorage-independent colony-forming abilities compared with untransfected cells or cells transfected with scrambled siRNA (Figure 3D). The siRNA-mediated knockdown of survivin expression in these cells was confirmed by western blot analysis. These findings suggest that survivin played an important role in the tobacco-induced transformation of HBE cells.

To help identify the mechanism by which survivin increased the viability of the HBE cells exposed to NNK, we cultured survivin siRNA-transfected BEAS2B and $BEAS2B_{N90d}$ cells in the absence of EGF and subjected them to cell cycle and apoptosis analyses. $BEAS2B_{N90d}$ cells revealed no detectable differences in their S and G_2/M cell populations; in contrast, the G_0/G_1 cell population was decreased in association with an increase in the $subG_0/G_1$ apoptotic population of (Figure 4A). TUNEL assay confirmed the significant increase in apoptosis in the survivin siRNA-transfected $BEAS2B_{N90d}$ cells (survivin siRNA versus scrambled siRNA, $P = 0.004$) (Figure 4B). Cell cycle and apoptosis were minimally changed in suvivin siRNA-transfected BEAS2B cells. These findings suggest that survivin expression played an important role in protecting the NNK-exposed HBE cells against apoptosis.

Survivin helps maintain the tumorigenicity of NNK-exposed HBE cells

We further explored the role of survivin in the tumorigenic potential of NNK-exposed HBE cells *in vitro* and *in vivo*. NNK-exposed $BEAS2B_{N75d}$ ($P < 0.001$) and $BEAS2B_{N90d}$ ($P < 0.001$) cells showed a significantly increased anchorage-independent colony-forming ability than did control BEAS2B cells (Figure 5A). In contrast, $BEAS2B_{N90d}$ and 1198 cells transfected with survivin siRNA showed significantly less anchorage-independent colony-forming ability than did untransfected or scrambled siRNA-transfected control cells ($BEAS2B_{N90d}$: survivin siRNA versus scrambled siRNA, $P < 0.001$; 1198: survivin siRNA versus scrambled siRNA, $P < 0.001$) (Figure 5B).

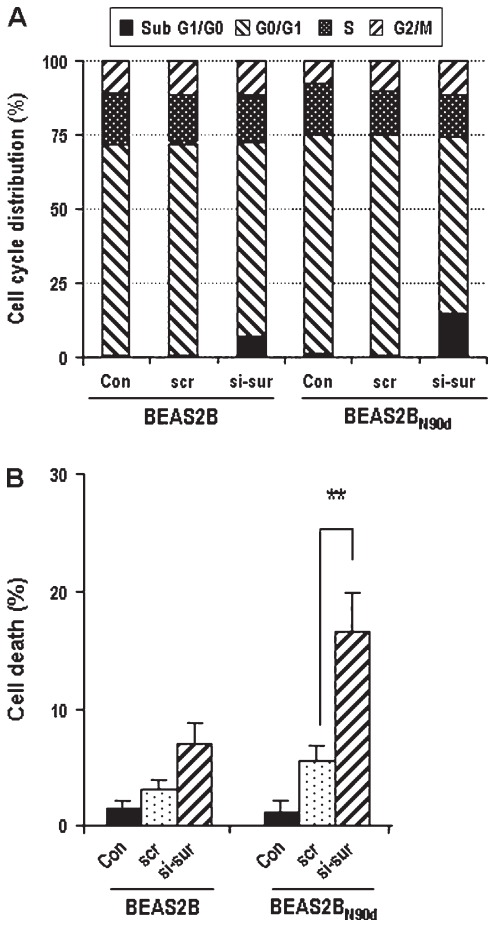

Fig. 4. Induction of apoptosis in NNK-exposed HBE cells by knockdown of survivin expression. BEAS2B and $BEAS2B_{N90d}$ cells were left untransfected (Con) or were transiently transfected with scrambled siRNA (scr) or survivin siRNA (si-sur) for 1 day and then incubated in the absence of EGF for a further 1 day. These cells were subjected to flow cytometric analysis of cell cycle (**A**) and cell death (**B**).

