

Transforming Growth Factor β Enhances Epithelial Cell Survival via Akt-dependent Regulation of FKHRL1

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The Forkhead family of transcription factors participates in the induction of death-related genes. In NMuMG and 4T1 mammary epithelial cells, transforming growth factor β (TGF β) induced phosphorylation and cytoplasmic retention of the Forkhead factor FKHRL1, while reducing FKHRL1-dependent transcriptional activity. TGF β -induced FKHRL1 phosphorylation and nuclear exclusion were inhibited by LY294002, an inhibitor of phosphatidylinositol-3 kinase. A triple mutant of FKHRL1, in which all three Akt phosphorylation sites have been mutated (TM-FKHRL1), did not translocate to the cytoplasm in response to TGF β . In HaCaT keratinocytes, expression of dominant-negative Akt prevented TGF β -induced 1) reduction of Forkhead-dependent transcription, 2) FKHRL1 phosphorylation, and 3) nuclear exclusion of FKRHL1. Forced expression of either wild-type (WT) or TM-FKHRL1, but not a FKHRL1 mutant with deletion of the transactivation domain, resulted in NMuMG mammary cell apoptosis. Evidence of nuclear fragmentation colocalized to cells with expression of WT- or TM-FKHRL1. The apoptotic effect of WT-FKHRL1 but not TM-FKHRL1 was prevented by exogenous TGF β . Serum starvation-induced apoptosis was also inhibited by TGF β in NMuMG and HaCaT cells. Finally, dominant-negative Akt abrogated the antiapoptotic effect of TGF β . Taken together, these data suggest that TGF β may play a role in epithelial cell survival via Akt-dependent regulation of FKHRL1.

INTRODUCTION

Transforming growth factor β (TGF β) is involved in various cellular processes, including cell division, differentiation, motility, adhesion, and apoptosis (Massagué, 1998). TGF β stimulates the proliferation of mesenchymal cells while inhibiting the growth of most normal epithelial cells (Massagué and Chen, 2000; Massagué and Wotton, 2000). TGF β signals are transmitted through a heterodimeric complex of two transmembrane serine/threonine kinases, the type I and

II TGF β receptors (Massagué, 1998; Massagué and Wotton, 2000). Receptor-associated Smads are intracellular signal transducers that associate with T β RI, become phosphorylated, and translocate to the nucleus where they regulate transcription of TGF β target genes (Lagna *et al.*, 1996; Massagué and Chen, 2000). TGF β modulates several signaling pathways in mammalian cells. The c-Jun NH₂-terminal kinase (JNK) can either be activated (Atfi *et al.*, 1997) or inhibited by TGF β (Imai *et al.*, 1999; Huang *et al.*, 2000). Rapid activation of extracellular signal-regulated kinase by TGF β has been reported in epithelial cells (Hartsough *et al.*, 1996). We previously reported that TGF β phosphorylates Akt in a phosphatidylinositol-3 kinase (PI3K)-dependent manner, response that was required for TGF β -mediated epithelial-to-mesenchymal transition and migration of mammary cells (Bakin *et al.*, 2000).

The Akt kinase is activated by phosphorylation at Thr308 and Ser473 mediated by 3-phosphoinositide-dependent protein kinase 1 and 2, respectively (Alessi *et al.*, 1997;

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Abbreviations used: dn, dominant-negative; FasL, Fas ligand; FCS, fetal calf serum; FHRE, Forkhead response element; JNK, c-Jun NH₂-terminal kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3 kinase; T β RI, TGF β type I receptor; Tet, tetracycline; TGF β , transforming growth factor β ; TM, triple mutant; WT, wild-type.

Stokoe *et al.*, 1997; Stephens *et al.*, 1998). Akt phosphorylates and inactivates glycogen synthase kinase3- β , an enzyme that regulates glycogen biosynthesis (Cross *et al.*, 1995). In addition to regulating cellular metabolism, Akt can promote enhanced cell survival (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Datta *et al.*, 1999). Bad was the first reported proapoptotic factor directly phosphorylated and inactivated by Akt (del Peso *et al.*, 1997). Akt also phosphorylates the proapoptotic molecule caspase 9 at Ser196 (Cardone *et al.*, 1998), which results in suppression of caspase 9-induced apoptosis in 293 cells (Datta *et al.*, 1999).

