

# Therapeutic Targeting of the Survivin Pathway in Cancer: Initiation of Mitochondrial Apoptosis and Suppression of Tumor-associated Angiogenesis<sup>1</sup>

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## ABSTRACT

**Purpose:** Molecular antagonists of the inhibitor of apoptosis protein survivin have shown promise as novel anticancer strategies for triggering tumor cell apoptosis, dysregulating mitotic progression, and inhibiting tumor growth in preclinical models. However, how survivin couples to the cell death machinery has remained elusive, and the relevant cellular targets of survivin antagonists have not been completely elucidated.

**Experimental Design:** Human umbilical vein and dermal microvascular endothelial cells were infected with replication-deficient adenoviruses encoding survivin (pAd-Survivin), green fluorescent protein (pAd-GFP), or a phosphorylation-defective survivin Thr<sup>34</sup>→Ala (pAd-T34A) dominant negative mutant. The effect of wild-type or mutant survivin was investigated on capillary network stability, endothelial cell viability, and caspase activation *in vitro* and on kinetics of tumor growth and development of angiogenesis in a breast cancer xenograft model *in vivo*. The cell death pathway initiated by survivin targeting was mapped with respect to cytochrome *c* release, changes in mitochondrial transmembrane potential, and apoptosome requirements using mouse embryonic fibroblasts deficient in Apaf-1 or caspase-9.

**Results:** Adenoviral transduction of endothelial cells with pAd-Survivin inhibited growth factor deprivation- or ceramide-induced apoptosis, reduced caspase-3 and -7 generation, and stabilized three-dimensional capillary networks *in vitro*. Conversely, expression of pAd-T34A caused apo-

ptosis in umbilical vein and dermal microvascular endothelial cells and resulted in caspase-3 activity. Cell death induced by survivin targeting exhibited the hallmarks of mitochondrial-dependent apoptosis with release of cytochrome *c* and loss of mitochondrial transmembrane potential and was suppressed in Apaf-1 or caspase-9 knockout mouse embryonic fibroblasts. When injected in human breast cancer xenografts, pAd-T34A inhibited growth of established tumors and triggered tumor cell apoptosis *in vivo*. This was associated with a ~60% reduction in tumor-derived blood vessels by quantitative morphometry of CD31-stained tumor areas, and appearance of endothelial cell apoptosis by internucleosomal DNA fragmentation *in vivo*.

**Conclusions:** Survivin functions as a novel upstream regulator of mitochondrial-dependent apoptosis, and molecular targeting of this pathway results in anticancer activity via a dual mechanism of induction of tumor cell apoptosis and suppression of angiogenesis.

## INTRODUCTION

The evasion from apoptosis, or programmed cell death, is an invariant molecular trait of human cancer (1), which may facilitate the acquisition of additional cancer traits (2) promoting resistance to therapy and disseminated disease. Cancer cells can achieve escape from apoptosis from allelic loss of cell death activators (3), deregulated overexpression of apoptosis inhibitors (4), or inactivating mutations in executioner molecules of cell death (5–7), *i.e.*, caspases. The net result is an increased antiapoptotic threshold that allows cancer cells to bypass internal surveillance checkpoints, thrive in unfavorable microenvironments, and acquire an invasive phenotype (1, 2).

Among the regulators of apoptosis that may participate in cancer (4), interest has been recently focused on survivin (8). A member of the IAP<sup>5</sup> gene family (9), survivin is expressed in most human tumors but is largely undetectable in normal differentiated tissues and correlates with reduced tumor cell apoptosis *in vivo*, abbreviated patient survival, accelerated rates of recurrences, and increased resistance to therapy (10). Molecular antagonists of the survivin pathway, including antisense, ri-

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<sup>5</sup> The abbreviations used are: IAP, inhibitor of apoptosis; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; HUVEC, human umbilical vein endothelial cell; DMVEC, dermal microvascular endothelial cell; PMA, phorbol 12-myristate 13-acetate; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; Tet, tetracycline; VEGF, vascular endothelial growth factor; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; CHX, cycloheximide; DAPI, 4',6-diamidino-2-phenylindole; PARP, poly(ADP-ribose) polymerase; DEVD-AMC, Asp-Glu-Val-Asp-aldehyde.

bozymes, or dominant negative mutants, have shown efficacy in causing spontaneous apoptosis in tumor cells, enhancement of cell death stimuli, including chemotherapy and irradiation, and catastrophic mitotic defects (10). Consistent with the idea that manipulation of apoptotic pathways could provide novel cancer therapeutics (11), molecular targeting of survivin showed promising results in preclinical models *in vivo*, suppressing *de novo* tumor formation and inhibiting growth of established tumors, alone or in combination with other anticancer approaches (reviewed in Refs. 10 and 12). However, critical gaps in our understanding of the survivin pathway still exist that have hampered its full exploitation for cancer therapeutics. Specifically, how survivin couples to the cell death machinery (13) has not been fully elucidated, and the potential cellular targets of survivin antagonists have not been clearly defined (10). The complexity of the survivin pathway may extend beyond the tumor cell population, and increased survivin expression has been demonstrated in endothelial cells during the proliferative (14, 15) and remodeling (16, 17) phases of angiogenesis, potentially acting as a cytoprotective mechanism for these cells (18).

To conclusively credential the survivin pathway for cancer therapeutics, we used a phosphorylation-defective survivin Thr<sup>34</sup>→Ala dominant negative mutant (19) and mapped the link between survivin and the cell death machinery and its implications for tumor growth *in vivo*. We found that survivin functions as a novel upstream regulator of mitochondrial-dependent apoptosis and that this pathway is required for preservation of tumor cell viability as well as maintenance of tumor-associated angiogenesis *in vivo*.

## MATERIALS AND METHODS

**Cell Cultures and Antibodies.** HUVECs or DMVECs were purchased from Clonetics (San Diego, CA) and maintained in culture on gelatin-coated 6-well clusters in M199 medium supplemented with 20% FCS, 50 µg/ml endothelial cell growth supplement, 100 µg/ml heparin, 100 µg/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Inc., Grand Island, NY) in 5% CO<sub>2</sub> at 37°C. The Tet-inducible human melanoma YUSAC-2 cell line expressing wild-type survivin or the phosphorylation-defective survivin Thr<sup>34</sup>→Ala mutant [survivin(T34A)] upon Tet removal (Tet-off system) was characterized previously (20). MEFs originated from wild-type, Apaf-1<sup>-/-</sup> (21), or caspase 9<sup>-/-</sup> (22) embryos have been described previously. MEFs were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.05% β-mercaptoethanol. Breast carcinoma MCF-7 and kidney epithelial HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture according to the supplier's specifications. An affinity-purified rabbit antibody to full-length recombinant survivin (1:1,000; NOVUS Biologicals, Littleton, CO) was characterized previously (23). Rabbit polyclonal antibodies to caspase-3 (1:5,000) or caspase-7 (1:750) were from Transduction Laboratories or NOVUS Biologicals, respectively. Antibodies to GFP (1:1,000; Clontech, Palo Alto, CA), β-actin (1:10,000; Sigma, St. Louis, MO), cytochrome *c* (1:1,000;

PharMingen, San Diego, CA), PARP (1:1,000; PharMingen), or Cox-4 (1:5,000; Clontech) were also used.

