MDM2 Is Required for Suppression of Apoptosis by Activated Akt1 in Salivary Acinar Cells[∇]

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Chronic damage to the salivary glands is a common side effect following head and neck irradiation. It is hypothesized that irreversible damage to the salivary glands occurs immediately after radiation; however, previous studies with rat models have not shown a causal role for apoptosis in radiation-induced injury. We report that etoposide and gamma irradiation induce apoptosis of salivary acinar cells from FVB control mice in vitro and in vivo; however, apoptosis is reduced in transgenic mice expressing a constitutively activated mutant of Akt1 (myr-Akt1). Expression of myr-Akt1 in the salivary glands results in a significant reduction in phosphorylation of p53 at serine¹⁸, total p53 protein accumulation, and p21^{WAF1} or Bax mRNA following etoposide or gamma irradiation of primary salivary acinar cells. The reduced level of p53 protein in myr-Akt1 salivary glands corresponds with an increase in MDM2 phosphorylation in vivo, suggesting that the Akt/MDM2/p53 pathway is responsible for suppression of apoptosis. Dominant-negative Akt blocked phosphorylation of MDM2 in salivary acinar cells from myr-Akt1 transgenic mice. Reduction of MDM2 levels in myr-Akt1 primary salivary acinar cells with small interfering RNA increases the levels of p53 protein and renders these cells susceptible to etoposide-induced apoptosis in spite of the presence of activated Akt1. These results indicate that MDM2 is a critical substrate of activated Akt1 in the suppression of p53-dependent apoptosis in vivo.

Glandular homeostasis requires a balance of cell proliferation and cell death. Head and neck irradiation causes secondary side effects in normal salivary gland tissues, resulting in severe salivary gland hypofunction and diminishment of the quality of life for patients (33). Perturbations of salivary gland homeostasis are likely to underlie the hypofunction observed following therapeutic irradiation of the head and neck region for treatment of cancer. The exquisite sensitivity of the salivary glands to radiation is characterized by a 50% reduction in salivary flow rate, loss of glandular weight and acinar cells, and morphological changes in gland structure (1, 18, 19, 29, 61). A single study has reported that a maximum of 2% apoptotic cells were observed in the salivary glands of rats receiving a single dose of 2 Gy and that no further increase was observed at higher doses of irradiation (up to 25 Gy) (68). This has suggested to some investigators that acute irradiation-induced damage cannot be responsible for the dramatic decrease in salivary gland function observed following irradiation; however, our studies suggest that irradiation-induced apoptosis is far more extensive than previously thought. Better understanding of radiation-induced damage is fundamental to cancer

treatment strategies as well as in reducing secondary side effects in normal tissues.

The serine/threonine protein kinase Akt, also known as protein kinase B, provides an important survival signal in many different tissues (17, 21, 42). Activation of Akt occurs in a phosphatidylinositol-3'-kinase-dependent manner following stimulation of cells with a variety of growth factors that induce cell proliferation and cell survival (20). Potential substrates for Akt include procaspase 9 (14), the proapoptotic Bcl-2 family member BAD (22, 23, 25), and members of the Forkhead family of transcription factors (13, 24). Other recently identified substrates for Akt include tuberous sclerosis complex (Tsc2), a regulator of the mammalian target of rapamycin (mTOR) (39, 70); Myt1, a regulator of G₂/M-phase transition (65); Yes-associated protein (YAP), a transcriptional coactivator of the p53 homologue p73 (9); and Chk1, a protein kinase activated by DNA damage, whose activation is inhibited when phosphorylated by Akt (44). Expression of activated Akt1 in the mammary glands of transgenic mice significantly delays involution of mammary epithelial cells, a process that requires apoptosis (38, 77). Other constitutively activated Akt transgenic mouse systems have confirmed other signaling molecules that lie downstream of Akt, including cyclin D, eIF4E, and mTOR (38, 79, 92). It is well known that activated Akt suppresses apoptosis, although the in vivo mechanisms of this suppression are largely unknown. Akt can phosphorylate a number of substrates in vitro; however, the critical substrate(s) in vivo may be cell type and stimulus specific.

Upon DNA damage, p53 undergoes several posttranslational modifications, including phosphorylation and acetylation, which increases the stability of p53 dramatically (2, 48, 55, 56, 93). The ATM (for "ataxia telangiectasia mutated") protein

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kinase senses double-stranded breaks or changes in the chromatin structure of the DNA (6) and forms a complex with p53, leading to the phosphorylation p53 on serine¹⁵ (55). In addition to ATM, ATR (for "ATM-Rad3-related") protein kinase also phosphorylates p53 on serine¹⁵, suggesting that p53 modification and accumulation may be controlled by multiple kinases that are activated by different types of DNA damage (7, 82). ATM and ATR are both able to phosphorylate and activate Chk1, which leads to phosphorylation and destruction of CDC25A and cell cycle arrest (8); phosphorylation of Chk1 by Akt inhibits Chk1 and prevents cell cycle arrest (44). DNA damage-induced activation of p53 causes cell cycle arrest, predominantly at the G₁/S transition, and activates DNA repair; however, if DNA damage is severe and repair is not effective, p53 also can induce apoptosis (86).

Recently, Akt has been shown to modulate the activity of p53 through its substrate MDM2 (for "murine double minute clone 2") (64, 67, 98). MDM2 is an E3 ubiquitin ligase that negatively regulates p53 transcriptional activity (95). Phosphorylation of MDM2 on serine¹⁶⁶ and serine¹⁸⁶ by Akt stimulates translocation of MDM2 to the nucleus, where it binds to p53 and targets p53 degradation by the proteasome (64, 67, 98). The correlation between Akt activation and MDM2 localization has also been extended in vivo to breast tumor tissues (98). To date, analysis of the Akt/MDM2/p53 pathway has been largely confined to studies utilizing transient transfection of tissue culture cells; here, we report that expression of MDM2 in primary cells that express activated Akt1 is critically important for the ability of Akt to suppress apoptosis following DNA damage.

We generated transgenic mice in which expression of a constitutively activated mutant of Akt1, myr-Akt1, was targeted to the mammary gland through the use of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (77). Expression of the MMTV-LTR in other tissues, such as salivary glands, lung, kidneys, prostate gland, and lymphoid tissue, has been described (36), and we identified a founder line that expresses the myr-Akt1 transgene in the salivary gland. Our studies suggest that myr-Akt1 suppresses p53-dependent apoptosis through the regulation of MDM2 phosphorylation. Improved understanding of the salivary gland-specific events involved in early gamma-irradiation damage will provide the foundation for potential elimination of the subsequent secondary chronic effects and improved care for patients undergoing head and neck irradiation.

MATERIALS AND METHODS

Mice. myr-Akt1 transgenic mice were generated by standard techniques, as described previously (77), by the Transgenic Mouse Core of the University of Colorado Cancer Center. FVB mice were purchased from Taconic Laboratories (Germantown, NY). Genomic tail DNA was extracted from the founder mice by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation (77). Transgenic mice were identified by PCR as previously described. The forward primer was from the sequence of Akt1 (5'-GCC GCT ACT ATG CCA TGA AGA-3'), and the reverse primer was targeted against the hemagglutinin (HA) epitope (5'-GTA ATC TGG AAC ATC GTA TGG GTA-3') (77). Animals were maintained in accordance with protocols approved by the University of Colorado Health Sciences Center Laboratory Animal Care and Use Committee.

