Survivin repression by p53, Rb and E2F2 in normal human melanocytes

Deepak Raj², Tong Liu³, George Samadashwily², Fengzhi Li⁴ and Douglas Grossman¹,2,5,6

¹Department of Dermatology, University of Utah, 30 North 1900 East, Salt Lake City, UT 84132, USA, ²Department of Oncological Sciences, ³Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, UT 84112, USA and ⁴Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

The inhibitor of apoptosis protein survivin is a dual mediator of apoptosis resistance and cell cycle progression and is highly expressed in cancer. We have shown previously that survivin is up-regulated in melanoma compared with normal melanocytes, is required for melanoma cell viability, and that melanocyte expression of survivin predisposes mice to ultraviolet-induced melanoma and metastasis. The mechanism of survivin up-regulation in the course of melanocyte transformation and its repression in normal melanocytes, however, has not been clearly defined. We show here that p53 and retinoblastoma (Rb), at basal levels and in the absence of any activating stimuli, are both required to repress survivin transcription in normal human melanocytes. Survivin repression in melanocytes does not involve alterations in protein stability or promoter methylation. p53 and Rb (via E2Fs) regulate survivin expression by direct binding to the survivin promoter; p53 also affects survivin expression by activating p21. We demonstrate a novel role for E2F2 in the negative regulation of survivin expression. In addition, we identify a novel E2F-binding site in the survivin promoter and show that mutation of either the p53- or E2F2-binding sites is sufficient to increase promoter activity. These studies suggest that compromise of either p53 or Rb pathways during melanocyte transformation leads to up-regulation of survivin expression in melanoma.

Introduction

Melanoma, a malignant tumor arising from melanocytes, is generally associated with poor response to conventional chemotherapy and radiation (1). Inherent resistance to apoptosis is largely responsible for treatment failure in melanoma, and there is considerable interest in developing apoptosis-based therapeutics to improve responses in patients with advanced disease (2). Among the many acquired genetic changes in melanoma, the best characterized involve: (i) activating mutations in BRAF, HRAS or NRAS (3); (ii) increased activity of telomerase (4); (iii) defects in retinoblastoma (Rb) signaling (5) and (iv) compromised p53 signaling (6–8). Two recent studies have shown that introduction of these four genetic events into normal human melanocytes is completely sufficient for transformation into invasive melanocytic lesions (9,10). It is therefore likely that apoptotic dysregulation in melanoma stems from one or more of these transforming events.

Among the many apoptotic regulators expressed in human melanoma, the inhibitor of apoptosis protein and mitotic regulator survivin (11,12) is notable for its low expression in normal melanocytes (13). Its importance in melanoma development and as a potential therapeutic target in this disease is indicated by several recent animal studies from our group (14,15). Furthermore, survivin expression has been associated with reduced survival in patients with advanced melanoma (16,17).

Although the mechanisms responsible for survivin up-regulation during melanocytic transformation have not been reported, epigenetic, genetic and post-translational mechanisms for survivin gene regulation have been described in other cell types. The survivin promoter was found to be methylated in ovarian cancers and unmethylated in normal ovarian tissues (18), although such differences in promoter methylation were not found in earlier comparisons of normal and malignant tissues (19). Although survivin mutations have not been described in cancer, a promoter mutation (C³¹ → G) was reported in various carcinoma cell lines (20). Up-regulation of survivin in tumor cells may also result from increased half-life of the survivin protein, which is regulated by ubiquitination (21) and may be stabilized through interactions with other binding partners (22). Multiple factors have been shown to effect survivin transcription in malignant cells. Survivin is transcriptionally up-regulated by Dec1 (23), Sp1 (19), c-myc (24) and KLF5 (25), all of which show aberrant activation in tumors as compared with normal tissues. Various transcription factors including Stat3 (26), HIF-1α (27) and Rb-E2F1 (28) have been shown to interact directly with the survivin promoter in breast cancer and lung cancer cell lines. While p53 was shown to repress survivin transcription by direct binding to the survivin promoter in a lung adenocarcinoma line (29), another study in an ovarian carcinoma cell line found that negative regulation of survivin by p53 was not affected by mutation or deletion of the putative p53-binding site in the survivin promoter (30). Most of the work on survivin gene regulation has been in malignant cells, and little is known regarding mechanisms of survivin repression in normal cells.

Here, we investigate the regulation of survivin expression in normal melanocytes by examining upstream factors involved in melanocyte transformation and show that both p53 and Rb are required for survivin repression in this cell type. We show that E2F2, acting downstream of Rb, plays a novel role in the negative regulation of survivin. We identify a novel functional E2F-binding site in the survivin promoter and show that mutation of either the p53- or E2F2-binding site increases survivin transcription. Thus, our data suggest that up-regulation of survivin expression in melanoma results from compromise of either p53 or Rb pathways.