**Adenoviral Transduction.** Replication-deficient adenoviruses encoding wild-type survivin (pAd-Survivin), GFP (pAd-GFP), or survivin Thr<sup>34</sup>→Ala mutant (pAd-T34A) were generated using the pAd-Easy system, as described previously (24). Viruses were propagated in HEK293 cells and purified by CsCl banding. With this protocol, no replication-competent adenovirus particles are generated (24). For adenoviral transduction, monolayers of proliferating endothelial cells or genetically modified MEFs were incubated with pAd-GFP, pAd-Survivin, or pAd-T34A at a multiplicity of infection of 50 in M199 medium plus 20% FCS for 10 h, washed with PBS (pH 7.4), and placed in fresh medium plus 20% FCS for 16 h at 37°C. Transduction efficiency (>95% of the cell population) was estimated by GFP fluorescence.

**Three-dimensional Capillary Formation.** HUVEC monolayers (80% confluence) in 6-well clusters were incubated with pAd-GFP or pAd-Survivin at a multiplicity of infection of 50 for 8 h at 37°C followed by an additional 24-h incubation in complete medium at 37°C. Rat tail type I collagen (3 mg/ml; Becton Dickinson, Bedford, MA) in 0.1 volume of 10× DMEM was neutralized with sterile 1 M NaOH and kept on ice. HUVECs were added to the collagen suspension to a final concentration of 1 × 10<sup>6</sup> cells/ml collagen. Ten drops (0.1 ml each) of the HUVEC-collagen mixture were added to a 35-mm plate. Plates were placed in a humidified incubator at 37°C, and the HUVEC-collagen mixtures were allowed to gel for 10 min, after which 3 ml of M199 medium containing 20% FCS, 50 µg/ml endothelial cell growth supplement, 100 µg/ml heparin, 100 µg/ml penicillin, and 100 µg/ml streptomycin were added to each plate. Cells were allowed to form capillary-like vascular tubes over a 7-day culture in the presence of 16 nM PMA (Sigma). Addition of PMA results in a potent morphogenic effect promoting the formation of three-dimensional vascular tube-like structures, which closely mimic capillary formation *in vivo* via a protein kinase C-, mitogen-activated protein kinase-, and phosphatidylinositol 3'-kinase-dependent pathway (25). As determined in previous studies, PMA withdrawal under these conditions results in rapid regression of capillary structures and HUVEC apoptosis *in vitro*. In other experiments, capillary tube formation was assessed in HUVEC or DMVEC cultures in Matrigel (200 µl) in the presence of VEGF (50 ng/ml) and assessed by phase-contrast microscopy during a 48–72-h culture at 37°C. To quantify tube formation, cells were washed three times in PBS (pH 7.4) and snap frozen in OCT embedding compound. Cryostat sections of the gels (6 µm) were placed on poly-L-lysine-coated glass slides, fixed with acetone for 10 min at -20°C, air-dried, and stained with H&E. Sections were examined by phase-contrast microscopy, and the total vessel area (in 3 fields/slide in each experiment) was quantified using the NIH (Bethesda, MD) Image program.

**Cell Viability and Apoptosis.** HUVECs transduced with pAd-GFP, pAd-Survivin, or pAd-T34A were incubated in 0% FCS for up to 72 h or, alternatively, treated with 25 µM C6 ceramide or the combination of TNF-α (10 ng/ml; Endogen, Woburn, MA) plus CHX (10 µg/ml; Sigma), for 6–12 h at 37°C (14). Cultures under the various conditions were analyzed for nuclear morphology of apoptosis after fixation in 4% paraform-

aldehyde containing 0.25% Triton X-100 for 10 min at 22°C, and staining of cell nuclei was performed with 6.5 µg/ml DAPI (Sigma) in 16% polyvinyl alcohol (Air Products and Chemicals, Allentown, PA) and 40% glycerol. The percentage of apoptotic cells was calculated by direct counting of nuclei with apoptotic morphology (condensed chromatin, fragmented DNA) in five independent high-power fields (×400; each field contained about 150 cells) in three independent experiments, using a Zeiss fluorescence microscope (26). For survivin targeting, exponentially growing HUVECs or DMVECs were transduced with pAd-GFP, pAd-survivin, or pAd-T34A; harvested after a 96-h culture at 37°C; and analyzed for DNA content by propidium iodide staining and flow cytometry. The hypodiploid (*i.e.*, apoptotic) cell fraction was quantified using CELL Quest software (Becton Dickinson), as described previously (27). Apoptosis in transduced wild-type or Apaf-1 or caspase-9 knockout MEFs was also assessed by DNA content analysis and flow cytometry (27). For quantification of caspase activity, virally transduced HUVECs were lysed in 0.5% Triton X-100, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 20 mM HEPES, and protease inhibitors. Protein-normalized aliquots of the various cell extracts were separated by SDS-gel electrophoresis, transferred to nylon membranes (Millipore Corp.), and immunoblotted with antibodies to caspase-3, caspase-7, survivin, GFP, or β-actin, followed by chemiluminescence and autoradiography. In parallel experiments, detergent-solubilized HUVEC extracts were assayed for caspase-3-dependent hydrolysis of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-AMC; PharMingen, San Diego, CA), with determination of fluorescence emissions on a spectrofluorometer with excitation wavelength of 360 nm and emission of 460 nm.