To further explore the role of survivin in the tumorigenicity of NNK-exposed malignant HBE cells *in vivo*, nude mice were injected with untransfected 1170-I cells or 1170-I cells transfected with WT or mut survivin siRNA. Immunoblot analysis was performed to confirm the loss of survivin expression in representative 1170-I cells transfected with WT survivin siRNA (Figure 5C). Nude mice injected with control or mut survivin siRNA-transfected 1170-I cells formed tumors that were measurable 1 week after injection (control: mean size, 30.87 ± 8.90 mm^3; mut survivin siRNA: mean size, 34.53 ± 8.33 mm^3). Six months after injection, tumors had grown in five of eight mice injected with control cells and six of eight mice injected with mut survivin siRNA-transfected cells. Subcutaneous injection of WT survivin siRNA-transfected 1170-I cells also resulted in the presence of a palpable nodule in all mice (mean size: 23.13 ± 4.40 mm^3) after 1 week. However, all these tumors (eight of eight) had completely regressed by 2 weeks, and no evidence of tumor regrowth was found during the entire observation period (6 months) (Figure 5C). Representative nude mice from these three groups are shown in Figure 5D. Taken together, these findings indicate a key role for survivin in the tumorigenicity of NNK-exposed HBE cells.

Discussion

The exposure of aerodigestive tract epithelium to tobacco carcinogens often leads to histologic changes over large areas of the tissue, resulting in field cancerization with potential multi-focal unsynchronized,