Materials and methods

Cells

Normal human melanocytes were prepared from discarded foreskins, as approved by the Institutional Review Board at the University of Utah (#8476). Melanocytes were isolated and propagated as described previously (13), and experiments were performed with cells that had been passed 2–4 times. The Mel-SV40ER line was generated by infection of melanocytes with an SV40ER-expressing retrovirus kindly provided by Stephen Lessnuck (Huntsman Cancer Institute, Salt Lake City, UT) in the presence of polybrene (8 μg/ml, Sigma, St. Louis, MO), followed by selection with zeocin (0.5 mg/ml, Invitrogen, Carlsbad, CA) as described (31). Immortalized and transformed melanocyte lines Mel-STV, Mel-STR and Mel-STM (10) were generously provided by Robert Weinberg (Whitehead Institute, Cambridge, MA). These transformed melanocyte lines and melanoma cell lines YUSAC2, YUGEN8, and LOX (32) were all maintained in Dulbecco’s minimal essential media containing 5% heat-inactivated fetal bovine serum and antibiotics.

Western blotting and cycloheximide chase assays

Melanocytes were grown in normal melanocyte medium or medium containing 50 μg/ml cycloheximide (Sigma) or various cell cycle inhibitors (described below). After washing with phosphate-buffered saline (PBS), cells were scraped into lysis solution containing 2% sodium dodecyl sulfate, 50 mM Tris (pH 7.4) and protease (P-2714) and phosphatase (P-2850) inhibitor cocktails (both from Sigma). The lysate was heated at 94°C for 5 min, protein quantitated using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).
and thereafter stored at −20°C. Lysates (20–50 µg) were electrophoresed on denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking in PBS containing 5% non-fat dry milk and 0.1% Tween, membranes were incubated with primary antibodies against survivin (1:2500 dilution, sc-193X, Santa Cruz Biotechnology, Santa Cruz, CA), survivin (1:1500 dilution, SIGMA-ALDRICH, St. Louis, MO) for 1 hr at room temperature and then probed with anti-rabbit secondary horseradish peroxidase-conjugated antibodies (PerkinElmer Life Sciences, Boston, MA), bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) and autoradiography.

**RNAi**

Oligonucleotides were designed to target human p53 (5'-GACUAUCUGUGGAAUCAUAGCGTdT3'-3'), 5'-GUAGAUACCAUCGGGUCUUCTT3'-3'), 5'-AAUGUUCACUGUAAGUGATdT3'-3'), 5'-UGCUAAACCAACGACUAGGUGGACUCdT3'-3'), 5'-GAAUGAUCUGGUAUCAUAGCGTdT3'-3'), 5'-GAUGAUACCAUCGGGUCUUCTT3'-3'), and 5'-GGUGGAAAAUCAUAGGUGGACUCdT3'-3'). Primer sets were synthesized and solubilized in RNA interference buffer (Qiagen, Valencia, CA), mixed to give a stock concentration of 20 µM, heated at 90°C for 1 min followed by annealing at 37°C for 1 h and then aliquoted and stored at −20°C. Oligonucleotides targeting p21 (sc-29427) were obtained from Santa Cruz Biotechnology. Transfections were performed in six-well plates. The RNAi duplexes targeting p53 or p21 (20 nM for melanocytes, 50 nM for HeLa, final concentration), Rh (two duplexes used in combination at 50 nM final concentration) or p21 (100 nM final concentration) were mixed with 2 µl Lipofectamine 2000 (Invitrogen) and 100 µl of Opti-MEM (Invitrogen) for each well, and after 25 min added drop-wise to melanocytes (80% confluent) and 1 h for HeLa cells. After 24 hr, the media was replaced with normal melanocyte media containing antibiotics.

Lentiviral vectors expressing control scrambled small interfering RNA (siRNA), or siRNA targeting human p53 or Rh, and packaging constructs pRSV-A, pRSV-B and pRSV-C were synthesized and solubilized in RNA interference buffer (Qiagen, Valencia, CA), mixed to give a stock concentration of 20 µM, heated at 90°C for 1 min followed by annealing at 37°C for 1 h and then aliquoted and stored at −20°C. Oligonucleotides targeting p21 (sc-29427) were obtained from Santa Cruz Biotechnology. Transfections were performed in six-well plates. The RNAi duplexes targeting p53 or p21 (20 nM for melanocytes, 50 nM for HeLa, final concentration), Rh (two duplexes used in combination at 50 nM final concentration) or p21 (100 nM final concentration) were mixed with 2 µl Lipofectamine 2000 (Invitrogen) and 100 µl Opti-MEM (Invitrogen) for each well, and after 25 min added drop-wise to melanocytes (80% confluent) in 100 mm dish. Approximately, 4 µg of each plasmid were added to 0.5 ml of a 250 mM CaCl2 solution, which was then added drop-wise (while blowing air bubbles) to an equal volume of 2× 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)-buffered saline and left to stand for 45 min to allow formation of a fine precipitate. This mixture was then added drop-wise to the cells in 9 ml media containing 25 µM chloroquine (Sigma). The media was replaced 24 hr later to remove the transfaction mixture and the chloroquine, and live virus was collected at 48–72 h. The virus was filtered sterilized (0.45 µm BioExpress syringe filter, Intermountain Scientific Corp., Kaysville, UT), aliquoted and stored at −80°C. For lentival infection, undiluted lentiviruses along with polybrene (8 µg/ml, Sigma) was added to melanocytes (80% confluent) and after 4–6 h, virus-containing medium was aspirated and replaced with normal melanocyte media.