Immunoblotting. Tissues were lysed in RIPA (150 mM NaCl, 50 mM Tris [pH 7.4], 2 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate) supplemented with protease inhibitor cocktail (Sigma Chemical Company, St. Louis,

MO), 100 µg/ml phenylmethylsulfonyl fluoride, and 100 U/ml aprotinin (Pierce Chemical Company, Rockford, IL). Tissues were then boiled for 10 minutes, chilled on ice, and disrupted by sonication until homogeneous (77). Primary salivary acinar cells were lysed in JNK lysis buffer (25 mM HEPES [pH 7.5], 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 10 mM NaF) supplemented with aprotinin (4 µg/ml), Prefabloc (0.5 mg/ml), and leupeptin (2 µg/ml) (Pierce Chemical Company), as well as 1 mM sodium orthovanadate, and the lysates were clarified by centrifugation at 4°C for 30 min at 13,000 rpm in a refrigerated Savant microcentrifuge (3). Protein concentrations were determined with the BCA Protein Assay kit (Pierce Chemical Company). For immunoblotting, 100 µg of whole cellular protein was resolved on an 8 or 10% polyacrylamide gel, transferred to an Immobilon membrane (Millipore Corporation, Bedford, MA), and immunoblotted. Anti-HA antibody was purchased from Roche Diagnostics (Indianapolis, IN). Anti-ERK antibody was purchased from Promega Corporation (Madison, WI), while anti-phosphorylated p53 (serine¹⁵), anti-phosphorylated GSK3α/β (serine⁹), anti-GSK3β and anti-phosphorylated MDM2 (serine¹⁶⁶) were acquired from Cell Signaling Technologies (Beverly, MA). Serine¹⁵ in human p53 corresponds to serine¹⁸ in mouse p53, and this antibody detects both species (80). Serine¹⁶⁶ in human MDM2 corresponds to serine¹⁶³ in the mouse genome, and this antibody also detects both species. Anti-p53 (DO-12) was obtained from Novacastra Laboratories (Burlingame, CA), anti-p73 (Ab-4) and anti-tubulin were purchased from Neomarkers (Fremont, CA), and anti-p63 and anti-MDM2 (Ab-4) were acquired from Oncogene Research Products (San Diego, CA). We have also used Imgenex antibodies IMG-246 and IMG-259 for detection of p73 with similar results, and these antibodies have been shown to detect endogenous p73 (75). Anti-phosphorylated ATM¹⁹⁸¹, anti-Chk1, and anti-phosphorylated Chk1 (serine³⁴⁵) were from Cell Signaling Technologies. Secondary antibodies were conjugated with horseradish peroxidase (HRP), and the anti-HRP-conjugated rabbit antibody was purchased from Bio-Rad (Hercules, CA), while the anti-HRP-conjugated mouse antibody was obtained from Amersham (Arlington Heights, IL). Enhanced chemiluminescence lighting (Pierce Chemical Company) was used according to the manufacturer's instructions to detect immunoblotted proteins. In some cases, membranes were then stripped, as previously described (35), reblocked in Tris-buffered saline (10 mM Tris [pH 7.4], 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk (Carnation), and probed with a second antibody. The activation of Akt kinase activity was quantitated with a radioactive Akt kinase assay kit (Upstate Biotechnology, Lake Placid, NY) using 300 µg of tissue lysates according to the manufacturer's instructions (50).

Histology. Tissues were fixed in 10% neutral buffered formalin and then embedded in paraffin. Tissue sections were cut at 4 µm and processed for standard staining with hematoxylin and eosin by the Histology Service of the Department of Pathology at the University of Colorado School of Medicine. Tissue sections were observed by standard light microscopy, and photomicrographs were taken with Nikon Eclipse E600 microscope with Spot Diagnostic imaging software.

DNA content. Genomic DNA was extracted from myr-Akt1 or FVB salivary glands by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Submandibular and parotid gland DNAs were diluted 1:1,000, and sublingual gland DNA was diluted 1:10 prior to analysis. DNA concentrations were determined with a PicoGreen double-stranded DNA quantitation kit (Molecular Probes) and analyzed in triplicate. Sample fluorescence (excitation, ~480 nm; emission, ~520 nm) was measured on a microplate reader (Molecular Devices, Sunnyvale, CA). A lambda DNA standard provided in the kit was used to generate a standard curve.

Saliva collection. Mice were injected intraperitoneally (i.p.) with 0.25 mg carbachol per kg body weight. Saliva was collected immediately following injection for 5 min and chilled on ice. Total proteins present in the collected saliva were analyzed by resolving 25 µg total protein on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and the gel was stained with Gel Blue reagent (Pierce Chemical Company) according to the manufacturer's instructions.

Immunofluorescence staining. Salivary glands to be examined for amylase staining were removed, fixed in 10% neutral buffered formalin overnight, and subsequently embedded in Tissue Freezing Medium (Fisher Scientific, Pittsburgh, PA). Sectioning (7 µm) and staining were performed by the Histology Core, Barbara Davis Center for Childhood Diabetes (Denver, CO) (40). Photomicrographs were taken with a Nikon Eclipse E600 microscope using fluorescein isothiocyanate (amylase) and UV (Hoerst) filters, and images were merged as described above.

Generation and cultivation of primary salivary acinar cells. Primary salivary acinar cells were prepared from 4- to 5-week-old female FVB or myr-Akt1 transgenic mice. Mice were anesthetized with avertin (0.4 to 0.6 mg/gm, i.p.), and

primary parotid or submandibular acinar cells were prepared under sterile conditions similar to previously published protocols (50, 51, 72). A 1% (vol/vol) cell suspension was seeded onto collagen-coated dishes or coverslips (Falcon/Becton Dickinson, Fairlawn, NJ), and cultures were approximately 80% confluent after 5 days in culture. Untreated cells were examined by light microscopy to ensure an enriched population of acinar cells. Photomicroscopy was accomplished with an Olympus CK2 inverted microscope and imaged on Kodak TMAX100 film. 5-Bromo-2'-deoxyuridine (BrdU) labeling was performed for 30 min with Labeling and Detection Kit I (Roche Diagnostics) according to the manufacturer's instructions, and immunofluorescent nuclei were imaged on a Nikon Eclipse E600 microscope using a fluorescein isothiocyanate filter. Cell counts were performed on a minimum of six fields of view per slide from three independent experiments (total cells counted ranged from 850 to 2,500 per group).

Induction and quantitation of apoptosis. Primary mouse salivary acinar cells were treated with various doses of etoposide (50 to 200 μ M) or ionizing radiation (0.25 to 5 Gy). Etoposide was purchased from Sigma Chemical Company. Exposure to ionizing radiation was performed with a cobalt source [model GB150, type B(U), serial no. 32R; Atomic Energy of Canada Ltd.]. Lysates were prepared in JNK lysis buffer, as described above, 18 h after treatment with etoposide or 24 h after treatment with gamma irradiation (3, 51). Activation of caspase 3 was quantitated with the BioMol QuantiZyme Colorimetric assay kit (Plymouth Meeting, PA). The adherent and floating cells were collected from a 100-mm² dish and lysed in caspase lysis buffer supplemented with 0.1% Triton X, aprotinin (4 μ g/ml), Prefabloc (0.5 mg/ml), and leupeptin (2 μ g/ml), according to the manufacturer's instructions and previously published reports (3, 53). Caspase 3 activity in 15 μ g of cellular lysate was measured by the cleavage of Ac-DEVD-pNA substrate, and absorbance at A_{405} was quantitated in a microtiter plate reader (Molecular Devices) at 10-min intervals for 7 h. Cells for the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay were fixed in 2% paraformaldehyde, and detection of apoptotic cells was performed with the Cell Death kit (Roche Diagnostics). Cell counts were performed on a minimum of six fields of view per slide from three independent experiments.

For in vivo experiments, 4-week-old female FVB and myr-Akt1 transgenic mice were anesthetized with avertin (0.4 to 0.6 mg/gm, i.p.), and the head and neck region was exposed to ionizing radiation with a cobalt source [model GB150, type B(U), serial no. 32R; Atomic Energy of Canada Ltd.]. The rest of the body was shielded with ~6 mm of lead to avoid the systemic effects of gamma irradiation. Animals were treated in accordance with protocols approved by the University of Colorado Health Sciences Center Laboratory Animal Care and Use Committee.

Salivary glands were removed 8 and 24 h postirradiation, fixed in 10% neutral buffered formalin, and then embedded in paraffin. Slides were heated to 60°C for 45 min and then rehydrated in citrosolve, graded alcohols, and distilled H₂O washes. For antigen retrieval, slides were placed in citrate buffer in a pressure cooker, heated in a microwave oven for 20 min, and allowed to cool for 20 min. After washes, the slides were blocked with avidin and biotin. Anti-activated caspase 3 (9661; Cell Signaling) was layered on at a 1:100 dilution in Biomedica primary antibody-diluting medium and allowed to incubate overnight at 4°C on a rotating plate. Nonspecific peroxidase activity was quenched with 1% H₂O₂ followed by secondary antibody (goat anti-rabbit at a 1:200 dilution) incubation at room temperature for 50 min. Vector ABC was incubated for 30 min at room temperature, and color development was achieved with Biogenex DAB incubation for 1 to 2 min. Slides were counterstained with Gill's hematoxylin, dehydrated, and mounted in Protexx. Cell counts were performed with a minimum of five fields of view per slide from three mice (total cells counted ranged from 1,800 to 2,500 per mouse).