**Characterization of Mitochondrial-dependent Apoptosis.** Tet-regulated YUSAC-2 cells ( $5 \times 10^7$  cells; Ref. 20) were washed in TD buffer containing 135 mM NaCl, 5 mM KCl, and 25 mM Tris-HCl (pH 7.6) and allowed to swell for 10 min in ice-cold hypotonic CaRSB buffer [10 mM NaCl, 1.5 mM CaCl<sub>2</sub>, 10 mM Tris-HCl (pH 7.5) and protease inhibitors]. Cells were Dounce-homogenized with 60 strokes, with addition of MS buffer [210 mM mannitol, 70 mM sucrose, 5 mM EDTA, and 5 mM Tris (pH 7.6)] to stabilize mitochondria (2 ml of 2.5× per 3 ml of homogenate). After removing nuclear contaminants by centrifugation at 3,000 rpm for 15 min on ice, the supernatants were layered over a 1–2 M sucrose step gradient [in 10 mM Tris (pH 7.6), 5 mM EDTA, 2 mM DTT, and protease inhibitors] and centrifuged at 110,000 × *g* for 30 min at 4°C. Mitochondria were collected at the 1–1.5 M interphase by lateral suction through the tube, washed in 4 volumes of MS buffer at 15,000 rpm, and suspended in a final volume of 200 µl of MS buffer. The top layer containing a cytosolic and free protein fraction was collected and used in parallel experiments. Samples from cytosolic or mitochondrial fractions harvested at various time intervals in the presence or absence of Tet were analyzed sequentially by Western blotting with antibodies to cytochrome *c*, PARP, Cox-4, or β-actin followed by chemiluminescence. To monitor changes in mitochondrial transmembrane potential, Tet– YUSAC-2 cells expressing wild-type survivin or survivin(T34A) were harvested at increasing time intervals (6–72 h) at 37°C, suspended in 600 µl of distilled deionized water, and homogenized. Cell homogenates were incubated with

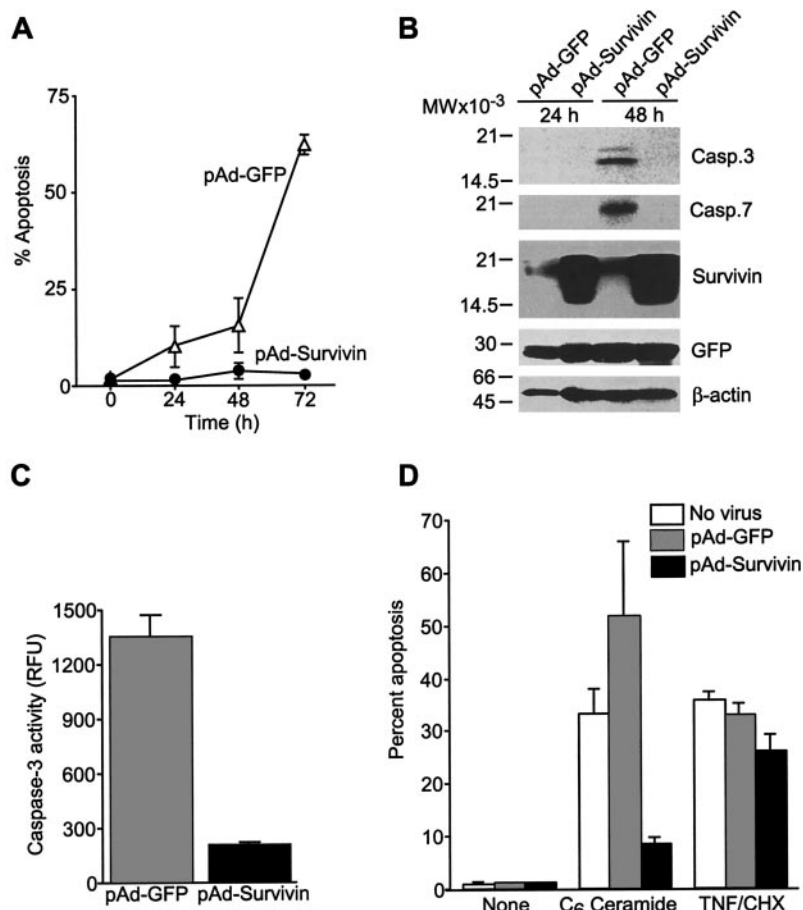
the green fluorescence dye JC-1 (10 µg/ml; Molecular Probes Inc., Eugene, OR) for 10 min in the dark, washed in ice-cold PBS, and analyzed by flow cytometry.

**Breast Cancer Xenograft Model.** Female 6–8-week-old C.B.-17 SCID/beige mice (Taconic Farms, Germantown, NY) received s.c. injection (one injection in each flank) with  $2.5 \times 10^6$  exponentially growing MCF-7 cells in 250 µl of sterile PBS (pH 7.4). Tumor growth was confined to local masses and did not affect animal survival over a 4-month observation period, as described previously (24). About 5 days after injection, tumors became palpable, and groups of three animals were randomized and distributed between treatment groups (6 tumors/group). Animals were treated with pAd-GFP or pAd-T34A by intratumoral injections ( $10^9$  green fluorescence units in 50 µl distributed between 3 sites/tumor mass) on 2 consecutive days followed by 5 days of interval (24). Tumor volume was monitored by measuring tumors in the three dimensions with a caliper every other day for up to 14 days after tumors became palpable (total of two pAd injections). Adenoviral gene transfer *in situ* was assessed for GFP expression by fluorescence microscopy, as described previously (24). All experiments involving animals were approved by the institutional animal care and use committee.

**Histological Assessment of Tumor-associated Angiogenesis.** Breast cancer tumors prepared as described above were harvested after 7 days of treatment, formalin-fixed, and paraffin-embedded. Five-µm tissue sections were cut, air-dried on glass slides, deparaffinized, rehydrated, and quenched with 1% hydrogen peroxide for 45 min at room temperature. For endothelial cell detection by CD31 (platelet-endothelial cell adhesion molecule-1) staining, a two-step antigen retrieval method was used with pressure cooking for 5 min in 10 mM sodium citrate buffer (pH 6.0) and tissue digestion with 0.05% trypsin at 37°C for 10 min. Tissue sections were blocked with 10% goat serum for 30 min at room temperature. A previously described rabbit polyclonal antibody to mouse CD31 [Sleet4; a kind gift of Dr. J. A. Madri, Yale School of Medicine, New Haven, CT (28, 29)] was applied for 14 h at 4°C at a 1:500 dilution in 0.5% goat serum. Naïve normal rabbit antiserum was used at an equivalent dilution and produced no staining (data not shown). Binding of the primary antibodies was detected using a biotinylated goat antirabbit secondary antibody, followed by the avidin-biotin-peroxidase system (NovaRed Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA) with 3-amino-9-ethyl carbazole (Vector Laboratories) as the chromophore. Total CD31 positivity was determined by measuring the percentage of positively stained areas within each field using a color-sensitive software imaging program based on MATLAB script (The MathWorks Inc., Natick, MA) and developed by B. Bourke in the laboratory of Prof. A. Sinusas (Yale School of Medicine, New Haven, CT). Quantitative image analysis was performed on 7 randomly selected fields/section (magnification, ×200), for each of 2 sections/tumor, for each of 4 tumors/treatment group ( $n = 56$ ). *In vivo* apoptosis was determined by TUNEL (Zymed, San Francisco, CA) as described previously (24), except that antigen retrieval was carried out by tissue digestion in 0.05% trypsin at 37°C for 30 min.