**Reverse transcriptase–polymerase chain reaction**

RNA was harvested from cells using TriReagent (Molecular Research Center, Cincinnati, OH) as per the manufacturer's instructions. Approximately, 0.5 µg total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen), and equal amounts of cDNA (1 µl of each 20 µl reaction) were subjected to polymerase chain reaction (PCR) using primers for survivin (5'-GTGTGGGGCTTCTTTCTTGTG-3' and 5'-AGGGAATAACCCCTGGGAAT-3'), p21 (5'-CTCTCTCTCTGCATGGCAAAC-3' and 5'-AAGGCAGAAGTTACGACG-3'), maspin (clone G167-70, BD Biosciences, San Diego, CA), p21 (100 ng/ml) (OP64, EMD Biosciences, San Diego, CA), p21 (200 ng/ml) (sc-193X, sc-633X, sc-878X and sc-866X, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After staining with species-appropriate secondary horseradish peroxidase-conjugated antibodies (PerkinElmer Life Sciences, Boston, MA), bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) and autoradiography.

**Electrophoretic mobility shift assay**

For preparation of nuclear extracts, cells were grown to 80% confluence in a six-well plate, washed twice with cold PBS, scraped into 0.5 ml cold PBS and microfuged at 350g for 5 min. Cells were washed with 0.5 ml Buffer A [10 mM HEPES, pH 7.8, 15 mM KCl, 2 mM MgCl2, 0.1 mM ethylenediaminetetra-acetic acid (EDTA) with freshly added 1 mM dithiothreitol (DTT)], 1 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitor cocktail (Sigma) and then re-suspended in 0.2 ml Buffer A. After holding on ice for 10 min, NP-40 was added to a final concentration of 0.5% and the tube was microfuged at 1330g for 15 min. The supernatant was discarded and the nuclear pellet was re-suspended in 30 µl of Buffer B (20 mM HEPES, pH 7.8, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, and 25% glycerol with freshly added 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitor cocktails), incubated at 4°C for 15 min and then microfuged at 16 300g for 10 min. The supernatant was transferred to a new tube and 50 µl Buffer C (20 mM HEPES, pH 7.8, 50 mM KCl, 0.2 mM EDTA with freshly added 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitor cocktails) was added to bring the total volume of nuclear extract to 80 µl. The nuclear extract was aliquotted and stored at −80°C.

To perform electrophoretic mobility shift assays, the following complementary pairs of high-performance liquid chromatography-purified oligos were obtained from the University of Utah DNA/Peptide Core Facility (mutant nucleotides in lower case): survivin promoter containing the p53-binding site (5'-CGCTTAAGAGGCTGCTTCCTTTCCAGTTCCCCTGTGGGGG-3' and 5'-CCTGGGCGTTCATTTGGGAGCCCCAAGCTTTTACATGAGGAGG-3'), the wild-type (5'-CAGTGGCTGTCATTTGGGAGCCCCAAGCTTTTACATGAGGAGG-3' and 5'-ACCCACGGCGGAGCCCTTCTTGCTTCTCGG-3') and the wild-type (5'-CAGTGGCTGTCATTTGGGAGCCCCAAGCTTTTACATGAGGAGG-3' and 5'-ACCCACGGCGGAGCCCTTCTTGCTTCTCGG-3') were used as non-specific competitors for p53-binding experiments. The reaction was allowed to stand at room temperature for 20 min. For binding reaction, 5 µl of nuclear extract was thawed on ice and added to labeled oligo duplex that had been diluted down to ~1 × 10^3 c.p.m. in 1× binding buffer (40 mM HEPES, pH 7.8, 10 mM MgCl2, 50 mM KCl, 0.05 mM EDTA, 20% glycerol with freshly added 5 mM DTT, 1 mg/ml bovine serum albumin and 0.1 mg/ml Poly dIdC). For competition reactions, 100-fold excess of oligo duplex were diluted similarly in 1× binding buffer and added to the reaction, which was brought up to a total volume of 20 µl in 1× binding buffer. Unlabeled duplexes (5'-CAGGAGCAGATATGATGATGATGATTCCCTGGTG-3' and 5'-CCTGGGCGTTCATTTGGGAGCCCCAAGCTTTTACATGAGGAGG-3') were used as non-specific competitors for p53-binding experiments. The reaction was allowed to stand at room temperature for 20 min. For supershift experiments, 2 µg antibody specific for p53 (pAb 4B12, BD Biosciences) or individual E2F factors (E2F1, sc-1932; E2F2, sc-633X; E2F3, sc-878X; E2F4, sc-866X; Santa Cruz Biotechnology) was also added. Each sample (18 µl) was run on a 4% non-denaturing polyacrylamide gel that had been pre-run for 1 h at 4°C. The gel was then subjected to vacuum drying at 80°C using a large gel dryer, exposed in a Phosphor Cassette (Molecular Dynamics, Sunnyvale, CA) and bands were visualized using a Storm PhosphorImager (Molecular Dynamics).
Transfection reporter assays