The role of Akt in gene transcription was first discovered by studies performed in *Caenorhabditis elegans*. DAF-16, a Forkhead transcription factor in *C. elegans*, is negatively regulated by Akt. DAF-16 is activated by DAF-2 and DAF-23, where DAF-2 is an insulin receptor-like protein, and DAF-23, a PI3K-like protein (Kops and Burgering, 1999). The mammalian orthologs of DAF-16 are AFX, Forkhead response element (FKHR), and FKHRL1. In *C. elegans*, mutations of *daf2* synergize with mutations of *daf1*, a type I TGF β receptor, in inducing dauer formation (Ogg *et al.*, 1997), suggesting that TGF β can interact with the DAF-2/DAF-16 pathway. All three DAF-16 mammalian homologs share a Forkhead 100-amino acid core domain, responsible for binding to DNA. AFX, FKHR, and FKHRL1, each contain three Akt phosphorylation sites (Datta *et al.*, 1999), which can be phosphorylated by Akt in mammalian cells (Brunet *et al.*, 1999; Guo *et al.*, 1999; Kops *et al.*, 1999; Nakae *et al.*, 1999). On phosphorylation by Akt, Forkhead factors translocate from the nucleus to the cytoplasm (Biggs *et al.*, 1999; Brunet *et al.*, 1999), where 14-3-3 proteins may sequester them and prevent their function (Brunet *et al.*, 1999). In their unphosphorylated state, Forkhead factors predominantly localize in the nucleus where they bind to insulin response elements and/or the Fas ligand (FasL) promoter and activate transcription of target genes that may induce cell death (Brunet *et al.*, 1999; Kops and Burgering, 1999; Tang *et al.*, 1999). Accordingly, overexpression of either FKHR or FKHRL1 results in apoptosis (Brunet *et al.*, 1999; Tang *et al.*, 1999). So far, FasL is the only known candidate gene to mediate FKHRL1-induced apoptosis (Brunet *et al.*, 1999). Therefore, by phosphorylating FKHRL1 and excluding it from the nucleus, the PI3K target Akt may prevent the transcriptional engagement of FKHRL1, inhibit Forkhead-induced apoptosis, and contribute to cell survival.

In this report, we show that treatment with TGF β results in phosphorylation and nuclear exclusion of endogenous and ectopic FKHRL1 in mammary epithelial cells and skin keratinocytes. This effect required PI3K and Akt function as inhibitors of PI3K or expression of dominant-negative Akt (dn Akt) prevented TGF β -mediated inhibition of FKHRL1. Moreover, both Forkhead-dependent transcription and cell death induced by either serum starvation or forced expression of FKHRL1 was partially blocked by TGF β . These results suggest that TGF β , via activation of Akt, may induce cytoplasmic retention of FKHRL1 and thus act as an antiapoptotic factor in epithelial cells. These mechanisms may be biologically relevant to TGF β -mediated tumor progression.

MATERIALS AND METHODS

Cell Lines, Inhibitors, and Antibodies

NMuMG nontumorigenic mouse mammary epithelial cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml insulin. 4T1 breast tumor cells, kindly provided by F. Miller (Karmanos Cancer Center, Detroit, MI), were maintained in DMEM with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HaCaT keratinocytes, stably transfected with a dn Akt mutant vector or vector alone (mock cells), have been described previously (Jost *et al.*, 2001). The HaCaT cells were maintained in DMEM supplemented with 10% FCS, 0.1 mg/ml hygromycin B, 2 μ g/ml tetracycline (Tet), 100 U/ml penicillin, and 100 μ g/ml streptomycin. To induce expression of the dn Akt protein, cells were washed two times with Tet-free medium and kept in Tet-free medium for 48 h. TGF β 1 was obtained from R & D Systems (Minneapolis, MN). Antibodies to FKHRL1, phospho-Ser-253 FKHRL1 and C-terminal phospho Smad2 were from Upstate Biotechnology (Lake Placid, NY) to Smad2 from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-conjugated anti-mouse IgG secondary antibody was purchased from Molecular Probes (Eugene, OR) and anti-hemagglutinin (HA)-fluorescein mouse monoclonal antibody from Roche Molecular Biochemicals (Indianapolis, IN). Akt and phospho-Ser-473 Akt polyclonal antibodies were from New England Biolabs (Beverly, MA). LY294002 was from Calbiochem (San Diego, CA).