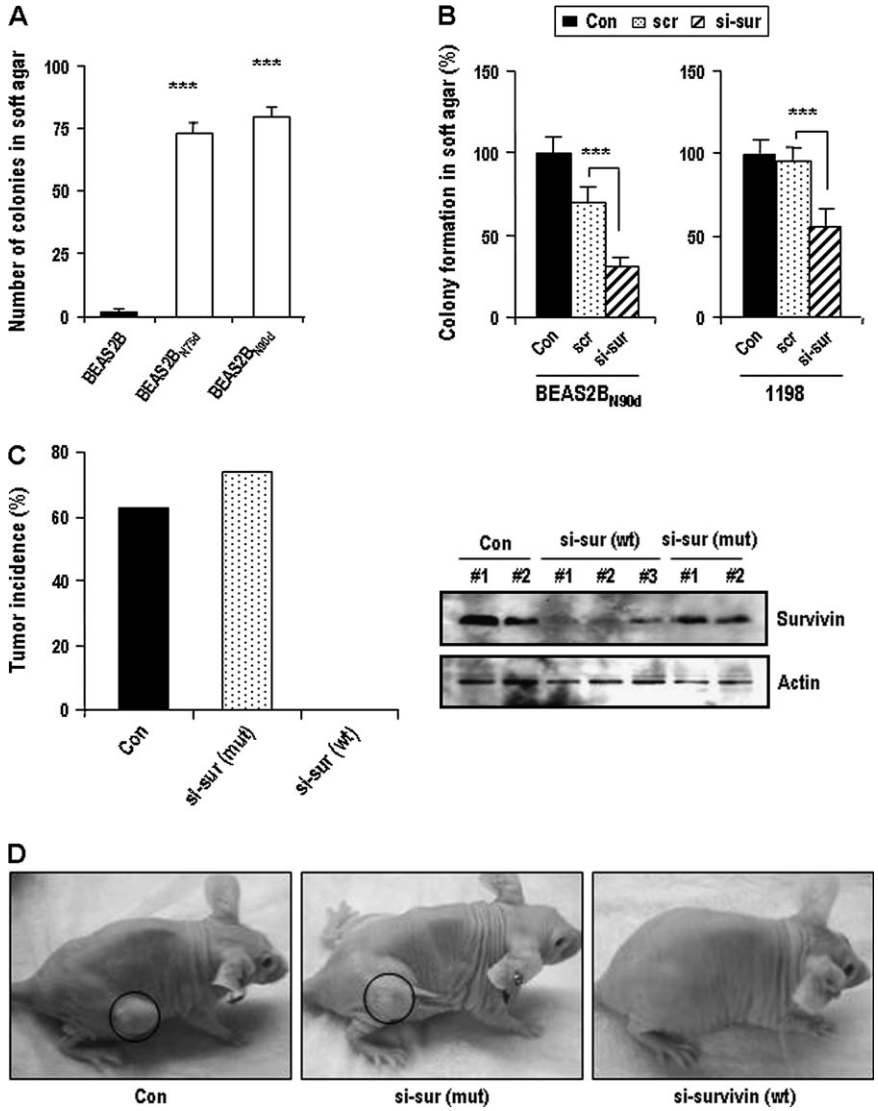

Fig. 5. Role of survivin in NNK-induced lung carcinogenesis. (**A** and **B**) BEAS2B, BEAS2B$_{N75d}$ and BEAS2B$_{N90d}$ cells were untransfected (**A**) or transfected (**B**) with scrambled siRNA (scr) or survivin siRNA (si-sur) for 1 day. These cells were subjected to soft-agar anchorage-independent colony-forming assays. The mean values from triplicate experiments are shown. The error bars represent 95% confidence intervals, $^{**}P < 0.001$. (**C** and **D**) 1170-I cells were untransfected (Con) or transfected with an expression vector containing WT or mut survivin siRNA (si-sur). Two or three representative colonies of each treatment group were analyzed for survivin expression (**C**, right). Single 1170-I cell colonies were amplified and injected subcutaneously into nude mice; the resulting tumor incidence is shown in the graph (**C**, left) and representative mice from each group are shown in (**D**).

premalignant and primary malignant lesions (27). Recent findings have demonstrated that the expression of survivin, one of the major regulators of cell division and apoptosis (8,28,29), is induced in NSCLC cells by nicotine (7), suggesting a role for survivin during tobacco-induced lung carcinogenesis. However, most previous reports of survivin over-expression have come from cancer cases in which such over-expression was associated with poor prognosis (8,11,30). To date, studies on human lung cancer have not validated a role for survivin expression in tobacco-induced lung carcinogenesis. In our recent study, 1198 and 1170-I cells exposed to cigarette smoke condensate showed higher level of survivin expression compared with 1799 control cells (23). Our present results suggest that (i) survivin mRNA is expressed in the bronchial epithelial cells of >40% of heavy smokers; (ii) tobacco components, including nicotine and NNK, induce survivin mRNA expression and stimulate Akt–mTOR-mediated *de novo* synthesis of survivin protein in NHBE cells and (iii) induced survivin expression plays a role in the malignant transformation of HBE cells by stimulating the survival and tumorigenic potential of tobacco-exposed HBE cells (Figure 6).

Although highly expressed during human embryonic and fetal development, survivin is rarely expressed in terminally differentiated adult tissues except for some proliferating cells (31,32). We recently observed survivin expression in a significantly high percentage of bronchial dysplasia specimens obtained from patients with NSCLC (23). Survivin protects cells from various apoptotic stimuli, including many anticancer agents (8). Suppressing survivin's expression induces cell cycle arrest and apoptosis and sensitizes cells to chemotherapy (29). Survivin has been shown to inhibit apoptosis by direct interaction with caspase-9 (33). More recent reports have described its indirect inhibition of caspases via physical interaction with Smac/Diablo, the release of which into the cytosol in response to apoptotic stimuli allows other IAPs to block caspases without being antagonized (20,34). These findings have raised the possibility of a role for survivin in the transformation of HBE cells.

The tobacco component-mediated genetic and epigenetic events that cause survivin gene expression have been defined. Frequent loss of heterozygosity has been observed in histologically normal or minimally altered bronchial epithelia of chronic smokers at chromosomal