The construct pLuc1430c contains the proximal 1.4 kb of the human survivin promoter upstream of the firefly luciferase reporter, and has been described previously (19). Individual mutant promoter constructs were generated by PCR-based segment overlap extension using this construct as a template. For the p53-binding site mutant, separate products were generated using each of the following primer sets (mutant nucleotides in lower case): 5′-CGGGGACCTCACTTAGGCG-3′, 5′-AGTGGATCCCTCTGAAAATGACA-3′, and 5′-GGGGGCTCTTCTCTATTC-3′. These products were annealed and used in a second PCR reaction with the primers 5′-CCAGCTCTCTTCTCTATTC-3′ and 5′-GGGGGCTCTTCTCTATTC-3′ to generate the final product, which was digested with HindIII and MluI and cloned into these sites of the wild-type construct. For the E2F-binding site mutant, the following primer sets were used: 5′-GGGGGCCCTTCTCTTATTC-3′, 5′-GCACCAGCAATCATTCAATC-3′, and 5′-GATAAGCTGATCTCTCGCAGCC-3′. These products were annealed and used in a second PCR reaction with the primers 5′-CCAGCTCTCTTCTCTATTC-3′ and 5′-GATAAGCTGATCTCTCGCAGCC-3′, and 5′-GGGGGCCCTTCTCTTATTC-3′ to generate the final product, which was digested with HindIII and MluI and cloned into these sites of the wild-type construct. To generate the double p53/E2F mutant, the p53 mutant construct was used as an initial template rather than wild-type construct. Specific mutations, as well as lack of spontaneous mutations, were confirmed by DNA sequencing.

Cells were plated in 12-well plates and transfected with 200 ng of firefly construct and 20 ng of pGL-4.9-D for knockdown of p53 or Rb with Lipofectamine 2000 as above. After 24 h, the cells were harvested and reporter assays performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. The lysates were pipetted into a microtiter plate (#2100, Dynex Technologies, Chantilly, VA) and luminescence was measured using the MLX Microplate Luminometer (Dynex Technologies). Readouts of firefly luminescence were normalized to Renilla luciferase activity (internal control) and expressed as arbitrary units.

Results

Oncogenic transformation of human melanocytes results in up-regulation of survivin

Consistent with previous reports (13,32), expression of survivin is low in normal human melanocytes compared with melanoma cells (Figure 1a). In order to determine the specific oncogenic event that causes up-regulation of survivin in melanoma, we used a previously described model system (10) in which introduction of hTERT and disruption of p53 and Rb activity [by introduction of the Simian virus 40 early region (SV40ER)] was sufficient to immortalize normal human melanocytes, and further addition of activated HRas (RasG12V) or constitutively active c-Met receptor (TPR-Met) resulted in tumorigenicity in vivo. We used a retroviral system to stably introduce SV40ER into human melanocytes, and then examined survivin expression in this cell line (Mel-SV40ER), in normal melanocytes, in a panel of immortalized and transformed melanocytes (10) and three human metastatic melanoma lines. We found that survivin expression in Mel-SV40ER was greatly increased over that in normal melanocytes, with levels approaching those seen in melanoma cell lines (Figure 1b). By comparison with Mel-SV40ER, there was some enhancement of survivin expression in cells transduced with SV40ER, RasG12V and hTERT, but not in cells transduced with SV40ER and either hTERT or hTERT plus TPR-Met (Figure 1a). Thus, the up-regulation of survivin in these melanocyte lines compared with normal melanocytes was primarily attributable to SV40ER. We concluded that the oncogenic events leading to up-regulation of survivin during melanocytic transformation must be downstream of SV40ER.