**Statistical Analysis.** The kinetics of tumor growth under the various conditions tested was analyzed by the unpaired





**Fig. 1** Survivin inhibits endothelial cell apoptosis. **A**, cytoprotection against growth factor deprivation. HUVECs were transduced with pAd-GFP or pAd-Survivin, incubated in 0% serum for up to 72 h, fixed, and stained with DAPI. The percentage of cells with apoptotic nuclear morphology was determined by fluorescence microscopy. **B**, caspase processing. Cell lysates of HUVECs treated as described in **A** and harvested after 24 and 48 h were analyzed by Western blotting with cleavage-specific antibodies to active caspase-3 and -7. **C**, caspase activity. Cell lysates of HUVECs treated as described in **A** and harvested after 48 h were analyzed for caspase-3 activity by hydrolysis of the fluorogenic substrate DEVD-AMC. **D**, cytoprotection against death-inducing stimuli. HUVECs were infected as described in **A** and incubated in 0% serum with or without C<sub>6</sub> ceramide or the TNF- $\alpha$ /CHX combination for up to 8 h, before analysis of nuclear morphology by DAPI staining.

two-tailed *t* test on a GraphPad Prism software package for Windows. A *P* of 0.05 was considered statistically significant.

## RESULTS

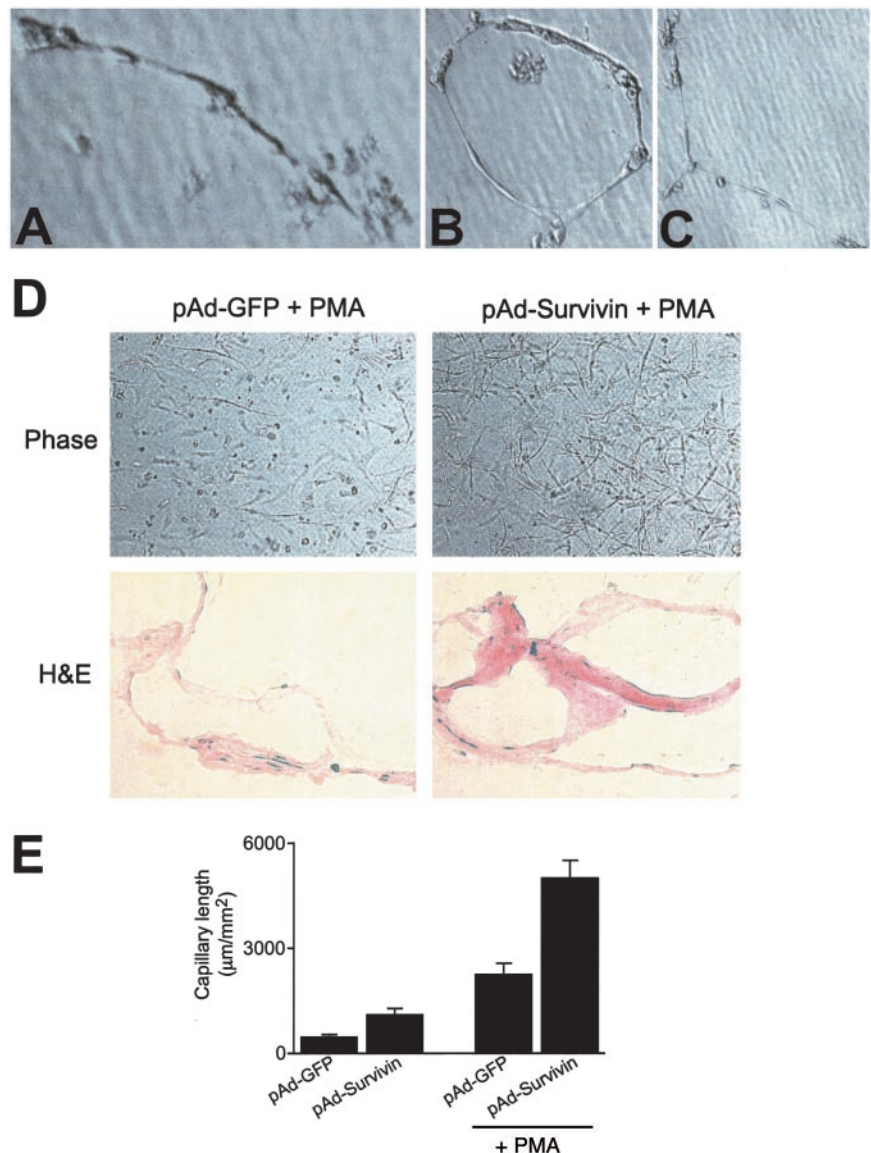
### Antiapoptotic Effect of Survivin in Endothelial Cells.

When deprived of nutrients, HUVECs transduced with pAd-GFP exhibited time-dependent loss of cell viability, with chromatin condensation and nuclear fragmentation. By 72 h of growth factor deprivation, ~60% of the HUVEC population expressing pAd-GFP showed morphological signs of apoptosis (Fig. 1A). In contrast, HUVECs transduced with pAd-Survivin were resistant to growth factor deprivation-induced apoptosis throughout a 72-h culture (Fig. 1A). A potential effect of survivin on caspase activation and catalytic activity was investigated. Incubation of pAd-GFP-transduced HUVECs in low serum resulted in time-dependent generation of active caspase-3 and -7, by Western blotting with activation-specific antibodies (Fig. 1B). This was associated with generation of caspase-3 catalytic activity by hydrolysis of the chromogenic substrate DEVD-AMC (Fig. 1C). In contrast, HUVECs transduced with pAd-Survivin did not exhibit caspase activation by Western blotting (Fig. 1B), and caspase-3 catalytic activity was largely suppressed in these cells (Fig. 1C). In addition to growth factor deprivation,

transduction of HUVECs with pAd-Survivin nearly completely suppressed ceramide-induced apoptosis, whereas cell death induced by the combination of TNF- $\alpha$  plus CHX was only minimally reduced (Fig. 1D).

**Survivin Promotes Capillary Formation *in Vitro*.** Cultivation of HUVECs or DMVECs in Matrigel pellets or rat collagen gels resulted in the formation of capillary-like tube structures by 48 h (Fig. 2A) that exhibited extensive elongation and branching after a 72-h culture at 37°C (Fig. 2, B and C). In the absence of PMA as a stabilizing agent, HUVECs transduced with pAd-GFP exhibited only a negligible degree of capillary formation after a 7-day culture at 37°C. Treatment of pAd-GFP-treated HUVECs with PMA increased capillary length by ~3-fold and stabilized tube formation by phase-contrast microscopy and H&E staining (Fig. 2, D and E). Transduction with pAd-Survivin prolonged capillary tube viability in the presence of PMA and supported the persistence of viable capillaries even in the absence of PMA throughout a 7-day culture at 37°C (Fig. 2, D and E).