Immunoblot Analysis

After washes with phosphate buffered saline (PBS), cell monolayers were lysed in a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin. Equal amount of protein in whole cell lysates [as measured by Bradford (1976) method] were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBST) containing 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20 (v/v) for 1 h at ambient temperature and then incubated overnight with primary antibodies in TBST in 1% skim milk at 4°C. After washing membranes with TBST three times, they were incubated with a 1:5000 dilution of horseradish peroxidase-linked secondary antibody in TBST for 1 h, followed by three washes in TBST. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

Immunocytochemistry and Transfections

Cells were grown in DMEM/10% FCS to ~60% confluence on glass coverslips in 12-well plates, washed with serum-free medium, incubated in serum-free medium for 24 h, and then stimulated with 2 ng/ml TGF β for 1 h in the absence or presence of 20 μ M LY294002, an inhibitor of PI3K (Vlahos *et al.*, 1994). In experiments involving ectopic FKHRL1 expression, cells in 60-mm dishes (10⁶ cells/dish) were transfected with 10 μ g of either WT-FKHRL1 or triple mutant (TM)-FKHRL1, each for 16 h with the use of FUGENE 6 (Roche Molecular Biochemicals). Cells were then transferred to coverslips on 12-well plates, incubated in serum-free medium for 24 h, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Coverslips were next blocked with 3% skim milk in PBS for 30 min and incubated with primary antibodies diluted in 1% skim milk/PBS (1:500 for FKHRL1 and P-Ser253 FKHRL1; 1:200 for anti-HA fluorescein). After three washes with PBS, samples were incubated with fluorescent secondary antibodies diluted in PBS (1:500) for 1 h at room temperature. Coverslips were mounted on glass slides with AquaPolyMount (Polysciences, Warrington, PA) and examined by laser scanning confocal microscopy (LSM 410);

Carl Zeiss, Thornwood, NY). For detection of apoptotic nuclei, cells were incubated in 1 $\mu\text{g/ml}$ Hoechst 33258 (Sigma, St. Louis, MO) in PBS for 10 min after incubation with secondary antibody. Fluorescent images of Hoechst-stained nuclei or HA-stained samples were recorded with a Zeiss Axiophot upright microscope.

Transcriptional Reporter Assays

Cells were seeded at the density of 10^5 cells/well (12-well plates). After 24 h, the cells were transfected with 0.5 $\mu\text{g/well}$ of either WT-FKHRL1-HA or TM-FKHRL1-HA, each with 0.5 $\mu\text{g/well}$ of Forkhead-responsive element (FHRE)-Luc and 0.005 $\mu\text{g/well}$ of pCMV-Rl (Promega, Madison, WI) with the use of 3 $\mu\text{l/well}$ of FUGENE6 reagent for 16 h. Transfected cells were then subjected to serum starvation either in the presence or absence of 2 ng/ml TGF β for 24 h. Firefly luciferase and *Renilla reniformis* luciferase activities in cell lysates were determined with the use of the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2001 luminometer (Analytical Luminescence Laboratory). Firefly luciferase activity was normalized to *R. reniformis* luciferase activity and presented as relative luciferase units.

Apoptosis Assays

Cells were seeded at the density of 5×10^5 cells/well in six-well dishes. The following day, the medium was changed to serum-free medium with or without 2 ng/ml TGF β . Both floating cells and adherent cells were harvested 72 h later. Pooled cells were washed with PBS and then subjected to Apo-5-bromo-2'-deoxyuridine (BrdU) analysis with the use of an Apo-BrdU assay kit (Phoenix Flow Systems, San Diego, CA) according to the manufacturer's protocol in a FACS/Calibur Flow Cytometer (BD Biosciences, Mansfield, MA). For evaluation of DNA fragmentation, 10^6 cells/dish in 60-mm dishes were incubated in serum-free medium \pm 2 ng/ml TGF β . After 72 h, floating and adherent cells were harvested, washed with PBS, and resuspended in 200 μl of cytosolic DNA extraction buffer (5 mM Tris, pH 7.4, 20 mM EDTA, 0.5% Triton X-100) followed by vortexing for 1 min. After centrifugation for 15 min at 12,000 rpm at 4°C, the supernatants were transferred into new tubes and subjected to phenol/chloroform extraction. The DNA fragments were pelleted by adding 3 M sodium acetate, washed with ethanol, resuspended in TE containing 200 $\mu\text{g/ml}$ RNase, and separated by 1.5% agarose gel electrophoresis.

Cell Cycle Analysis by Flow Cytometry

Cells were harvested by trypsinization, fixed in ethanol, and labeled with 50 $\mu\text{g/ml}$ propidium iodide (Sigma) containing 125 U/ml protease-free RNase (Calbiochem) as described previously (Busse *et al.*, 2000). Cells were filtered through a 95- μm pore size nylon mesh (Small Parts, Miami Lakes, FL) and a total of 15,000 stained nuclei was analyzed in a FACS/Calibur Flow Cytometer (BD Biosciences).