RNA isolation and quantitative RT-PCR. Adherent and floating primary cells were collected from a 100-mm² dish and lysed in RLT buffer (QIAGEN, Valencia, CA). Cells were homogenized with QiaShredder columns (QIAGEN) and frozen at -80°C until the isolation procedure. Cellular homogenates were thawed at 37°C, and RNA was isolated by the RNeasy QIAGEN column procedure. For preparation of RNA from tissue, salivary glands were isolated from 4-week-old FVB or myr-Akt1 female mice, cleaned of connective tissue, and stored in RNA Later (QIAGEN). Samples were lysed in RLT lysis buffer and homogenized with a polytron (74). Total RNA was treated with DNase 1 using the DNA-free kit according to the manufacturer's instructions (Ambion, Austin, TX) or using the on-column digestion protocol (QIAGEN). Quantitative reverse transcriptase PCR (RT-PCR) was conducted in triplicate for each RNA sample, according to DeRyckere and DeGregori (26), with the DNA Engine Opticon 2 system (MJ Research Inc., Waltham, MA). Briefly, 25 ng of total RNA was added to the RT-PCR buffer containing 0.2 μ M 5' and 3' primers, 305 mU/ μ l of anti-RNase (Ambion), 76 mU/ μ l of Moloney murine leukemia virus RT (In-

vitrogen), and 1 $\times$ SYBR green MasterMix (Applied Biosystems, Warrington, United Kingdom) at a final volume of 25 μ l. The 96-well microplate was capped and centrifuged at 3,300 $\times$ g for 4 min. RNA was reverse transcribed at 48°C for 30 min, and the RT was inactivated by a 10-min incubation at 95°C. DNA amplification was performed with 40 cycles of 95°C for 15 s, 60°C for 60 s, and fluorescence detection at 72°C. A melting curve following DNA amplification was obtained by increasing the temperature from 72 to 99.9°C, with fluorescence detection every 0.1°C/s to ensure a single amplification product. Background fluorescence was determined between cycles 1 and 18 and was used to calculate the threshold value, 5 standard deviations greater than background. Differences in starting concentrations were determined by the cycle number at which the fluorescence intensity crossed the threshold for each sample (C_T). The C_T values were subtracted for the total number of cycles (40 cycles) and normalized with ribosomal protein S15 RNA to produce a relative abundance of starting RNA concentrations (45). Homogeneity of variation was tested with an *F* test, and difference was determined with an unpaired Student's *t* test for equal sample variation. Primer sequences for S15 were as follows: forward, 5'-ATC ATT CTG CCC GAG ATG GTG-3'; reverse, 5'-TGC TTT ACG GGC TTG TAG GTG-3'. Primer sequences for p21^{WAF1} were generated according to previously published protocols (90) (forward, 5'-GCC ACA GCG ACC ATG TCC AA-3'; reverse, 5'-GCG TCT CCG TGA CGA AGT CAA A-3'), and all primers were synthesized by Invitrogen. Quantitation of p73 and p63 RNA levels by real-time RT-PCR used the following primers: p73 forward, 5'-TCT TCC TCC TCC ACC TT-3'; p73 reverse, 5'-TGC TGA GCA AAT TGA ACT GC-3' (90); p63 forward, 5'-CAG CAC CAG CAC CTA CTT CA-3'; p63 reverse, 5'-GAT AAG CTG GCT CAC GGA AG-3'. Validated primer sets for p53 and Bax quantitative RT-PCR were purchased from QIAGEN (QuantiTect Primer Assays) and used with QuantiTect SYBR green RT-PCR reagents according to the manufacturer's instructions.

Transduction of cells with recombinant human adenoviruses. Recombinant adenoviruses expressing a kinase-inactive mutant of Akt (Ad-KD-Akt1) or LacZ (Ad-LacZ) were used as previously described (50). Briefly, primary cells were transduced with the desired recombinant adenovirus at equal multiplicities of infection for 1 hour in medium lacking fetal calf serum. The culture medium was then changed to complete medium with fetal calf serum, as described above. Cells were harvested for immunoblotting 24 h after transduction, as described above.

siRNA transfection. Custom small interfering RNA (siRNA) constructs were designed by QIAGEN based on murine MDM2 (GenBank accession no. NM_010786) or purchased from Ambion (catalog no. 67963 and 68152). Four days after initiation of primary salivary acinar cultures, 5 μ g of MDM2 siRNA was complexed with RNAiFect (QIAGEN) at a ratio of 1:9 according to the manufacturer's instructions. Twenty-four to 72 h after transfection, MDM2 protein expression in primary myr-Akt1 or FVB salivary acinar cells was determined by immunoblotting, as described above. For analysis of apoptosis, primary myr-Akt1 salivary acinar cells were transfected with MDM2 siRNA for 30 h and then treated with 150 μ M etoposide for 18 h. Apoptosis was quantified by caspase 3 activity and TUNEL staining, as described above.

RESULTS

Identification of transgenic mice expressing myr-Akt1 in the salivary gland. Three different founder lines of myr-Akt1 transgenic mice that transmitted the transgene to their offspring were initially established and were designated 1173, 1176, and 1699. The effects of the myr-Akt1 transgene upon mammary gland biology in lines 1173 and 1176 have previously been described (76, 77). The MMTV-LTR promoter is also expressed in the salivary gland (36); to determine whether the transgene was expressed in this tissue, the salivary glands (submandibular, sublingual, and parotid) were removed from 4-week-old female animals from each of the founder lines and immunoblot analysis was used to detect the transgene. Transgene expression in the salivary glands was shown to be significantly higher in the 1699 line (Fig. 1A, top panel, lanes 1 to 5) than in the two previously described founder lines (Fig. 1A, top panel, lanes 6 to 15). Consistent with our previous observation, two HA-containing bands (77), which may differ in their phos-

FIG. 1. Identification of myr-Akt1 transgenic mice. Tissue lysates were prepared from 4-week-old female mice. (A) Protein lysates (100 μ g) isolated from the salivary glands of mice in each founder line were electrophoresed on an 8% SDS-polyacrylamide gel and immunoblotted with an antibody that detects the HA tag. Membranes were stripped and immunoblotted with a total ERK antibody to confirm equal loading of lanes (bottom panel). (B) Total Akt levels were analyzed in FVB salivary glands and compared to salivary glands from myr-Akt1 founder line 1699. Membranes were stripped and immunoblotted with a total ERK antibody to confirm equal loading of lanes (bottom panel). (C) Total Akt was immunoprecipitated from tissue lysates (300 μ g) ($n = 4$ [each genotype]) and used in an immune complex protein kinase assay containing [γ - 32 P]ATP and a specific substrate peptide (RPRAATF). The incorporation of 32 P into the substrate peptide was determined by liquid scintillation counting and was plotted as average counts per minute. (D) Phosphorylation of GSK3 α/β on serine⁹ was analyzed in tissue lysates by immunoblot analysis with anti-phospho-GSK3. Membranes were stripped and immunoblotted with a total GSK3 β antibody to confirm equal loading of lanes (bottom panel).

phorylation states (K. Schwertfeger, unpublished results), were detected. Based on the elevated level of transgene in the salivary glands, the 1699 line was chosen for further characterization. In female mice, total Akt protein levels were increased

1.7-fold in submandibular (Fig. 1B, lanes 1 and 2), 11.5-fold in sublingual (Fig. 1B, lanes 3 and 4), and 4.9-fold in parotid (Fig. 1B, lanes 5 and 6) salivary glands compared to FVB controls. Akt kinase activity was examined by measuring the phosphor-

FIG. 2. Histological examination of salivary gland structure ($\times 200$). Submandibular (a and d), sublingual (b and e), and parotid (c and f) salivary glands from 4-week-old female animals were isolated from myr-Akt1 transgenic (a to c) and FVB control (d to f) mice. Tissues were fixed in 10% formalin, sectioned, stained with hematoxylin and eosin, and examined by bright-field microscopy. Magnification bars, 100 μm .

ylation of a substrate peptide (50). In myr-Akt1 female mice, Akt kinase activity increased 1.56-fold in the submandibular gland, 1.78-fold in the sublingual gland, and 1.33-fold in the parotid gland relative to endogenous Akt (Fig. 1C), with some variation between different transgenic animals. Although the kinase assay reveals a rather modest increase in Akt kinase activity, the increase is consistent with the increase in the amount of phosphorylated glycogen synthase kinase 3, a known Akt substrate, observed in the salivary glands of transgenic mice compared to wild-type mice (Fig. 1D). Therefore,

both Akt protein and kinase activity were increased in the salivary glands of myr-Akt1 transgenic mice.