**Survivin Targeting Induces Endothelial Cell Apoptosis.** Transduction of exponentially proliferating HUVECs or DMVECs (expressing endogenous survivin) with a phosphorylation-defective survivin mutant pAd-T34A (20) resulted in induction of apoptosis in both cell types in the absence of other

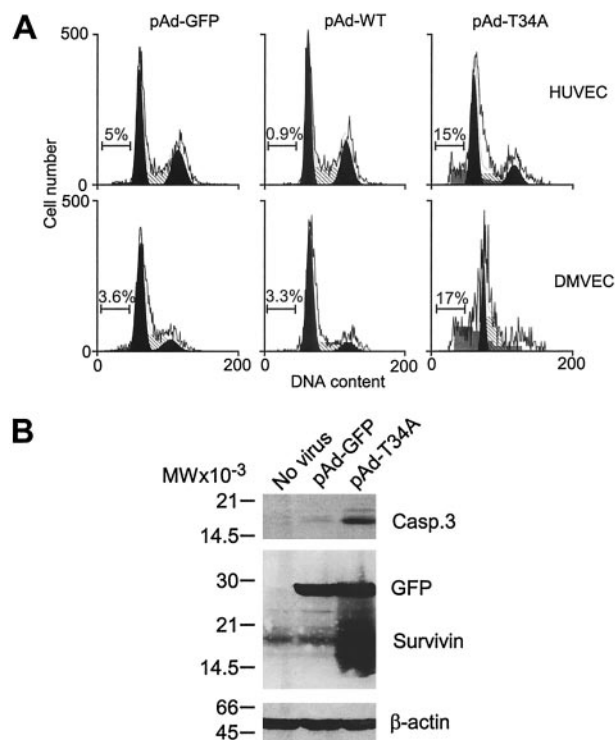


**Fig. 2** Survivin stabilizes three-dimensional capillary networks. *A–C*, three-dimensional capillary formation. HUVECs were grown in Matrigel pellets in the presence of VEGF (50 ng/ml) at 37°C and analyzed for capillary formation by phase-contrast microscopy. Organized tube-like structures became visible after 48 h (*A*) and progressed to form elongated and branching capillaries by 72 h (*B* and *C*). Original magnifications,  $\times 400$  (*A*) and  $\times 200$  (*B*, *C*). *D*, stabilization of capillary formation by survivin. HUVECs were transduced with pAd-GFP (*left panels*) or pAd-Survivin (*right panels*) for 10 h in 20% serum, mixed with collagen gels, and allowed to form capillary-like tube structures in the presence of PMA. The gels were maintained in culture for 7 days and analyzed by phase-contrast microscopy (*top panels*) or stained with H&E to quantify capillary-like tube length (*bottom panels*). Original magnifications,  $\times 100$  (*top panels*) and  $\times 200$  (*bottom panels*). *E*, quantification of capillary-like tube length. The experimental conditions are as described in *D*, and capillary-like tube length in the presence or absence of PMA was determined by NIH Image software analysis. Data are representative of one experiment of three independent determinations.

cell death stimuli by DNA content analysis and flow cytometry (Fig. 3A). Consistent with genuine induction of apoptosis, survivin targeting by pAd-T34A resulted in *de novo* generation of active caspase-3 by Western blotting (Fig. 3B). In contrast, HUVECs or DMVECs transduced with pAd-GFP or pAd-Survivin did not exhibit induction of apoptosis or caspase-3 cleavage (Fig. 3, *A* and *B*).

**Survivin Targeting Initiates Mitochondrial-dependent Apoptosis.** To map the position of survivin in cell death pathway(s) (13), we used stably transfected YUSAC-2 melanoma cells in which expression of survivin(T34A) is conditionally induced upon withdrawal of Tet (Tet-off system; Ref. 20). Conditional expression of survivin(T34A) in YUSAC-2 cells resulted in rapid and progressive accumulation of mitochondrial cytochrome *c* in the cytosol by Western blotting (Fig. 4A). In contrast, Tet-regulated expression of wild-type survivin in

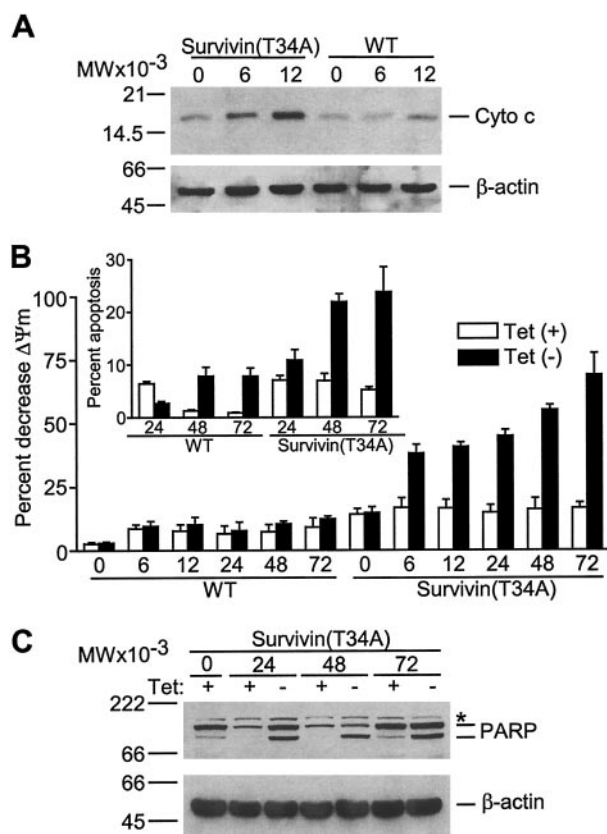
YUSAC-2 cells did not result in cytochrome *c* increase in the cytoplasm (Fig. 4A). Conditional expression of survivin(T34A) was also associated with time-dependent loss of mitochondrial transmembrane potential, beginning at 6 h after Tet removal and steadily increasing over a 72-h time interval (Fig. 4B). This coincided with time-dependent induction of apoptosis in YUSAC-2 cells by DNA content analysis (Fig. 4B, *inset*), in agreement with previous observations (20). In contrast, Tet-regulated expression of wild-type survivin did not result in significant changes in mitochondrial transmembrane potential (Fig. 4B) and did not affect YUSAC-2 cell viability throughout a 72-h culture (Fig. 4B, *inset*). Consistent with genuine induction of apoptosis, Tet-regulated expression of survivin(T34A) resulted in time-dependent cleavage of  $M_r \sim 115,000$  proform PARP to an apoptotic  $M_r \sim 85,000$  fragment, whereas no PARP cleavage was observed in the presence of Tet (Fig. 4C).