RESULTS

TGF β Induces Phosphorylation and Nuclear Exclusion of Endogenous FKHRL1

We have previously observed that TGF β can phosphorylate and activate Akt (Bakin *et al.*, 2000), which is known to phosphorylate FKHRL1 both in vivo and in vitro (Brunet *et al.*, 1999). Thus, we first determined whether TGF β can induce FKHRL1 phosphorylation. As shown in Figure 1A, treatment with 2 ng/ml TGF β for 30 min increased the phosphorylation of FKHRL1 in 4T1 and NMuMG cells as determined by immunoblot with a P-Ser253 FKHRL1 antibody. Akt can phosphorylate FKHRL1 at Thr32, Ser253, and Ser315. Brunet *et al.* (1999) showed that the Akt-dependent

shift in FKHRL1 mobility on SDS-PAGE is primarily due to phosphorylation at Ser315, suggesting that the slower migrating band in the lysates from TGF β -treated cells (Figure 1A) may represent P-Ser315 FKHRL1 also recognized by the P-Ser253 FKHRL1 antibody. Cotreatment with LY294002 inhibited TGF β -mediated phosphorylation of FKHRL1, suggesting that this effect required PI3K function.

Others have reported that Forkhead transcription factors translocate from the nucleus to the cytoplasm after Akt-mediated phospho-phosphorylation (Biggs *et al.*, 1999; Brunet *et al.*, 1999; del Peso *et al.*, 1999; Takaishi *et al.*, 1999). To determine whether TGF β can induce translocation of endogenous FKHRL1, we stimulated serum-starved cells with 2 ng/ml TGF β for 30 min and then performed immunofluorescence analysis. Figure 1B shows FKHRL1 staining in cytoplasm and nucleus in both NMuMG and 4T1 cells. TGF β treatment results in exclusion of the nuclear FKHRL1 staining, which was prevented by LY294002. These results suggest that TGF β -mediated phosphorylation and subsequent nuclear exclusion of FKHRL1 are both PI3K-dependent.

TGF β Inhibits Nuclear Translocation of Exogenous FKHRL1 and Forkhead-dependent Transcription

We next determined whether TGF β -mediated FKHRL1 regulation required function of the PI3K effector kinase Akt. Cells were transfected with HA-tagged WT-FKHRL1 or a triple mutant TM-FKHRL1, in which all three Akt phosphorylation sites (Thr32, Ser253, and Ser315) (Brunet *et al.*, 1999) had been mutated to alanine, and examined the subcellular localization of ectopic FKHRL1 under various conditions. In serum-containing medium and in both 4T1 and NMuMG mammary cells, WT-FKHRL1 primarily localized in the cytoplasm (Figure 2A, first column of each cell line). On removal of serum for 24 h, WT-FKHRL1 protein localized predominantly in the nucleus. Treatment with TGF β promoted WT-FKHRL1 translocation to the cytoplasm, which was prevented by cotreatment with the PI3K inhibitor LY294002. 4T1 and NMuMG cells transfected with the TM-FKHRL1 construct exhibited exclusive nuclear localization of the HA-tagged mutant under any experimental condition (Figure 2A, third row). The results imply that the phosphorylation status of the three Akt sites determines the subcellular distribution of FKHRL1: unphosphorylated FKHRL1 is localized mainly in the nucleus; phosphorylation of the three Akt-consensus sites upon the addition of TGF β results in nuclear exclusion of FKHRL1.

We next asked whether Forkhead-dependent transcription was regulated by TGF β signaling. We cotransfected cells with either a WT- or TM-FKHRL1 constructs and a FHRE-Luc vector in which a Forkhead response element is linked to a luciferase reporter gene. FHRE contains the binding site of the FasL promoter and FKHRL1 is known to bind this site and enhance transcription of the FasL gene (Brunet *et al.*, 1999). Withdrawal of serum increased FKHRL1-dependent luciferase expression in both NMuMG and 4T1 cells (Figure 2B). However, the addition of TGF β reduced luciferase activity to levels obtained in the presence of serum, suggesting that TGF β down-regulates Forkhead-dependent transcription possibly by a mechanism involving nuclear exclusion of FKHRL1. In cells transfected with the Akt-insensitive TM-FKHRL1, transcriptional activity was higher