Histological analysis was performed on hematoxylin- and eosin-stained paraffin sections of control and transgenic mice to determine if transgene expression modified the structure and/or the development of the different salivary glands. Recent studies have reported increases in cell and tissue size in animals overexpressing Akt1 either in the pancreas or in the heart (79, 83). No gross morphological changes were detected in the myr-Akt1 salivary glands at low magnification (Fig. 2). To

TABLE 1. Salivary gland weights and salivary flow rate of myr-Akt1 transgenic mice

Mouse strain	Gland wt (% BW) $\pm$ SEM (<i>P</i>) ^a			Salivary flow rate $\pm$ SEM (<i>P</i>) ^b
	SMG	SBL	PAR	
FVB	0.342 $\pm$ 0.01	0.100 $\pm$ 0.004	0.354 $\pm$ 0.014	4.38 $\pm$ 0.76
myr-Akt	0.411 $\pm$ 0.015 (0.0007)	0.105 $\pm$ 0.008 (0.56)	0.404 $\pm$ 0.011 (0.013)	4.85 $\pm$ 1.48 (0.78)

^a Glandular wet weights were determined in 4-week-old transgenic (*n* = 11) and control female (*n* = 9) animals. Tissue weights (in milligrams) were divided by total body weights (in grams) and multiplied by 100 to represent the glands' percentage of body weight. Abbreviations: BW, body weight; SMG, submandibular gland; SBL, sublingual gland; PAR, parotid gland. *P* values (in parentheses) were determined by a *t* test.

^b To determine salivary flow rates, mice were injected i.p. with 0.25 mg carbachol per kg body weight. Induced saliva (*n* = 10/genotype) was collected in preweighed Eppendorf tubes with a micropipette for 5 minutes and chilled on ice. *P* values (in parentheses) were determined by a *t* test.

determine whether there was a change in cell size, glandular wet weights and DNA contents were analyzed (Tables 1 and 2). Slight, but consistent (*P* values are displayed in the bottom row of Table 1), increases in submandibular and parotid glandular wet weights relative to body weight (20% and 14%, respectively) could be identified at 4 weeks of age (Table 1), and this size difference remained constant as the animals aged (>8 months [data not shown]). Sublingual glands from myr-Akt1 mice were not significantly different in size from age-matched FVB control glands, due possibly to the small size of the tissue (12 to 16 mg). DNA content analysis was used to determine whether the increases in glandular size were due to an increase in the cell number or cell size (Table 2). No differences in total DNA content were detected in the myr-Akt1 submandibular, sublingual, or parotid glands compared to FVB salivary glands (*P* values are displayed in the bottom row of Table 2), suggesting that the increase in wet weight of the submandibular and parotid glands resulted from a slight increase in cell volume or size that was not distinguished histologically. Due to slight physical differences in the sizes of the salivary glands between the FVB and myr-Akt1 transgenic mice, we examined whether changes in salivary flow rate and salivary protein composition could be detected. Saliva was collected from animals after carbachol stimulation over a 5-minute period; no differences in salivary flow rates were detected between myr-Akt1 transgenic mice and nontransgenic controls (Table 1). No change in salivary protein composition could be detected between both groups of animals by SDS gel electrophoresis (data not shown). An important component of saliva is amylase, which is primarily produced by the parotid gland and serves to hydrolyze starches to simple sugars (97). Expression of amylase was analyzed in all three salivary glands by immunofluorescence staining, and the distributions of amylase in the myr-Akt1 transgenic and FVB control animals were very similar (data not shown). Overall, the physiology of the myr-Akt1 salivary glands appears to be similar to that of FVB control animals,

and, importantly, no defects in salivary gland function due to transgene expression could be detected.

Suppression of apoptosis induced by DNA damage in primary salivary acinar cells from myr-Akt1 transgenic mice. Transfection of established cell lines with constitutively activated Akt suppresses apoptosis induced by a variety of stimuli (28, 34, 37, 43, 46); therefore, we wished to determine whether primary salivary acinar cells prepared from myr-Akt1 transgenic mice were resistant to apoptotic stimuli. We focused on DNA-damaging agents because they induce apoptosis in a p53-dependent manner and because of the clinical relevance of gamma-irradiation-induced damage to salivary epithelial cells (33). Etoposide is a genotoxin that inhibits topoisomerase II, resulting in double-stranded DNA breaks (84), and gamma irradiation induces both single- and double-stranded DNA breaks (41). Primary cultures of parotid and submandibular cells were prepared as previously described (50, 51, 73), and the proliferation of these cells was confirmed by incorporation of BrdU (data not shown). Etoposide-induced apoptosis was significantly reduced in primary salivary acinar cells from myr-Akt1 transgenic mice as indicated by activation of caspase 3 at 18 hours after treatment (Fig. 3A and B). Although etoposide did induce some apoptosis in the myr-Akt1 salivary acinar cells, the amount of caspase 3 activated was diminished significantly at all concentrations of etoposide examined (Fig. 3A and B). Suppression of apoptosis in the myr-Akt1-expressing cells was confirmed by TUNEL (data not shown).

Exposure of primary salivary acinar cells from FVB control mice to gamma irradiation resulted in an induction of apoptosis (Fig. 4A and B), and the extent of caspase 3 activation 24 h after exposure was similar in both irradiated submandibular and parotid primary cells. In contrast, primary salivary acinar cells from myr-Akt1 transgenic mice were resistant to apoptosis based on quantitation of caspase 3 activity (Fig. 4A and B). Even at the highest dose of irradiation used (5 Gy), no caspase 3 activation was observed in either primary parotid or submandibular acinar cells from the myr-Akt1 transgenic mice (Fig. 4A and B). Analysis of the responses of primary parotid and submandibular cells to gamma irradiation by the TUNEL assay substantiated the results obtained with the caspase 3 assay (data not shown). Therefore, primary salivary acinar cells from myr-Akt1 transgenic mice are less responsive to apoptosis induced by two different DNA-damaging agents, etoposide and gamma irradiation.

Diminished apoptosis in myr-Akt1 transgenic mice following in vivo gamma irradiation. It has been previously demonstrated that parotid salivary glands are sensitive to the effects of gamma irradiation, resulting in severe reductions in gland-

TABLE 2. DNA content of salivary glands

Mouse strain	Total DNA content (mg) $\pm$ SEM (<i>P</i>) ^a		
	SMG	SBL	PAR
FVB	1,246 $\pm$ 83.7	2.5 $\pm$ 0.6	1,372 $\pm$ 131.8
myr-Akt	1,240 $\pm$ 68.5 (0.97)	3.6 $\pm$ 0.3 (0.86)	1,330 $\pm$ 75.1 (0.78)

^a DNA content from salivary glands was determined with a PicoGreen assay kit. Total genomic DNA from 4-week-old transgenic (*n* = 6) and control (*n* = 4) female animals is shown for each gland. Abbreviations: SMG, submandibular gland; SBL, sublingual gland; PAR, parotid gland. *P* values (in parentheses) were determined by a *t* test.