**Fig. 3** Induction of endothelial cell apoptosis by survivin targeting. **A**, DNA content. Exponentially growing HUVECs or DMVECs were transduced with the indicated adenoviral constructs and analyzed for DNA content by propidium iodide staining and flow cytometry after a 96-h culture at 37°C. The percentage of cells with hypodiploid, *i.e.*, apoptotic, DNA content is indicated for each condition tested. **B**, caspase activation. HUVECs transduced with pAd-GFP or pAd-T34A were analyzed for expression of active caspase-3 by Western blotting. The reactivity of antibodies to survivin, GFP, or β-actin with HUVEC extracts is shown.

**Genetic Requirements of Apoptosis Induced by Survivin Targeting.** To map more precisely the genetic requirements of apoptosis induced by survivin targeting, we used MEFs isolated from mouse embryos deficient in the initiating components of mitochondrial-dependent apoptosis, Apaf-1 and caspase-9 (13). Transduction of wild-type MEFs expressing endogenous survivin (data not shown) with pAd-T34A resulted in time-dependent induction of apoptosis by DNA content analysis and flow cytometry (Fig. 5, *A* and *B*). In contrast, apoptosis induced by pAd-T34A was completely suppressed in MEFs isolated from Apaf-1 or caspase-9 knockout embryos (Fig. 5, *A* and *B*). In control experiments, transduction with pAd-GFP did not reduce cell viability of wild-type, Apaf-1<sup>-/-</sup>, or caspase 9<sup>-/-</sup> MEFs (Fig. 5, *A* and *B*).

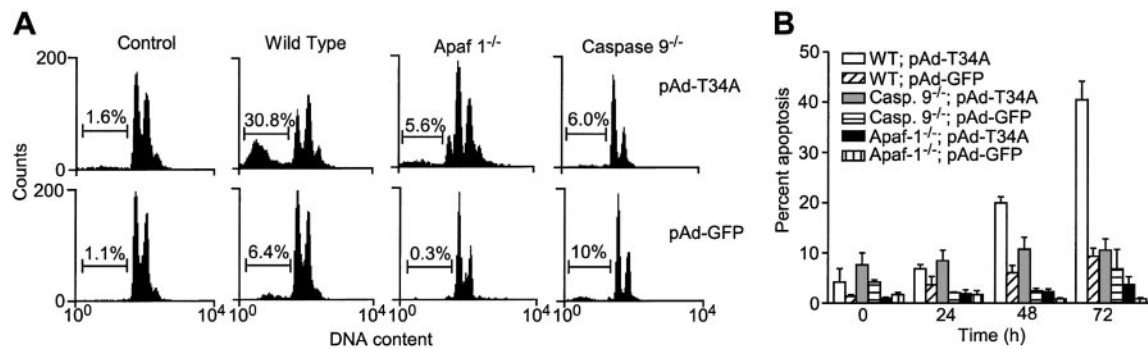
**Cytoprotective Effect of Survivin on the Tumor Microenvironment.** Intratumoral injection of pAd-T34A in human MCF-7 breast cancer xenografts resulted in GFP expression *in situ* (Fig. 6*A*, *inset*) and inhibition of tumor growth (Fig. 6*A*), in agreement with previous observations (24). This was associated with massive induction of tumor cell apoptosis *in vivo* by internucleosomal DNA fragmentation (Fig. 6*B*). In contrast, MCF-7 xenografts injected with pAd-GFP exhibited exponen-



**Fig. 4** Survivin targeting induces mitochondrial-dependent apoptosis. **A**, cytochrome *c* release. Cytosolic extracts were isolated from YUSAC-2 melanoma cells expressing wild-type survivin (WT) or survivin Thr<sup>34</sup>→Ala mutant [*Survivin(T34A)*] at the indicated time intervals upon Tet removal and analyzed by Western blotting with antibodies to cytochrome *c* or β-actin. **B**, mitochondrial transmembrane potential. YUSAC-2 cells expressing survivin or survivin(T34A) were analyzed for mitochondrial depolarization (ΔΨ<sub>m</sub>) by loss of JC-1 staining and flow cytometry. *Inset*, induction of apoptosis. The experimental conditions are as described above, except that Tet- YUSAC-2 cells expressing survivin (T34A) were analyzed at the indicated time intervals for DNA content by propidium iodide staining and flow cytometry. **C**, PARP cleavage. Extracts from survivin(T34A)-YUSAC-2 cells in the presence or absence of Tet were analyzed for cleavage of PARP by Western blotting. The position of uncleaved (*M<sub>r</sub>* 115,000) or cleaved (*M<sub>r</sub>* 85,000), *i.e.*, apoptotic, PARP is indicated. Asterisk, nonspecific band.