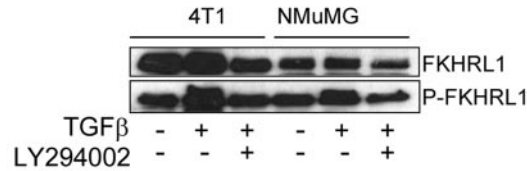
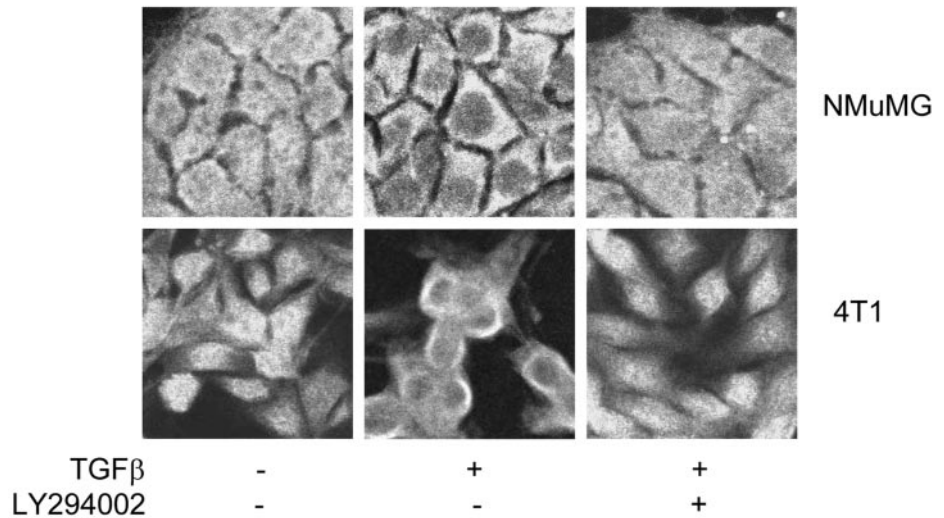
A

Figure 1. TGF β induces cytoplasmic retention and phosphorylation of FKHRL1 in Ser253. (A) Serum-starved, subconfluent 4T1 and NMuMG cells were treated with 2 ng/ml TGF β for 30 min with or without 20 μ M LY294002. After lysis, protein extracts (50 μ g/lane) were subjected to SDS-PAGE followed by immunoblot procedures as described in MATERIALS AND METHODS. Dilution ratio for primary antibodies is 1:500 for both FKHRL1 and P-Ser253 FKHRL1. (B) 4T1 and NMuMG cells grown to 60% confluence on coverslips in 12-well plates, serum-starved for 24 h, and then treated with 2 ng/ml TGF β for 30 min with or without 20 μ M LY294002. Cells were washed, fixed as described in MATERIALS AND METHODS, and stained with antibodies against FKHRL1 (1:500). Texas Red-conjugated anti-rabbit IgG (1:500) was used as secondary antibody. Fluorescent images were captured with the use of a laser scanning confocal microscope.

B

than in cells transfected with WT-FKHRL1. Although to a lesser degree than in cells transfected with the WT construct, TGF β also reduced the transcriptional activity mediated by TM-FKHRL1 (Figure 2B).

Dominant-Negative Akt Inhibits TGF β -induced FKHRL1 Phosphorylation, Nuclear Exclusion of FKHRL1, and Forkhead-dependent Transcription

The causal role of Akt in TGF β -mediated regulation of FKHRL1 was investigated with the use of HaCaT keratinocytes expressing Tet-suppressible dn Akt (Jost *et al.*, 2001). The construct used in this system encodes a kinase-inactive version of Akt in which Lys179 in the catalytic domain has been mutated to Met (Dudek *et al.*, 1997). Withdrawal of Tet from the cell culture medium for 48 h induced expression of dn Akt (Figure 3A). Treatment with TGF β also induced Akt activity, as measured by P-Ser473 Akt and P-Ser253 FKHRL1 immunoblot analyses in both mock HaCaT cells \pm Tet and in dn Akt HaCaT cells in the presence of Tet (Figure 3A). However, when the dn Akt transgene was expressed by withdrawing Tet from dn Akt HaCaT cell culture medium for 48 h, TGF β -induced phosphorylation at Ser253 of FKHRL1, Ser473 of Akt (Figure 3A) were all reduced. Consistent with transcriptional reporter activity data from NMuMG and 4T1 cells, in mock-

transfected HaCaT cells \pm Tet and in dn Akt HaCaT cells treated with Tet, TGF β reduced FKHRL1-dependent transcription (Figure 3B, left). In dn Akt HaCaT cells, however, removal of Tet blocked the inhibitory effect of added TGF β on FKHRL1-induced reporter activity (Figure 3B, right), suggesting that Akt is indeed responsible for mediating this TGF β response. To exclude the possibility that dn Akt may be blocking TGF β effects on FKHRL1 via inhibition of the TGF β type I receptor (T β RI) kinase, we tested the effect of dn Akt on phosphorylation of Smad2. As shown in Figure 3C, TGF β -mediated C-terminal phosphorylation of Smad2 in HaCaT cells was not altered by dn Akt.