FIG. 3. Induction of apoptosis by etoposide is diminished in salivary acinar cells from myr-Akt1 transgenic mice. Four days after initiation of the cultures, primary salivary acinar cells were treated with increasing doses of etoposide for 18 h. (A) All adherent and nonadherent primary parotid acinar cells were collected and lysed in caspase lysis buffer (BioMol QuantiZyme Colorimetric assay kit), and 15 μ g of cell lysate was used to analyze the level of enzyme activity for each sample in quadruplicate. The ability of the enzyme to cleave a chromogenic substrate was read at A_{405} every 10 minutes for 7 hours, and the increase relative to untreated parotid cells is plotted. No statistical differences were detected between untreated FVB and untreated myr-Akt1 parotid lysates ($P > 0.62$). (B) Primary submandibular acinar cells were collected in caspase lysis buffer as described above, and the increase relative to untreated submandibular cells is plotted. No statistical differences were detected between untreated FVB and untreated myr-Akt1 submandibular lysates ($P > 0.95$). Graphs of caspase activity represent all data points collected from five independent experiments, and Student's t test P values, comparing two different treatment groups, were calculated by Microsoft Excel. Asterisks designate significant differences ($P \leq 0.05$) between myr-Akt1 and FVB mice.

dular function (49, 88). The head and neck region of FVB and myr-Akt1 transgenic mice was exposed to a single dose of 5 Gy, and parotid glands were collected 8 or 24 h after gamma irradiation (Fig. 5). The number of apoptotic cells was quan-

FIG. 4. Salivary acinar cells from myr-Akt1 transgenic mice are resistant to gamma-irradiation-induced apoptosis. Four days after initiation of the cultures, primary salivary acinar cells were treated with increasing doses of gamma irradiation, and the extent of apoptosis was quantitated after 24 h. (A) All adherent and nonadherent primary parotid acinar cells were collected, the activation of caspase 3 was determined as described in the legend to Fig. 3A, and the increase relative to untreated parotid cells is plotted. (B) Primary submandibular acinar cells were exposed to various concentrations of gamma irradiation, the activation of caspase 3 was quantitated as described in the legend to Fig. 3A, and the increase relative to untreated submandibular cells is plotted. Caspase graphs represent all data points collected from five independent experiments (mean $\pm$ standard error of the mean), and Student's t test P values, comparing two different treatment groups, were calculated by Microsoft Excel. Asterisks designate significant differences ($P \leq 0.05$) between myr-Akt1 and FVB calculated with a two-sample t test.

titated by immunostaining with anti-active caspase 3 antibody (Fig. 5A) and graphed as a percentage of total cells as determined with hematoxylin counterstaining (Fig. 5B). In FVB mice, apoptosis could be detected as early as 8 h postirradiation, with 7.4% of the cells positive for activated caspase 3, which increased to 27.4% 24 h postirradiation. In contrast, the level of apoptosis was diminished in the myr-Akt1 mice, with 2.5% of the cells positive for activated caspase 3 at 8 h postirradiation, which increased to 8.2% at 24 h. These data indicate that in contrast to previously published data with rats, in which

FIG. 5. Reduced apoptosis in myr-Akt1 transgenic mice following targeted head and neck gamma irradiation in vivo. (A) Four-week-old female FVB (left panel) and myr-Akt1 transgenic (right panel) mice were exposed to 5 Gy irradiation, and the number of apoptotic cells in the parotid glands was quantitated by anti-activated caspase 3 immunohistochemistry at 24 h postirradiation. Magnification bars, 100 μ m. (B) At 8 and 24 h after irradiation, the percentage of activated caspase 3-positive cells was calculated from parotid glands isolated from FVB and myr-Akt1 transgenic mice. The graph represents all data points from three mice per group (mean $\pm$ standard error of the mean). Cell counts were performed on a minimum of five fields of view per slide from three mice (total cells counted ranged from 1,800 to 2,500 per mouse). Asterisks designate significant differences ($P \leq 0.05$) between myr-Akt1 and FVB calculated with a two-sample t test in Microsoft Excel. (C) At 8 and 24 h after irradiation, tissue lysates were prepared as described for Fig. 1 and immunoblotted with anti-phospho-p53 (serine¹⁸, top panel) or anti-HA to detect transgene (second panel). Membranes were stripped and immunoblotted with an anti-ERK antibody to confirm equal loading of lanes (bottom panel).

a maximum of 2% apoptosis was detected, irradiation of the mouse salivary glands results in a more significant level of apoptosis. These data demonstrate that expression of the myr-Akt1 transgene suppresses DNA damage-induced apoptosis in primary salivary acinar cells in vitro as well as in the salivary gland in vivo.

p53 activity induced by DNA damage results in an increase in total p53 protein levels and in the modification of p53 by phosphorylation and/or acetylation (2, 48, 55, 56, 93). Phosphorylation of p53 on serine¹⁸ has been hypothesized to be required for its stabilization and activation following gamma irradiation (93); however, this is not universal in all cell types or for the corresponding serine site in the mouse (4, 10, 15, 80). We were interested in determining whether p53 was phosphorylated on serine¹⁸ in the salivary glands of FVB and myr-Akt1 transgenic mice exposed to irradiation. The parotid gland from FVB and myr-Akt1 transgenic mice, as well as from unirradiated control mice, was dissected 8 and 24 h after exposure to 5 Gy gamma irradiation. Exposure of FVB mice to gamma irradiation resulted in a dramatic increase in the level of serine¹⁸ phosphorylated p53 at 8 h after irradiation, and the level of phosphorylated p53 dramatically decreased by 24 h postirradiation (Fig. 5C). In contrast, the level of phosphorylated p53 was significantly lower at 8 h postirradiation in the myr-Akt1 transgenic mice examined than in the FVB control mice (Fig. 5C). At 24 h after irradiation of the myr-Akt1 transgenic mice, phosphorylated p53 could be detected but it was not consistently elevated compared to that observed in the FVB mice at either 8 or 24 h postirradiation. This suggests that the activation of p53 is dramatically reduced in myr-Akt1 transgenic mice following exposure to gamma irradiation.

Basal expression of p53 family members is reduced in the salivary glands of myr-Akt1 transgenic mice. As noted above, p53 plays a significant role in apoptosis induced by DNA damage. The results shown in Fig. 5C suggest that activation of p53 is blocked or diminished in myr-Akt1 transgenic mice, and there are many mechanisms that could account for this change. Akt has been reported to phosphorylate and activate MDM2, causing MDM2 to translocate to the nucleus where it aids in p53 degradation (64, 67). For this reason, we determined the levels of total p53 in salivary gland tissue lysates isolated from untreated 4-week-old myr-Akt1 and FVB female mice (Fig. 6A). Expression of constitutively activated Akt1 resulted in dramatically reduced levels of total p53 (Fig. 6A, top panel, lanes 1 to 3), which correlated with an increase in the phosphorylation of MDM2 at serine¹⁶³, a putative Akt phosphorylation site (Fig. 6A, second panel). No differences in p53 RNA expression in the salivary glands between untreated FVB and myr-Akt1 female mice could be detected by microarray analysis or quantitative RT-PCR (data not shown). Levels of endogenous total MDM2 (Fig. 6A, third panel) are also increased in all three salivary glands from transgenic mice, suggesting that phosphorylation of MDM2 may increase its stability (30). The decrease in total basal p53 levels clearly explains why there is a reduced level of serine¹⁸ phosphorylated p53 in the salivary glands of irradiated myr-Akt1 transgenic mice (Fig. 5C).

To determine whether the decrease in p53 protein levels led to a decrease in the expression of a p53 responsive gene, we used quantitative RT-PCR to determine the basal concentra-

tion of p21^{WAF1} RNA in parotid salivary glands of FVB and myr-Akt1 transgenic mice (Fig. 6B). Expression of constitutively activated Akt1 in the parotid glands resulted in significantly diminished levels of p21^{WAF1} mRNA (Fig. 6B). Consistent with the changes in p21^{WAF1} RNA levels, there is a decrease in p21^{WAF1} protein (Fig. 6C, top panel, lane 1). Significant reductions in total p53 protein levels along with decreased expression of p21^{WAF1} mRNA in the salivary glands of transgenic mice indicate that Akt-dependent regulation of p53 by MDM2 might function in vivo in a manner similar to that shown in vitro with transfected cells (54, 64).