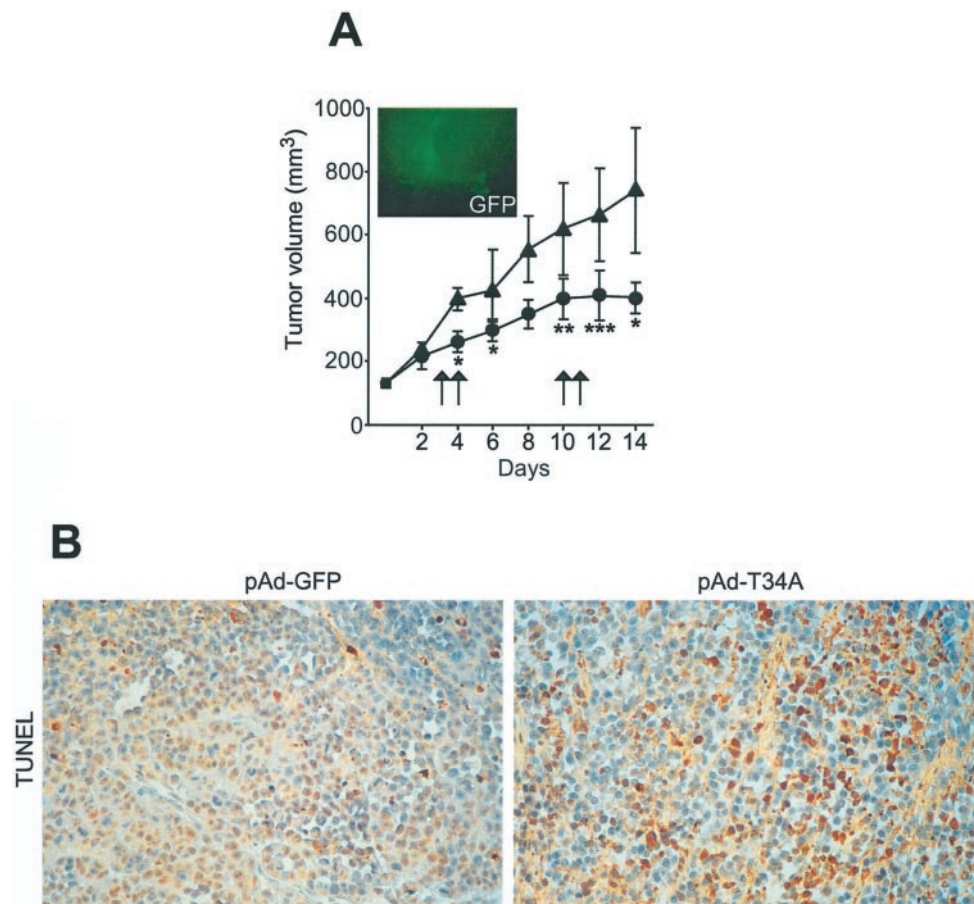
tial tumor growth (Fig. 6*A*) and no significant decrease in tumor cell viability by TUNEL staining (Fig. 6*B*). Immunohistochemical analysis of endothelial cell-derived CD31 in MCF-7 xenografts injected with pAd-GFP revealed an extensive network of viable blood vessels, consistent with tumor-associated angiogenesis (Fig. 7*A*). In contrast, intratumor injection of pAd-T34A resulted in a significant reduction in the number of viable blood vessels by CD31 staining (Fig. 7*A*). As determined by quantitative morphometric analysis of CD31-stained areas, injection of pAd-T34A resulted in a ~3-fold suppression of blood vessel density in MCF-7 xenografts, as compared with pAd-GFP (Fig. 7*B*). Reduction in blood vessel density in pAd-T34A-injected tumors was associated with appearance of apoptosis in endothelial cells lining tumor-derived blood vessels by internucleosomal DNA fragmentation *in vivo* (Fig. 7*C*).





**Fig. 5** Genetic requirements of apoptosis induced by survivin targeting. *A* and *B*, apoptosome requirement. MEFs from Apaf1<sup>-/-</sup> or caspase 9<sup>-/-</sup> knockout mice were infected with pAd-GFP or pAd-T34A and analyzed for DNA content after a 72-h culture at 37°C by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid, *i.e.*, apoptotic, DNA content under the various conditions tested is indicated. *Control*, uninfected MEFs. *B*, summary of the results. The experimental conditions are as described above. Data are the mean  $\pm$  SE of three independent experiments.

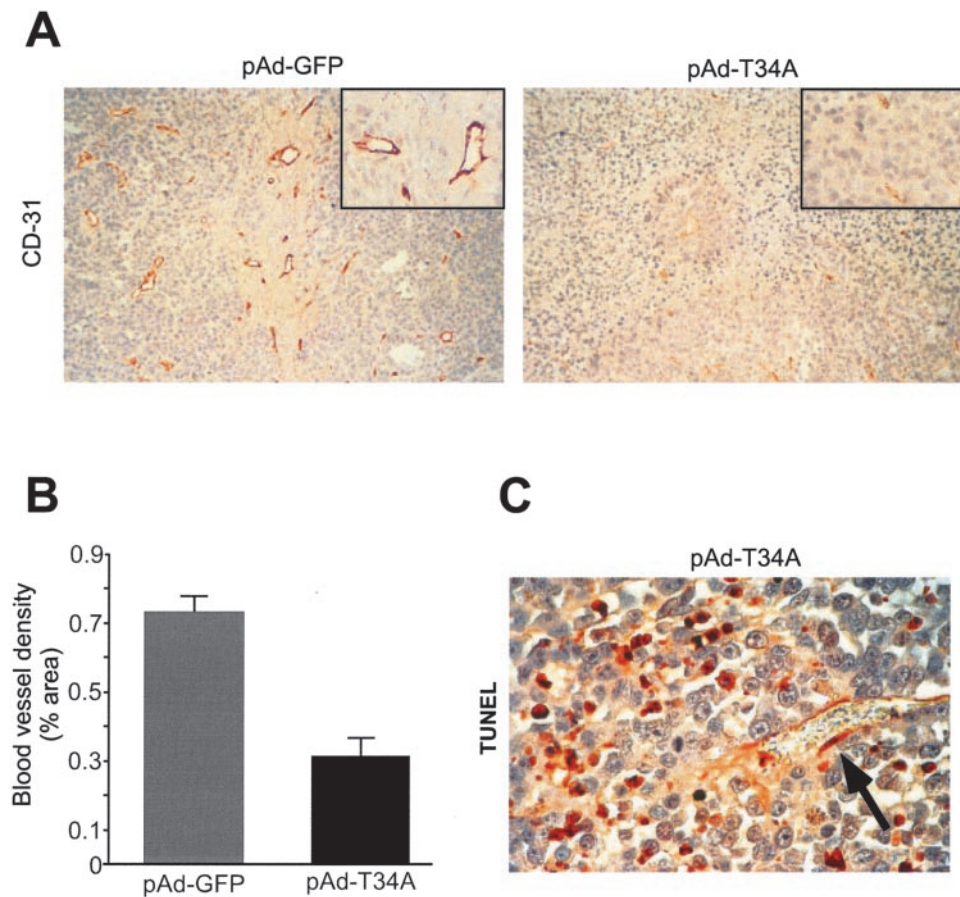
**Fig. 6** Anticancer efficacy of survivin targeting. *A*, kinetics of tumor growth. Human breast carcinoma MCF7 cells were implanted s.c. in the flanks of immunocompromised C.B.-17 SCID/beige female mice and allowed to form tumors for 5 days. Tumors (6 tumors/group) were injected locally with pAd-GFP ( $\blacktriangle$ ) or pAd-T34A ( $\bullet$ ) on three sites at the indicated time intervals (arrows). Tumor size was measured every other day for 14 days after initiation of treatment. Data are the mean  $\pm$  SE of two independent experiments. \*,  $P = 0.032$ – $0.01$ ; \*\*,  $P = 0.004$ ; \*\*\*,  $P = 0.0005$ . *Inset*, adenoviral gene transfer *in vivo*. Tumors were harvested after 7 days of treatment, processed for histology, and analyzed for GFP expression by fluorescence microscopy. GFP-positive cells surrounded the needle tracks at the putative injection sites. Original magnification,  $\times 100$ . *B*, tumor cell apoptosis *in vivo*. Tumors treated with pAd-GFP (*left panel*) or pAd-T34A (*right panel*) were prepared as described in *A*, harvested after 7 days, processed for histology, and analyzed for TUNEL staining. Magnification,  $\times 200$ .