Immunocytochemical analysis (Figure 4) shows that induction of dn Akt can block nuclear exclusion of exogenously expressed FKHRL1 in HaCaT keratinocytes. In mock HaCaT cells, serum starvation resulted in nuclear localization of WT-FKHRL1; treatment with TGF β for 30 min promoted translocation of WT-FKHRL1 from the nucleus to the cytosol regardless of the presence of Tet. Expression of kinase-inactive Akt by withdrawal of Tet from culture medium, blocked the ability of TGF β to induce nuclear exclusion of WT-FKHRL1. As expected, TM-FKHRL1 localized in the nucleus of both dn Akt and control cells regardless of the presence of Tet (Figure 4).

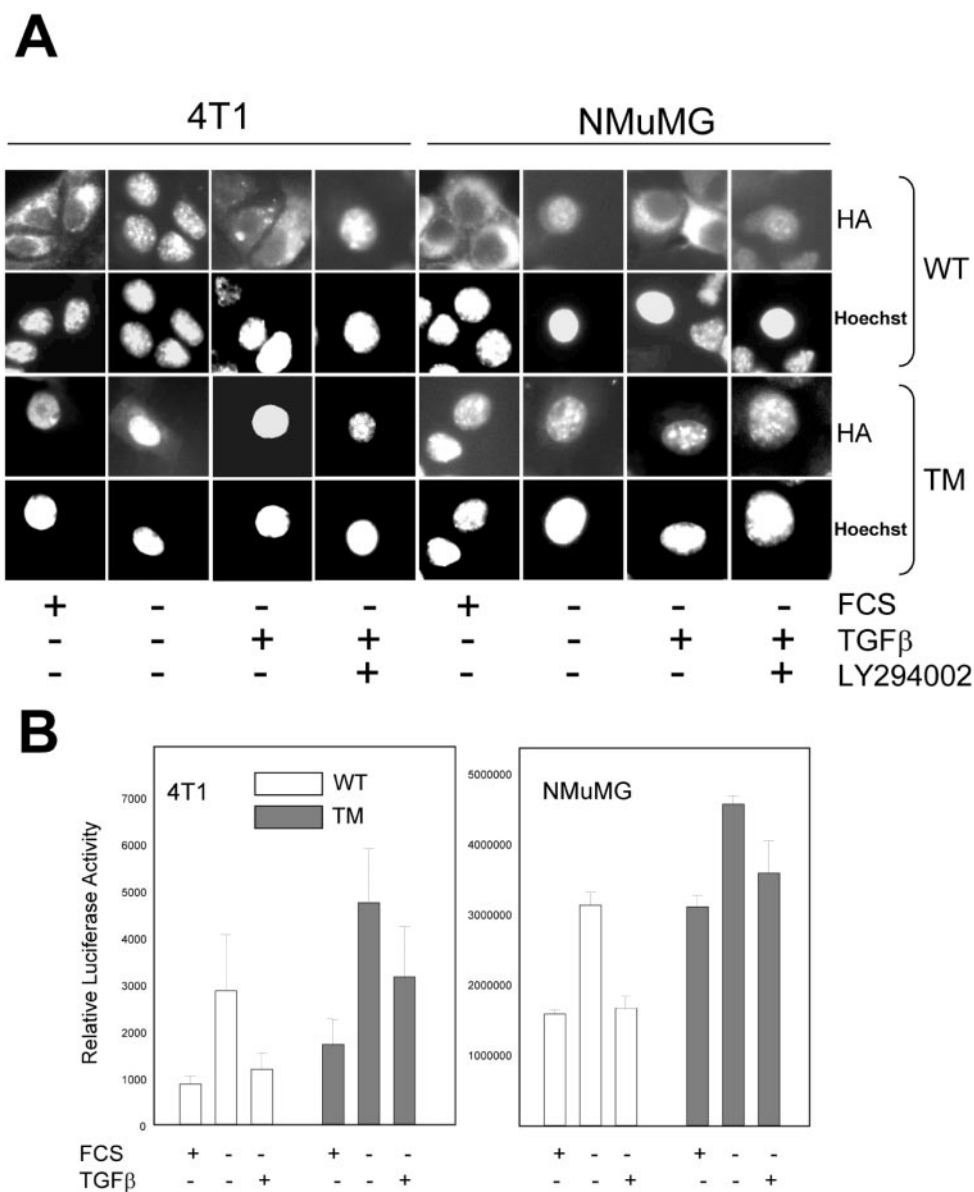


Figure 2. TGFβ inhibits nuclear translocation of exogenous FKHL1 and Forkhead-dependent transcription. (A) Cells seeded in 60-mm dishes (10^6 cells/dish) were transfected with $10 \mu\text{g}/\text{dish}$ of either WT- or TM-FKHRL1-HA for 16 h and transferred to coverslips on 12-well plates. The cells were then incubated in serum-free medium for 24 h followed by stimulation with 2 ng/ml TGFβ for 30 min with or without $20 \mu\text{M}$ LY294002. Fixation and staining were performed as described in MATERIALS AND METHODS. Fluorescence intensities associated with anti-HA fluorescein and Hoechst 33258 were observed with a Zeiss Aiophot upright microscope. (B) Cells were transfected with WT- or TM-FKHRL1, each with FHRE-Luc and pCMV-Rl vectors followed, where indicated, with removal of serum and the addition or not of 2 ng/ml TGFβ for 24 h. Dual luciferase assay was performed as described in MATERIALS AND METHODS. Relative luciferase units represents firefly luciferase activity normalized to *Renilla* luciferase activity. Each data point represents mean \pm SD of four wells.