Recently two p53 homologues, p63 and p73, have been described, and expression of these homologues is required for p53-dependent apoptosis (32). However, the importance of p63 and p73 in p53-dependent processes may not be universal in all tissue types (78). It is intriguing to note that loss of either p63 or p73 cooperates with the loss of p53 in accelerating tumor development at many sites, including the salivary gland (31). Clearly, these studies suggest that the combined role of p53, p63, and p73 is complex and may be cell type or stimulus type specific. We also evaluated the levels of p63 and p73 by immunoblot analysis to complete our analysis of the p53 family members in the salivary glands of myr-Akt1 transgenic mice. Densitometric analysis of total p63 levels revealed a 43% reduction in the submandibular glands of myr-Akt1 transgenic mice (Fig. 6D, top panel, lane 1), a 78% reduction in the sublingual glands of myr-Akt1 transgenic mice (Fig. 6D, top panel, lane 3), and a 36% reduction in the parotid glands of myr-Akt1 transgenic mice (Fig. 6D, top panel, lane 5) compared to the respective FVB control salivary glands (Fig. 6D, lanes 2, 4, and 6, respectively). Total p73 levels also were reduced in the myr-Akt1 transgenic mice: a 61% reduction in the submandibular glands (Fig. 6D, middle panel, lane 1), an 83% reduction in the sublingual glands (Fig. 6D, middle panel, lane 3), and a 48% reduction in the parotid glands (Fig. 6D, middle panel, lane 5) compared to the respective FVB control salivary glands (Fig. 6D, lanes 2, 4, and 6, respectively). The antibodies used to detect p63 and p73 should detect all isoforms; however, only one band corresponding to full-length p63 (~63 kDa) or p73 (~73 kDa) was detected. The reduction in p63 and p73 proteins appears to result from a decrease in the expression of these genes in the salivary gland of myr-Akt1 transgenic mice as determined by quantitative real-time RT-PCR (FVB versus myr-Akt, two-sample *t* test: *P* < 0.05 for RNA or protein concentration [real-time data not shown]). To date there is no evidence that MDM2 regulates the degradation of either p63 or p73 (91).

Diminished levels of total p53, phosphorylated p53, and p53 target genes in myr-Akt1 salivary acinar cells following DNA damage. In order to evaluate the effect of myr-Akt1 expression on p53 activation following DNA damage, we used primary salivary acinar cells. Phosphorylation of p53 on serine¹⁵ has been hypothesized to be required for its stabilization following gamma irradiation (93); however, this is not universal in all cell types or for the corresponding serine site in mice (4, 10, 15, 80). Levels of total p53 and phosphorylated p53^{serine18} were determined by immunoblot analysis of etoposide- and gamma-irradiation-treated primary salivary acinar cells (Fig. 7). Total p53 levels increased following etoposide treatment of FVB primary cells at all concentrations of etoposide (Fig. 7A, middle panel,

FIG. 6. Expression of p53 family members and p21^{WAF1} is reduced in salivary glands of myr-Akt1 transgenic mice. (A) Tissue lysates were immunoblotted with anti-p53 (DO12) to detect total levels of p53 (top panel) or levels of phosphorylated MDM2^{serine163} (second panel) or total levels of MDM2 (third panel). Membranes were stripped and immunoblotted with an anti-ERK antibody to confirm equal loading of lanes (bottom panel). (B) Total cellular RNA isolated from untreated parotid glands ($n = 4/\text{genotype}$) was analyzed by real-time RT-PCR for basal concentrations of p21^{WAF1}. p21^{WAF1} C_T values were normalized by S15 C_T values and are expressed as the ratio of p21^{WAF1} expression to S15 expression. The Student's t test P value, comparing two different treatment groups, was calculated with Microsoft Excel ($P \leq 0.025$). (C) Parotid tissue lysates were analyzed for total levels of p21^{WAF1}. The membrane was stripped and immunoblotted with an anti-ERK antibody to confirm equal loading of lanes (bottom panel). (D) Tissue lysates were analyzed for total levels of p63 (top panel) and p73 (middle panel). Membranes were stripped and immunoblotted with an anti-ERK antibody to confirm equal loading of lanes (bottom panel). Immunoblots are representative of three individual mice.

lanes 7 to 10); however, phosphorylation of p53 at serine¹⁸ could be only detected at 150 and 200 μM etoposide (Fig. 7A, top panel, lanes 9 and 10). This difference may reflect a difference in the sensitivity of the anti-p53 antibody versus the anti-phosphorylated serine¹⁸ antibody, or it may indicate that posttranslational modifications of p53 other than phosphorylation of serine¹⁸ are important in stabilizing p53 following DNA damage. In contrast, etoposide treatment of myr-Akt1 primary cells did not result in demonstrable increases in total p53 or p53 phosphorylation at serine¹⁸ (Fig. 7A, lanes 2 to 5).

Treatment of FVB-derived submandibular salivary acinar cells with gamma irradiation also led to an increase in p53 phosphorylation and an increase in the level of total p53 compared to untreated or starved salivary acinar cells at all doses of irradiation (Fig. 7B, lanes 7 and 8 versus lanes 9 to 12). The amounts of phosphorylated p53^{serine18} were dramatically re-

duced in irradiated primary salivary acinar cells from myr-Akt1 transgenic mice compared to irradiated cells from FVB mice (Fig. 7B, top panel, lanes 3 to 6 versus lanes 9 to 12). An increase in total p53 protein was detected only after treatment of myr-Akt1 primary salivary acinar cells with the highest dose of irradiation examined (5 Gy) (Fig. 7B, middle panel, lane 6); however, this did not correspond with an increase in serine¹⁸ phosphorylated p53 (Fig. 7B, top panel, lane 6). This also may indicate that posttranslational modifications of p53 other than phosphorylation of serine¹⁸ are important in stabilizing p53 following DNA damage. Alternatively, a decrease in transgene expression may occur at high doses of gamma irradiation. Similar results were obtained with primary cultures derived from either the parotid or the submandibular glands. In contrast to the myr-Akt1 tissue lysates, the expression of p53 can be detected in the primary salivary acinar cells isolated from the

FIG. 7. Diminished levels of p53 in primary myr-Akt1 salivary acinar cells following DNA damage. (A) Cell lysates were collected as described in Materials and Methods and immunoblotted with an anti-phospho-p53 (serine¹⁸, top panel) or anti-p53 (DO12, middle panel) antibody 18 hours after exposure to different concentrations of etoposide. Membranes were also stripped and immunoblotted with a β -tubulin antibody to confirm equal loading of lanes (bottom panel). (B) Cell lysates were collected 24 h after exposure to various doses of gamma irradiation, and lysates were collected and immunoblotted with an anti-phospho-p53 (serine¹⁸, top panel) or anti-p53 (DO12, middle panel) antibody. Membranes were also stripped and immunoblotted with a β -tubulin antibody to confirm equal loading of lanes (bottom panel). Results shown are representative of three independent experiments.

myr-Akt1 transgenic mice, suggesting that placing these cells in culture may alone be enough stress to induce stabilization of p53 (compare Fig. 6 and 7). Expression of the myr-Akt1 transgene, however, still results in lower levels of p53 protein levels following DNA damage than in FVB controls. The data presented in Fig. 6 and 7 suggest that the down-regulation of p53, perhaps mediated by Akt-dependent phosphorylation of MDM2, may be responsible for the resistance of myr-Akt1 salivary acinar cells to DNA damage-induced apoptosis.

Activation of p53 has been associated with the transcription of numerous genes involved in cell cycle arrest, such as p21^{WAF1} (11), 14-3-3 σ (89), and GADD 45 (52), as well as proteins that regulate apoptosis, such as Bax (58), PUMA (96), Noxa (62), and PERP (5). The best-characterized p53 target gene that is induced following gamma irradiation of a wide

variety of cells is the cell cycle arrest gene p21^{WAF1} (12, 16). Expression of p21^{WAF1} can also be stimulated by p63 and p73 and thus may represent a universal target for all p53 family members (27). p21^{WAF1} RNA concentrations were determined by real-time RT-PCR (90) following treatment of cells with different doses of etoposide (Fig. 8A). The amount of p21^{WAF1} RNA was normalized to the ribosomal protein S15 RNA and was found to be significantly reduced in primary salivary acinar cells from myr-Akt1 mice compared to FVB control cells 18 h after etoposide treatment (Fig. 8A). We also evaluated the induction of Bax by quantitative RT-PCR. Bax expression increased in FVB control cells treated with higher concentrations of etoposide (150 to 200 μ M), while myr-Akt1 primary cells demonstrated no induction of Bax expression following etoposide treatment (Fig. 8B). Expression of p21^{WAF1} was induced in salivary cells from FVB mice at lower concentrations of etoposide than was expression of Bax. This differential induction may represent biphasic transcriptional activity of p53 with regard to genes that mediate cell cycle arrest versus those that are proapoptotic (63).