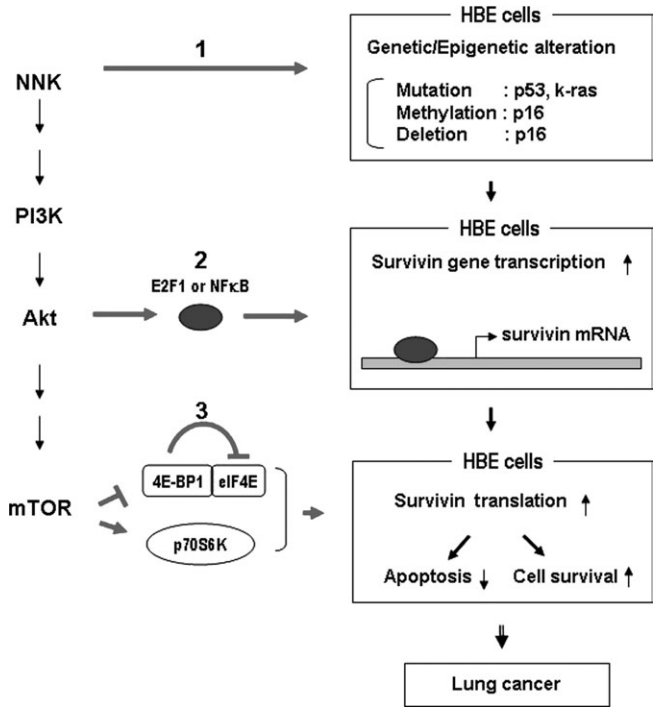

Fig. 6. Schematic model of tobacco-mediated induction of survivin expression and its involvement in lung carcinogenesis. Tobacco components, including NNK, can induce survivin gene expression by causing genetic and epigenetic alterations of tumor suppressor genes and oncogenes involved in the transcriptional regulation of the survivin gene. They can also increase survivin gene expression by activating the PI3K–Akt pathway, which is known to stimulate certain transcription factors involved in survivin expression (i.e. E2F and nuclear factor κB). Tobacco component-induced Akt activation stimulates mTOR, which in turn activates $p70^{S6K}$ and inhibits 4E-BP1, thereby increasing survivin translation. This cascade of events protects tobacco-exposed HBE cells against apoptotic stimuli during tobacco component-induced lung carcinogenesis.

sites 3p14, 9p21 and 17p13, in which the FHIT, p53 and p16 tumor suppressor genes reside (2,35). Tobacco components can also induce inactivating mutations in p53 (2), activating mutations in oncogene Ki-ras (36) and methylation or deletion mutations in p16 (37,38), all of which can contribute to the transcriptional up-regulation of survivin gene expression (22,39,40). Alternatively, survivin gene expression can be up-regulated by tobacco components via activation of the PI3K–Akt pathway, which stimulates E2F and nuclear factor κB, an important pair of transcription factors involved in survivin expression (7,41,42). In addition, as we have shown in our current study, tobacco components can activate Akt–mTOR-mediated *de novo* synthesis of survivin protein in NHBE cells.

The tobacco component-mediated survivin expression we observed *in vitro* and *in vivo* and the role of survivin in cell division and inhibition of apoptosis support the idea that survivin plays a role in tobacco-induced lung cancer development and progression. Indeed, findings from *in vitro* models have indicated that survivin expression induced by tobacco components promotes the survival and tumorigenic potential of HBE cells. When cultured in the absence of EGF, NHBE, premalignant and malignant HBE cells that had been exposed to tobacco components showed increased cell survival. However, siRNA knockdown of survivin expression was sufficient to suppress the survival potential of these cells. Suppression of survivin expression also reduced the colony-forming abilities of tobacco component-exposed HBE and NSCLC cells in soft agar. Furthermore, exponentially growing 1170-I malignant HBE cells, in which survivin expression was inhibited, could not grow when xenografted into athymic nude mice. Thus, survivin appears to promote the transformation of HBE cells and lung cancer progression by inhibiting apoptosis and stimulating tumorigenic potential in premalignant and malignant HBE cells. Survivin has been known to play a role in regulating cell cycle progression (43). However, in the present study, knockdown of survivin expression caused a negligible change in cell cycle distribution in $BEAS2B_{N90d}$ cells grown in the absence of EGF.

Taken together, our results suggest that tobacco components can induce survivin expression in NHBE cells during early lung tumorigenesis. The data from heavy smokers suggest that survivin is not normally expressed in bronchial epithelial cells but may be induced as the consequence of genetic and epigenetic changes induced in the cells by tobacco carcinogens. Because individuals have different susceptibilities to these carcinogens and the changes are different among the individuals, we were not surprised that survivin expression was detected in the epithelium of only some of the individuals. Whether those individuals with survivin expression carry a higher risk for lung cancer development remains to be determined. We observed increases in both cytosolic and nuclear survivin expression levels in the bronchial epithelium of A/J mice administered oral NNK and BaP. Because cytosolic survivin plays an essential role in protecting cells from apoptotic stimuli and promoting tumorigenesis (24), the biologic significance of increased expression of nuclear and cytoplasmic survivin is not assessed in the current study and remains to be addressed. The fact that no significant correlation between patient smoking history and survivin expression was observed by Monzo *et al*. (11) could be due to survivin expression in non-smokers being induced by indoor exposure to secondhand smoke (40) or from other mechanisms, such as human papillomavirus infection (44), an EGF receptor active mutation (45) that has been shown to induce survivin expression (46,47), or other as-yet unknown mechanisms involved in the regulation of survivin expression. We further provide evidence that survivin has an important role in tobacco-induced lung carcinogenesis. Our present findings provide a strong rationale for the use of agents that inhibit survivin expression for lung cancer chemoprevention and treatment. Since several IAP family members are often over-expressed in premalignant HBE cells and lung cancer cells (48), further studies are required to investigate the role of other IAPs in lung carcinogenesis. In addition, studies on the mechanisms underlying the induction of survivin expression in non-smokers are necessary to substantiate our hypothesis.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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