Knockdown of p53 or Rb in melanocytes causes transcriptional up-regulation of survivin

The SV40ER encodes the large T antigen, which contributes to cellular transformation through inhibition of p53 and Rb (36). Earlier studies (9) showed that knockdown of p53 (with dominant-negative p53) and Rb (with dominant active CDK4) in conjunction with hTERT and activated NRas were sufficient to transform human melanocytes, which suggested that p53 and Rb were the mediators downstream of SV40ER that caused up-regulation of survivin expression. In order to test this, we transiently transfected siRNAs against p53 or Rb into normal human melanocytes, and examined survivin levels by western blotting over a 24–72 h period. Substantial knockdown of p53, which was evident by 24 h and sustained for 72 h, was associated with increased survivin expression as early as 24 h following transfection (Figure 1b). Similarly, Rb knockdown, which increased over 24–72 h following siRNA transfection, was associated with robust induction of survivin expression (Figure 1b). Thus, knockdown of either p53 or Rb was sufficient for induction of survivin expression.

Fig. 1. Survivin expression is negatively regulated by p53 and Rb in human melanocytes. (a) Cell lysates were obtained from normal human melanocytes (Mel), human melanocyte lines infected with retrovirus expressing SV40ER (Mel-SV40ER); SV40ER and hTERT (Mel-STV); SV40ER, HRasG12V and hTERT (Mel-STR); and SV40ER, TPR-Met and hTERT (Mel-STM); and human metastatic melanoma lines YUSA2, YUGEN8 and LOX. Lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blotting with antibodies against survivin and actin. (b) Knockdown of p53 or Rb leads to up-regulation of survivin expression. Melanocytes were transfected with control scrambled siRNA (Scr) or siRNA targeting p53 or Rb. Cell lysates were prepared 24, 48 or 72 h following transfection and blotted for p53, Rb, survivin and actin as indicated. (c) RNA was prepared from melanocytes 24 h following transfection with control scrambled siRNA (Scr) or siRNA targeting p53 or Rb, and used for reverse transcriptase–PCR with primers specific for survivin or actin.
in normal melanocytes, suggesting that inactivation of either p53 or Rb may be the crucial event up-regulating survivin expression during melanocyte transformation.

To determine whether p53 and Rb affected survivin messenger RNA levels, we targeted p53 and Rb using RNAi and looked for changes in survivin messenger RNA levels at 24 h by semiquantitative reverse transcriptase–PCR. As shown in Figure 1c, there was a significant increase in survivin messenger RNA levels following knockdown of either p53 or Rb, indicating that these factors regulate survivin expression transcriptionally. Cycloheximide chase assays indicated that the half-life of survivin protein (2–4 h) is unchanged after knockdown of either p53 or Rb (supplementary Figure 1 is available at Carcinogenesis Online). These data suggest that both p53 and Rb repress survivin by transcriptional mechanisms and do not affect survivin protein stability.

Absence of epigenetic regulation of survivin in melanocytes

Given the previous report of methylation-dependent repression of survivin in normal ovarian tissue (18), we examined whether methylation was involved in regulation of survivin expression in normal melanocytes and melanoma cells. There are a total of 15 restriction sites for the methylation-sensitive enzymes HpaII and HhaI within the 250 bp canonical CpG island and upstream sequences of the survivin promoter and the early exonic region (Figure 2a). Genomic DNA isolated from normal melanocytes and two melanoma cell lines was treated with HpaII or HhaI, and then subjected to PCR using primers flanking overlapping segments of this region (−650 to +555, Figure 2a). In both melanocytes and melanoma cells, enzymatic digestion disrupted PCR amplification of survivin but not actin sequences (Figure 2b), suggesting that the survivin promoter and early exonic region is unmethylated in these cells. To confirm lack of survivin promoter methylation, melanocytes were grown in the absence or presence of the demethylating agent 5-aza-2′-deoxycytidine and then examined for survivin expression. While treatment with 5-aza-2′-deoxycytidine induced expression of the maspin gene, shown previously to be repressed by methylation in melanocytes (37), there was no corresponding up-regulation of survivin expression (Figure 2c). These data suggest that the survivin promoter is unmethylated in normal melanocytes and that repression of survivin expression is not due to promoter methylation.