Expression of p21^{WAF1} also was determined 12 h following treatment of primary salivary acinar cells from FVB and myr-Akt1 transgenic mice with 0.25 to 5 Gy gamma irradiation. (Fig. 8C). There was no statistical difference in the abundance of p21^{WAF1} RNA between starved myr-Akt1 and starved FVB primary submandibular acinar cells. In comparison to starved controls, gamma irradiation of FVB primary salivary acinar cells significantly increased the amount of p21^{WAF1} RNA at each dose of irradiation. However, the levels of p21^{WAF1} were not increased following gamma irradiation of myr-Akt1 cells. As observed above, expression of Bax was induced in FVB control cells exposed to higher concentrations of radiation (1 to 5 Gy) compared to the level of irradiation required to induce expression of p21^{WAF1}, while there was no induction of Bax expression following radiation of primary cells from myr-Akt1 mice (Fig. 8D). These data suggest that reduced stabilization and phosphorylation of p53 on serine¹⁸ in myr-Akt1 primary salivary acinar cells prevents induction of downstream gene targets such as p21^{WAF1} or Bax following gamma irradiation.

MDM2 is required for the ability of myr-Akt1 to suppress apoptosis. It has been previously demonstrated, with tissue culture cells transfected with constitutively activated mutants of Akt1, that MDM2 is a substrate for Akt (64, 67). In order to confirm that MDM2 phosphorylation is dependent on Akt kinase activity in myr-Akt1 salivary glands, primary cells were transduced with a recombinant adenovirus encoding a kinase-inactive mutant of Akt1 (Ad-KD), as in previous studies (50). Primary myr-Akt1 cells transduced with kinase-inactive Akt1 demonstrated reduced levels of phosphorylation of MDM2 at serine¹⁶³ compared to untreated or Ad-LacZ controls (Fig. 9A). There was no decrease in the amount of total MDM2 (Fig. 9A, second panel), indicating that inhibition of Akt kinase activity in myr-Akt1 primary cells directly decreases the phosphorylation of MDM2. The reduction of MDM phosphorylation at serine¹⁶³ correlated with an increase in total p53 levels (Fig. 9A, third panel, lane 3). To determine whether MDM2 is required for suppression of apoptosis in myr-Akt1 salivary acinar cells, we depleted these cells of MDM2 by using siRNA molecules specific for murine MDM2. Lipid transfection

FIG. 8. Diminished expression of p21^{WAF1} following DNA damage in myr-Akt1 salivary acinar cells. Total RNA was isolated from salivary acinar cells as described in Materials and Methods. (A) RNA was isolated from primary FVB or myr-Akt1 parotid acinar cells 18 h after treatment with etoposide, and the amount of p21^{WAF1} RNA was determined by quantitative RT-PCR. (B) Total cellular RNA was isolated from primary FVB or myr-Akt1 parotid acinar cells 18 h after treatment with etoposide, and the amount of Bax RNA was determined by quantitative RT-PCR. (C) Total cellular RNA was extracted from primary FVB or myr-Akt1 submandibular acinar cells 12 hours following gamma irradiation treatment and analyzed for p21^{WAF1} expression by quantitative RT-PCR. (D) Total cellular RNA was extracted from primary FVB or myr-Akt1 submandibular acinar cells 12 hours following gamma irradiation treatment and analyzed for Bax expression by quantitative RT-PCR. Bax and p21^{WAF1} C_T values were normalized by the ribosomal protein S15 C_T values and are expressed as the ratio of Bax or p21^{WAF1} expression to S15 expression. Asterisks designate significant differences ($P \leq 0.05$) between myr-Akt1 and FVB, as calculated with a two-sample t test. In panel B, comparisons between myr-Akt1 and FVB cells untreated or treated with 100 μ M etoposide did not reach significance (P , 0.07 and 0.13, respectively).

tion reagent alone or transfection with an siRNA that targeted ERK1 had no effect on the total amount of MDM2 protein (Fig. 9B, top panel, lanes 1 to 3). The ability of two different siRNA molecules to reduce the level of MDM2 protein 24, 48, and 72 h after transfection of primary salivary acinar cells is shown in Fig. 9B (top panel, lanes 4 to 9). Reduction of MDM2 expression correlated with the increase in total p53 protein at 48 and 72 h after transfection with siRNA targeting MDM2 (Fig. 9B, middle panel, lanes 5, 6, 8, and 9).

Primary salivary acinar cells isolated from myr-Akt1 transgenic mice were transfected with various siRNA molecules for 30 h prior to treatment with etoposide for 18 h. The extent of caspase 3 activation was used to quantitate induction of apoptosis. No significant differences were detected between primary salivary acinar cells from myr-Akt1 mice transfected with control siRNA molecules and etoposide-treated primary salivary acinar cells expressing myr-Akt1 (Fig. 9C). In contrast, three siRNA molecules directed against MDM2 (MDM2 Qia4, MDM2 Amb1, and MDM2 Amb2) rendered primary salivary acinar cells from myr-Akt1 mice sensitive to etoposide-induced

apoptosis (Fig. 9C). In addition, there was no statistical difference between the amount of caspase 3 activity induced in etoposide-treated FVB control primary acinar cells and the etoposide-treated myr-Akt1 primary cells transfected with MDM2 Amb2 siRNA (Fig. 9C, bar 7 versus 8 [$P \leq 0.49$]). We also investigated the activation of ATM and Chk1 by using phosphorylation-specific antibodies following etoposide treatment (Fig. 9D). These key molecules in the DNA damage response lie upstream of p53 activation and may also be modified by Akt (44, 71, 87). Etoposide treatment of primary acinar cells from myr-Akt1 transgenic mice resulted in the phosphorylation of ATM at serine¹⁹⁷⁸ regardless of whether the cells had been transfected with siRNA that targeted MDM2 (data not shown). This suggests that ATM is activated in the primary cells from myr-Akt1 transgenic mice following treatment of the cells with DNA-damaging agents. Increased levels of Chk1 phosphorylated on serine³⁴⁵ were observed in myr-Akt1 primary salivary cells transfected with siRNA targeting MDM2 (Fig. 9D, lanes 4 to 7), with a slight increase (34%) in the amount of phosphorylation following etoposide treatment rel-

FIG. 9. MDM2 expression is required for myr-Akt1-mediated suppression of apoptosis in salivary acinar cells. (A) Primary salivary acinar cells isolated from myr-Akt1 mice were transduced with recombinant adenoviruses encoding LacZ (Ad-LacZ) or a kinase-inactive mutant of Akt1 (Ad-KD-Akt1) at a multiplicity of infection of 150. Cell lysates were collected 24 h after transduction and immunoblotted with an anti-

ative to that seen in the cells not treated with etoposide (Fig. 9D, lanes 4 and 5 versus lanes 6 and 7). The total levels of Chk1 were largely unaffected by treatment of these primary cells. A higher level of p53 phosphorylation on serine¹⁸ was detected in myr-Akt1 primary cells transfected with siRNA targeting MDM2 following etoposide treatment compared to the level of phosphorylated p53 observed in cells treated with the control siRNA (Fig. 9D, lane 3 versus lanes 4 and 5). Reducing the level of MDM2 by siRNA also significantly increased total p53 protein levels (Fig. 9D, bottom panel, lanes 4 to 7); however, phosphorylation of p53 on serine¹⁸ was observed only following etoposide treatment (Fig. 9D, third panel, lanes 4 to 5). Moreover, these data provide further evidence that MDM2 is required for the ability of myr-Akt1 to suppress etoposide-induced apoptosis by regulating the level of total p53.

DISCUSSION

Tissue homeostasis results from a balance in the amounts of cellular proliferation and programmed cell death, or apoptosis. Irradiation of the head and neck region of rodents has been shown to result in salivary gland hypofunction similar to that observed in humans receiving ionizing irradiation (49, 60). In the rat model, salivary gland function is diminished 50 to 70% at 6 to 9 months following exposure to gamma irradiation; however, previous studies reported that only 2% of cells were apoptotic as evidenced by the presence of condensed nuclei on hematoxylin- and eosin-stained sections (61, 68). Because of the disparity between the extent of apoptosis and the extent of salivary gland hypofunction, some authors have concluded that apoptosis is not causally related to radiation-induced salivary gland hypofunction (68). Although the apoptotic index observed in a tissue reflects the balance between the induction of apoptosis and the clearance of apoptotic cells, our data indicate that there is far more apoptosis at 8 and 24 h following irradiation with a single dose of 5 Gy than previously suggested by quantifying the number of condensed nuclei. Better understanding of gamma-irradiation-induced damage to salivary glands is clinically relevant to improving the care of patients undergoing treatment for head and neck cancer.