TGFβ Partially Suppresses Apoptosis Induced by Serum Starvation or by Forced Expression of WT-FKHRL1 but not by TM-FKHRL1

Because TGFβ can induce phosphorylation and subsequent nuclear exclusion of FKHL1, which is known to be responsible for the expression of death-related genes (Brunet *et al.*, 1999; Kops and Burgering, 1999), we investigated whether expression of ectopic FKHL1 and/or serum starvation can induce apoptosis in NMuMG cells and whether TGFβ can reverse this process. As shown in Figure 5A, forced expression of either WT- or TM-FKHRL1 resulted in NMuMG cell apoptosis. Cells transfected with either construct were double-labeled with the nuclear stain Hoechst 33258 and an HA antibody. The same cells expressing WT- or TM-FKHRL1, as measured by HA staining, exhibited fragmented nuclei, sug-

gesting that FKHL1 causes NMuMG cell death. Of ~ 150 HA-positive nuclei, 45% of WT-FKHRL1- and 67% TM-FKHRL1-expressing nuclei were apoptotic.

To further determine whether nuclear localization of FKHL1 was causal to apoptosis, we examined the temporal correlation of nuclear localization of FKHL1 with the onset of DNA double-strand breaks as measured by Apo-BrdU assay. Initial experiments showed that $\geq 2 \mu\text{g/ml}$ exogenous WT-FKHRL1 was required to induce apoptosis of NMuMG cells (our unpublished results). Therefore, to minimize a possible contribution of the ectopic FKHL1 to NMuMG cell apoptosis, we used a >10 -fold lower concentration ($0.2 \mu\text{g/ml}$) of HA-tagged WT-FKHRL1. Nuclear localization of FKHL1 was first evident at 3 h after serum starvation, whereas a low level