## DISCUSSION

In this study, we have positioned the survivin pathway as a novel regulatory mechanism of mitochondrial-dependent apoptosis. Secondly, we have shown that targeting survivin exerts anticancer activity by combining enhanced tumor cell apoptosis with suppression of tumor-associated angiogenesis *in vivo*.

Despite the considerable interest in survivin for its bifunctional role in cell viability and regulation of mitosis (10) and the dramatic exploitation of this pathway in human tumors (12), critical aspects of the survivin pathway have remained elusive. In particular, how survivin couples to the cell death machinery has not been conclusively elucidated. Earlier claims that this



**Fig. 7** Suppression of tumor-derived angiogenesis by survivin targeting *in vivo*. **A** and **B**, survivin targeting inhibits tumor-associated angiogenesis. The experimental conditions are the same as those described in the Fig. 6 legend, except that human breast cancer xenografts injected with pAd-GFP or pAd-T34A were harvested after 7 days of treatment and analyzed for expression of CD31 (platelet-endothelial cell adhesion molecule-1) by immunohistochemistry. Magnification,  $\times 100$ . *Insets*,  $\times 200$ . **B**, morphometric quantification of CD31 positivity. Blood vessel density in pAd-GFP- or pAd-T34A-injected tumors shown in **A** was determined by image color analysis from CD31 reactivity. **C**, induction of endothelial cell apoptosis *in vivo*. Tumors were prepared as described in **A** and analyzed for apoptosis by internucleosomal DNA fragmentation. *Arrow*, TUNEL-positive endothelial cells. Magnification,  $\times 400$ .

may involve suppression of caspase catalytic activity (30), similar to other antiapoptotic IAP proteins (9), were disputed on both functional (31) and structural grounds (32). More recent arguments favored an indirect model of survivin-mediated cell viability, in which interference with survivin expression/function resulted in cell death merely as a consequence of catastrophic cell division defects (33, 34), potentially involving mistargeting of Aurora B kinase (35).

Here, we obtained clear evidence to unambiguously rule out these speculations (34) and, for the first time, to firmly position the survivin pathway as a novel upstream regulator of mitochondrial-dependent apoptosis (13). Interference with survivin function using a phosphorylation-defective survivin Thr<sup>34</sup>→Ala mutant (20) caused all of the earliest cellular and biochemical events of mitochondrial-dependent apoptosis (13), including release of cytochrome *c*, loss of mitochondrial transmembrane potential, and cleavage of caspase substrates. This cell death response was abolished in MEFs deficient in the upstream apoptosome components, Apaf-1 and caspase-9 (13). This model for survivin function is consistent with the ability of survivin to reduce the generation of active caspase-3 and -7 (Fig. 1), rather than suppressing the activity of the mature enzymes, and with the previously reported physical interaction between survivin and the upstream mitochondrial initiator, caspase-9 (19). Several possibilities for how survivin could influence the

upstream initiation of mitochondrial-dependent apoptosis could be envisioned, including its recently reported association with Smac/DIABLO (36), a mitochondrially released protein, which relieves the inhibitory function of IAP on caspase-9 activation (32). Clearly, the present data do not support the preliminary claims of Chen *et al.* (35) suggesting that survivin function could be recapitulated by its potential interaction with Aurora B kinase. Although critical for cell division, Aurora B is not believed to couple to the mitochondrial cell death machinery, and the phenotype of reduced spindle microtubule density and apoptosis induced by microinjection of antibodies to survivin (37) is quite distinct from the defect of microtubule bundling and astral microtubule extension observed after interference with Aurora B function (38, 39).

The Thr<sup>34</sup>→Ala mutation used here to map the survivin pathway abolishes a phosphorylation site for the main mitotic kinase p34<sup>cdc2</sup>-cyclin B1 (19) and has been used previously to interfere with the function of endogenous survivin, resulting in apoptosis (19) and anticancer activity *in vitro* and *in vivo* (24). Recently, Temme *et al.* (40) used an overexpression approach with a large, tetrameric DsRed-survivin fusion protein to investigate the subcellular distribution and function of wild-type survivin and survivin(T34A). In that study, expression of DsRed-survivin(T34A) caused mitotic defects, inhibited cell proliferation, and induced apoptosis (40), similar to the findings



presented here. Surprisingly, however, Temme *et al.* (40) also reported that survivin(T34A) inhibited apoptosis in HeLa cells. The contradictory results of Temme *et al.* (40) may reflect their highly artificial overexpression system, which is unlikely to recapitulate the dynamic intracellular trafficking and association with multiple protein partners of endogenous survivin (23). Also contrary to the claims of Temme *et al.* (40) that mislocalization of survivin(T34A) may cause the observed cellular phenotype, published data have demonstrated that this survivin mutant exhibits accelerated degradation *in vivo* (41), suggesting that its dominant negative mode of action may involve dimerization with endogenous survivin and premature destruction of the heterocomplex.

Consistent with previous observations (14, 15, 17), expression of survivin in endothelial cells representative of different vascular beds resulted in a broad cytoprotective mechanism counteracting apoptosis, reducing the generation of active caspases, and preserving cellular survival. This translated in a productive proangiogenic response with stabilization of three-dimensional capillary networks *in vitro*. It was recently proposed that expression of survivin in the endothelium could have profound repercussions for tumor growth, reducing the effectiveness of metronomic, antiangiogenesis chemotherapy (18). The data presented here fit well with that model and demonstrate that survivin expression during angiogenesis may provide a pivotal advantage factor to maintain a florid blood supply during tumor growth. Previous studies have demonstrated that this may involve up-regulated survivin expression during the proliferative phase of angiogenesis as a transcriptional target of VEGF (14) as well as during the nonproliferative, remodeling of blood vessels contributed, among others, by angiopoietin-1 (16, 17). Although increased survivin expression in the transformed tumor cell population or angiogenic endothelium may involve distinct signaling pathways that may include loss of p53, activation of phosphatidylinositol 3'-kinase, or phosphorylation of signal transducers and activators of transcription 3 (14–17, 42), the data presented here demonstrate that survivin provides a broad cytoprotective mechanism for the tumor microenvironment as a whole. This may explain, in part, the unfavorable outcome associated with survivin expression in embryologically disparate tumors and their propensity to progress to an invasive phenotype (10), as characterized recently in a transgenic mouse model of stepwise skin cancer (43). On the other hand, these data provide a rational basis for targeting survivin as a novel cancer therapeutic strategy aimed at lowering a general antiapoptotic threshold in tumor cells and favoring the collapse of tumor-associated angiogenesis.

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