The ability of Akt to suppress apoptosis induced by various stimuli is well established (28, 34, 37, 43, 46). Primary salivary acinar cells isolated from myr-Akt1 transgenic mice consis-

tently had lower levels of DNA damage-induced apoptosis than did control cells isolated from age-matched FVB mice. Importantly, this suppression of apoptosis by myr-Akt1 was also observed *in vivo* following exposure of the head and neck region to gamma irradiation treatment (Fig. 5). Following DNA damage, p53 undergoes several posttranslational modifications, and the stability of the p53 protein increases dramatically (55). We determined the levels of total p53 and serine¹⁸ phosphorylated p53 by immunoblot analysis and quantitated the levels of p53-responsive genes (p21^{WAF1} and Bax) by RT-PCR. Levels of total p53 and phosphorylated p53 (serine¹⁸) were increased in primary cells from FVB mice following exposure to either etoposide or gamma irradiation (Fig. 6), resulting in the induction of p21^{WAF1} and Bax expression in these cells (Fig. 7). The induction of p21^{WAF1} was observed following low doses of etoposide or gamma irradiation, and this reaction could contribute to the sensitivity of the salivary glands to ionizing radiation *in vivo*. In contrast, primary salivary acinar cells from myr-Akt1 transgenic mice were less sensitive to etoposide and gamma irradiation-induced apoptosis and had reduced levels of total p53, phosphorylated p53, p21^{WAF1} RNA, and Bax RNA following exposure to DNA-damaging agents.

It has recently been reported that Akt negatively regulates the levels of p53 protein via activation of MDM2 (54, 64, 67). Normally, p53 is maintained at low levels in the cell due to its short half-life, which is in part regulated by MDM2 (66). MDM2 is an E3 ubiquitin ligase that targets p53 for ubiquitination and subsequent degradation by the proteasome (56, 59). Analysis of the Akt/MDM2/p53 pathway has largely been accomplished by transfection of established cell lines with activated mutants of Akt. In transgenic mice expressing constitutively activated Akt1, there is an increase in total MDM2 protein and MDM2 phosphorylation at serine¹⁶³ which correlates with a dramatic reduction in total p53 protein, suggesting that Akt regulates p53 protein expression *in vivo*. We extended this observation to show that phosphorylation of MDM2 is Akt dependent and that MDM2 is required for myr-Akt1-induced suppression of apoptosis induced by DNA damage in primary salivary acinar cells from myr-Akt1 transgenic mice. It is interesting to note that phosphorylation of Chk1 at serine³⁴⁵, the putative phosphorylation site for ATM/ATR, occurs under conditions of reduced MDM2 expression in myr-Akt1 cells.

phosphorylated MDM2^{serine163} (top panel), anti-MDM2 antibody (second panel), or anti-p53 (DO12, third panel). The membrane was stripped and immunoblotted with a β -tubulin antibody to confirm equal loading of lanes (bottom panel). (B) Primary salivary acinar cells isolated from myr-Akt1 mice were transfected with various control or MDM2-specific siRNA molecules. Cell lysates were collected 24 to 72 h after transfection and immunoblotted with an anti-MDM2 antibody (top panel) or anti-p53 (DO12, middle panel). Membranes were also stripped and immunoblotted with a β -tubulin antibody to confirm equal loading of lanes (bottom panel). (C) Primary salivary acinar cells isolated from myr-Akt1 mice were transfected with siRNA molecules as described for panel A for 30 h and then treated with 150 μ M etoposide for 18 h. Activation of caspase 3 was determined as described in Materials and Methods and plotted as the increase relative to untreated parotid cells. The last lane depicts the induction of caspase 3 activity in FVB primary cells following treatment with 150 μ M etoposide for 18 h. Data were collected from four independent experiments (mean $\pm$ standard error of the mean), and Student's *t* test *P* values, comparing two different treatment groups, were calculated with Microsoft Excel. Asterisks designate significant differences ($P \leq 0.05$) between myr-Akt1 etoposide and individual siRNA molecules, as calculated with a two-sample *t* test. (D) Primary salivary acinar cells isolated from myr-Akt1 mice were transfected with various control or MDM2-specific siRNA molecules for 30 h followed by 150 μ M etoposide for 18 h, as described for panel B. Cell lysates were collected and immunoblotted with a phosphorylation-specific anti-Chk1 antibody (serine³⁴⁵, top panel), phosphorylation-specific anti-p53 (serine¹⁸, third panel), or anti-total p53 (bottom panel). Membranes were also stripped and immunoblotted with a total Chk1 antibody to confirm equal loading of lanes (second panel). In lanes 6 and 7, primary salivary acinar cells isolated from myr-Akt1 mice were transfected with siRNA molecules targeting MDM2 for 48 h.

This may indicate a feedback mechanism by which phosphorylation of Chk1 is dependent on the level of total p53 protein.

It has been suggested that the p53 homologs p63 and p73 are required for p53-dependent apoptosis induced following DNA damage; however, p63 and p73 are not required for p53-dependent apoptosis in T cells (32, 78). Full-length versions of p73 and p63 (termed TA to indicate presence of a transactivation domain) have been shown to induce apoptosis. Deletion of the N-terminal transactivation domain of p73 or p63, through utilization of the second transcriptional start site (termed ΔN), produces a protein that serves as a dominant-negative molecule that suppresses action of the respective full-length isoforms, as well as of p53 (94). Loss of p63 and p73 cooperates with the loss of p53 to accelerate tumor formation, suggesting that p63 and p73 may function as tumor suppressors in some tissues, including salivary glands (31). For these reasons it was important to examine the levels of all p53 family members in the salivary glands of myr-Akt1 transgenic mice. The basal levels of p53, p63, and p73 are all reduced in myr-Akt1 mice (Fig. 6). Regulation of p73 activity may occur by MDM2-dependent translocation without degradation or may involve another putative Akt substrate, YAP (9, 81); however, neither of these molecules can explain the reduced levels of p73 RNA we have observed. Modulation of p63 protein levels by activated Akt has not been previously observed and cannot be explained by the phosphorylation of MDM2, as p63 and MDM2 do not interact (91). We are currently conducting additional studies to understand the regulation of p63 by Akt and to investigate whether YAP is phosphorylated in the salivary glands of myr-Akt1 transgenic mice.

Significant efforts have focused on the identification of Akt substrates that suppress apoptosis in tissue culture cells (13, 14, 22, 42, 43, 57). Analysis of transgenic mice that express activated Akt1 has confirmed the importance of some of these substrates. For example, Wendel et al. demonstrated the chemoresistance of a B-cell lymphoma that overexpresses E μ -Myc; activated Akt1 was dependent on mTOR and eIF4E, and an mTOR inhibitor, rapamycin, reversed the Akt-induced chemoresistance in these cells (92). This result was somewhat surprising given the number of Akt substrates regulating apoptosis and emphasizes that the critical substrates for Akt may differ with the stimuli and cell type examined. Our analysis of activated Akt1 overexpression in vivo has revealed a universal reduction in the expression of the p53 family of proteins in salivary acinar cells, although the mechanism underlying this observation may differ for each p53 family member. It is well known that mutations in p53 and/or overexpression of MDM2 occurs in many cancers, and numerous studies have focused on the dysregulation of p53 in cancer (47). Disruption of the MDM2/p53 regulation pathway shows great promise in reactivating wild-type p53 in cancer cells (85), as well as sensitizing tumors to radiation therapy (69). Our studies have demonstrated that MDM2 is a critical substrate of Akt in suppression of apoptosis caused by DNA damage. These studies also suggest that the resistance of tumors expressing activated Akt to chemotherapy and radiation therapies could be reversed by targeting MDM2's function or expression. Future studies will determine whether targeted disruption of MDM2 in the salivary glands sensitizes myr-Akt1 transgenic mice to gamma irradiation in vivo.

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