Cell cycle-dependent regulation of survivin

Survivin plays a role in mitosis (12), and has been shown to be up-regulated in G2/M in malignant cell lines (38), although its expression may be cell cycle-independent (39). In order to examine cell cycle-specific changes in survivin expression in normal melanocytes, we enriched for cells at different phases of the cell cycle using specific chemical inhibitors. Melanocytes were treated with mimosine, thymidine or nocodazole to arrest cells in the G1, S or G2/M phases, respectively (Figure 3a). Western blotting showed that survivin expression was relatively decreased during G1, but increased during the S and G2/M phases (Figure 3b). To determine whether Rb and p53 activity correlated with the cell cycle-dependent variation in survivin expression, we assessed activity of p53 and Rb at various stages of the cell cycle. As Rb is inactivated by phosphorylation, its activity correlates inversely with levels of its phosphorylated forms (40). A direct transcriptional target of p53 is p21, whose protein levels reflect p53 activity (41). The phosphorylated forms of Rb (indicating Rb inactivation) were increased during S and G2/M and diminished during G1, though significantly, total Rb levels still remained high during G1. Levels of p21 (indicating active p53) were high during G1 and G2/M, but virtually undetectable during S phase (Figure 3b). Thus, increased activity of p53 and Rb during G1 is associated with decreased survivin expression, whereas decreased p53 and Rb activity during S phase correlate with higher survivin levels. These data are consistent with our earlier experiments in which survivin was up-regulated in cells with either Rb or p53 knocked down (Figure 1b). However, during G2/M, increased survivin levels correspond to inactivation of Rb, even though p53 activity (as indicated by p21 levels) continues to be high. This suggests that in normal melanocytes, Rb inactivation can up-regulate survivin even in the presence of elevated amounts of active p53.

Interaction of p53 and Rb pathways in the regulation of survivin

As we were unable to knock out p53 and Rb simultaneously using transient transfection (due to significant cellular toxicity), we used a lentiviral system to deliver siRNAs stably into human melanocytes, allowing for more efficient knockdown of p53 and Rb singly or in combination. We found that the negative regulatory effects of p53 and Rb on survivin expression were not additive, as knockdown of p53 or Rb singly or in combination resulted in similar increased levels of survivin (Figure 4a). Knockdown of p53 was associated with down-regulation of p21, but did not significantly alter total or phosphorylated Rb levels (Figure 4a). On the other hand, knockdown of Rb was...
associated with a corresponding decrease in the phosphorylated forms of Rb, but no change in p53 or p21 expression levels (Figure 4a). These data suggest that p53 and Rb do not influence each other’s basal activity in normal melanocytes but rather act independently to repress survivin expression and that basal activity of both p53 and Rb is simultaneously required for survivin repression in this cell type.

As noted above, p21 is a direct transcriptional target of p53, and is responsible for the regulation of several p53 target genes. While p21 exerts its effects primarily by influencing Rb activity, it may also independently affect other cellular signaling pathways (42). To determine whether p53 exerts its negative effects on survivin expression via p21, we examined survivin levels following transfection of melanocytes with siRNA against p21. Partial knockdown of p21 was associated with modest up-regulation of survivin (Figure 4b), suggesting that at least part of the effect of p53 on survivin expression may be mediated through p21.

Binding of p53 and E2F factors to the survivin promoter

We next investigated direct binding of p53 to the canonical p53-binding site in the proximal survivin promoter (Figure 2a) by electrophoretic mobility shift assay. Since the DNA-binding activity of p53 requires a conformational change induced by specific antibody binding (43), EMSAs were performed in the presence of the activating anti-p53 antibody Pab421. We constructed a radiolabeled probe corresponding to the putative p53-binding site in the survivin promoter, which formed supershifting complexes when combined with melanocyte nuclear extracts (Figure 5a). These complexes were disrupted by 100-fold excess of cold probe but not similar excess of non-specific probe (Figure 5a).

Since the transcriptional effects of Rb are mediated through modulation of the activity of E2F factors (40), we examined the survivin promoter for potential E2F-binding sites, and identified a novel E2F-binding site 120 bp upstream of the p53-binding site in the survivin promoter. In order to test its functionality, we prepared radiolabeled probes corresponding to this site and flanking sequences, incubated them with melanocyte nuclear extracts and observed complexes that could be competed away with 100-fold unlabeled wild-type probe but not with similar excess of mutant probe (Figure 5b). Taken together, these data suggest that both p53 and Rb (via particular E2F factors) may modulate survivin expression through direct interactions with the promoter in melanocytes.

We further investigated the importance of these particular E2F- and p53-binding sites in the regulation of survivin expression by transient reporter assay using YUSAC2 and YUGEN8 cells. Cells were transfected with a construct consisting of 1.4 kb survivin promoter sequence driving luciferase expression or similar constructs containing discreet mutations in the p53 and/or E2F sites. In YUSAC2 cells, we found a significant increase in survivin promoter activity upon mutation of either site, suggesting that both sites are repressive for survivin transcription (Figure 5c). When both sites were mutated...
in the same construct, a similar increase in activity was seen and an additive effect on promoter activity was not observed (Figure 5c). Similar results were obtained in YUGEN8 cells (Figure 5d). These data confirm the importance of both the p53 and E2F sites in repressing survivin expression and are consistent with our findings above that both p53 and Rb are required for repression of survivin in normal melanocytes.

**E2F2 is a negative regulator of survivin expression**

Finally, we screened melanocytes and YUSAC2 cells for expression of particular E2F factors that may be acting downstream of Rb and controlling survivin expression. While E2F2 and E2F4 were expressed in both cell types (Figure 6a), other E2F factors, namely E2F1 and E2F3, were not expressed in either cell type at detectable levels (not shown). Our data (Figure 5c and d) suggest that one or more of the E2F factors binding to the survivin promoter play a negative role in transcription. E2F2 has been characterized previously as a positive regulator of gene transcription (44). However, since it has also been shown to act as a negative regulator of cellular proliferation in certain contexts (45), we investigated the possibility that E2F2 may negatively regulate survivin expression in normal melanocytes. Using RNAi we achieved significant knockdown of E2F2, which was associated with up-regulation of survivin expression (Figure 6b). Similar results were obtained following E2F2 knockdown in HeLa cells (Figure 6c). Thus, E2F2 is an important negative regulator of survivin acting downstream of Rb.

**Discussion**

Our previous studies in melanoma cell lines (32,46) and animal models (14,46) have implicated survivin as an important molecule in melanoma pathogenesis. Its clinical relevance in this disease is underscored by studies demonstrating a correlation between survivin expression and poor outcome in patients (16,17). To better understand the role of survivin in human melanoma and to design optimal therapies targeting survivin, it will be important to elucidate the mechanisms of survivin up-regulation during melanocyte transformation, as well as mechanisms of repression of survivin expression in normal melanocytes. Thus, our approach taken here was to examine upstream factors known to be involved in melanocyte transformation (9,10),

---

**Fig. 5.** Binding of p53 and E2F factors negatively regulates the survivin promoter. (a) Melanocyte nuclear extracts were incubated with 32P-labeled duplexes corresponding to the consensus binding site for p53 in the survivin promoter and anti-p53 antibody pAb 421 (p53 Ab), in the absence or presence of 100-fold excess cold wild-type (WT) or non-specific (NS) competitor, as indicated. Asterisks indicate non-specific bands. (b) Melanocyte nuclear extracts were incubated with duplexes corresponding to the E2F-binding site in the survivin promoter, in the absence or presence of 100-fold excess cold wild-type (WT) or mutant (MUT) competitor, as indicated. (c) YUSAC2 cells were transfected with luciferase reporter constructs driven by wild-type (WT) survivin promoter sequences (open bar) or WT sequences containing mutations in the E2F- and/or p53-binding sites (shaded bars). Firefly fluorescence was normalized to Renilla. Error bars represent SEM of triplicate determinations. P values for comparisons between each mutant construct and the WT construct are indicated by asterisks (*P < 0.01; **P < 0.001; ***P = 0.002). (d) YUGEN8 cells were transfected with the same constructs as in (c). Error bars represent SEM of triplicate determinations. P values for comparisons between each mutant construct and the WT construct are indicated by asterisks (*P < 0.01; **P = 0.01).
and we found that p53 and Rb independently repress survivin expression in normal melanocytes.

Our results suggest that compromise of either p53 or Rb pathways in melanoma would be sufficient for up-regulation of survivin expression. Genetic alterations are common in human melanoma and usually involve mutational inactivation or deletion of tumor suppressors or mutational activation or amplification of oncogenes, leading to aberrant signaling (47). Interruption of the p16INK4A-CDK4-Rb pathway, due to germ line mutations in p16 or CDK4 (8) or p16 mutations in sporadic melanomas (48), promotes cell cycle progression through hyper-phosphorylation of Rb and consequent activation of E2F transcription factors (40). The p14ARF-HDM2-p53 pathway promotes p53-dependent apoptosis, and may be disrupted by germ line mutations (8) or deletions (7) in p14 that lead to increased HDM2-mediated degradation of p53 or acquisition of inactivating p53 mutations (6). The role of p53 in tumor suppression has been well characterized, acting in a gene-specific manner as either transcriptional activator (for pro-apoptotic genes such as bax) or repressor (for various oncogenes such as c-myc) (49). Induction of wild-type p53 by DNA-damaging agents such as ultraviolet or doxorubicin (29), or introduction of exogenous p53 (30), have been shown to down-regulate survivin expression in malignant cells. However, the role of p53 in the regulation of survivin under basal conditions in normal cells has yet to be defined. We found that endogenous p53, in the absence of DNA-damaging agents or other stimuli known to activate p53, plays a critical role in the repression of survivin expression in normal human melanocytes.

Earlier studies have suggested that p53 does not bind to the survivin promoter (30,50). In both these studies, performed in malignant cell lines, mutational analysis of the survivin promoter suggested that the p53-binding site was non-functional and that p53 acted through other mechanisms to repress survivin. In contrast, we found in normal melanocytes that the p53-binding site is functional as evidenced by direct binding of p53 to the survivin promoter and increased promoter activity upon mutation of the p53-binding site. While it has been suggested that p53 exerts its effects on survivin via the p21-Rb-E2F pathway (50), our data in melanocytes suggest that the effect of p53 on survivin is at least partially independent of p21. In addition, we show that siRNA-mediated silencing of p53 in melanocytes has no apparent effect on Rb activity, suggesting that in melanocytes p53 exerts its actions on survivin expression directly. The discrepant findings regarding p53 binding to the survivin promoter may be due to the fact that malignant cells often exhibit a variety of defects upstream and downstream of p53 which may compromise p53 function (such as DNA binding) even in the absence of p53 mutation (49).

As the effects of Rb are mediated through multiple E2F transcription factors, repression of individual genes by Rb depends on the nature of particular Rb-E2F interactions and the activity of the Rb-bound proteins recruited to the promoter (40). It has been suggested that E2F factors bind adjacent to the p53-binding site in the survivin promoter (29,51). We have, however, identified a novel E2F-binding site 120 bp upstream of the p53-binding site and shown using reporter assays that this site plays a repressive role in survivin transcription. Earlier studies reported that Rb represses survivin expression by binding to and inactivating E2F3 in mouse fibroblasts (51) or E2F1 in lung carcinoma cells (28). In contrast, we could not detect expression of E2F1 and E2F3 in human melanocytes, whereas E2F2 was highly expressed in this cell type. Although previous studies implicated E2F2 as a positive transcriptional regulator (44), our finding that E2F2 knockdown led to survivin up-regulation identifies it as a novel negative regulator of survivin expression. Thus, the mechanism of E2F-mediated regulation of survivin expression may be tissue specific and differ between melanocytes and other cell types.

While the p53 gene is commonly mutated in other cancers, melanomas generally exhibit far lower rates of p53 mutation (6), although p53 may be compromised in melanoma by deletions in p14ARF (7). Nevertheless, the high levels of p53 seen in some melanoma tumors (52) may at first appear paradoxical given that they consistently express high levels of survivin as we have observed previously (13). Our data may resolve this paradox in that p53 and Rb are both required to repress survivin expression in melanocytes, as inactivation of either factor is sufficient to derepress survivin expression. Furthermore, we did not observe any additive effect of knocking down p53 and Rb by RNAi or mutating both the p53- and E2F2-binding sites in the promoter. These findings suggest that p53 and Rb are both required to repress survivin expression. Consistent with this notion, Rb inactivation (by phosphorylation) in G2/M was sufficient to up-regulate survivin expression, even in the presence of high amounts of activated p53. In melanoma, the near-ubiquitous defects in Rb signaling (due to inactivation of p16, mutation of CDK4 or over-expression of cyclin D1) (40) would be predicted to up-regulate survivin expression even in the context of wild-type p53.

The secondary events leading to repression of the survivin promoter, following p53 and Rb (via E2F factors) binding, have not been clearly defined. It has been suggested that p53 recruits DNMT1 causing methylation of the survivin promoter and transcriptional repression (53). We found, somewhat surprisingly given earlier studies (18), that the survivin promoter is unmethylated in normal melanocytes, suggesting that the mechanism of p53 repression of survivin may be tissue specific. Several transcription factors, including Dec1 (23), Sp1 (19), KLF5 (25) and c-myc (24), have been shown to facilitate trans-activation by binding to the proximal part of the survivin promoter—interestingly in the same region where we have demonstrated p53 and E2F2 binding in this study. It is possible that p53 and Rb physically displace these transcriptional activators from the survivin promoter. Alternatively, given that histone deacetylases may be recruited to target promoters by p53 and Rb, these factors may promote repressive modifications of chromatin and thereby block transactivation. Possible interactions between p53 and Rb on the survivin promoter, histone modifications secondary to p53 and Rb binding and recruitment of other repressive factors by p53 and Rb may underlie tissue-specific differences in the regulation of survivin expression and merit further study.

Supplementary material

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

Funding

National Institutes of Health (AR050102); Huntsman Cancer Foundation.
Acknowledgements

We thank Robert Weinberg for transformed melanocyte lines Mel-STV, Mel-STR and Mel-STM; Stephen Lessnick for the SV40/ER-expressing retrovirus; Maria Soengas for the p53 and Rb RNAi lentiviruses; Sancy Leachman for helpful discussions and Ed Levine and Murray Cotter for reviewing the manuscript.

Conflict of Interest Statement: None declared.

References


Received July 24, 2007; revised September 17, 2007; accepted September 23, 2007.

Transcriptional repression of survivin