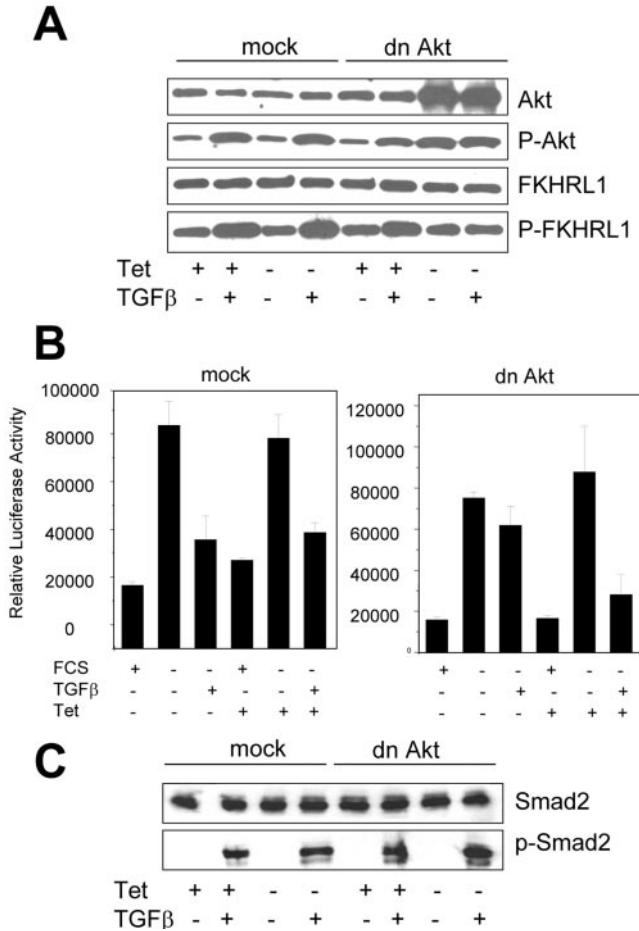


Figure 3. Dominant-negative Akt blocks TGFβ-induced FKHRL1 phosphorylation and Forkhead-dependent transcription but not phosphorylation of Smad2. (A) Mock-transfected and dn Akt-transfected HaCaT cells were incubated in medium with or without 2 μg/ml Tet for 48 h. For the last 24 h of incubation, medium was replaced by serum-free medium ± Tet. The cells were then treated with 2 ng/ml TGFβ for 30 min, harvested, and lysed. Protein extracts (30 μg/lane) were subjected to SDS-PAGE followed by immunoblot as described in MATERIALS AND METHODS. Dilution ratios for primary antibodies are 1:500 for P-Ser473 Akt, FKHRL1, and P-Ser253 FKHRL1, and 1:1000 for total Akt. (B) HaCaT cells were transfected with WT- or TM-FKHRL1, each with FHRE-Luc and pCMV-Rl vectors, and incubated in medium with or without 2 μg/ml Tet for 48 h. For the last 24 h where indicated, medium was replaced with serum-free DMEM ± Tet with or without 2 ng/ml TGFβ. Dual luciferase assay was performed as described in MATERIALS AND METHODS. Each data point represents mean ± SD of four wells. (C) In the presence or absence of Tet, cells were incubated with 2 ng/ml TGFβ for 30 min and lysed as in Figure 3A. Cell lysates were resolved by SDS-PAGE and subjected to immunoblot procedures for total and phosphorylated Smad2. Each lane contains 50 μg of protein. Dilution ratios for primary antibodies are 1:1000 for total Smad2 and 1:500 for P-Smad2.

of apoptosis above baseline was first detectable at 12 h reaching a maximum at 48 h, implying that the nuclear localization of FKHRL1 was not secondary to the onset of apoptosis.

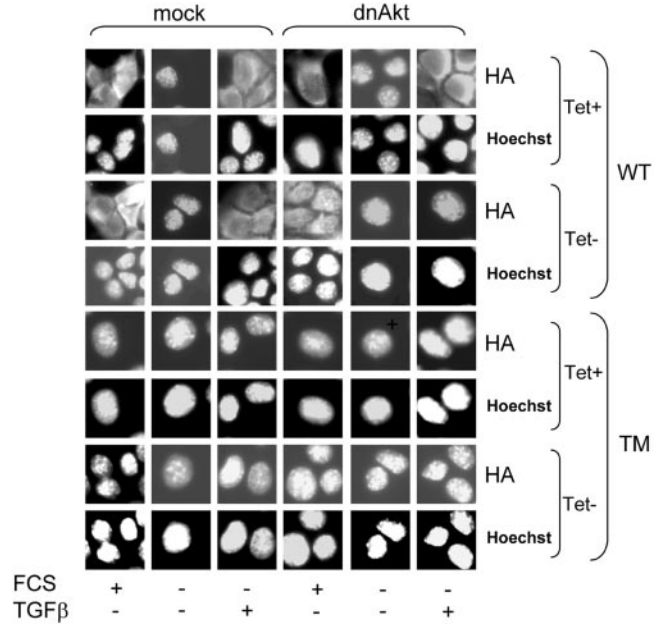


Figure 4. Dominant-negative Akt blocks TGFβ-induced nuclear exclusion of FKHRL1. Mock and dn Akt HaCaT cells seeded in 60-mm dishes (10⁶ cells/dish) ± Tet were transfected with 10 μg/dish of either WT- or TM-FKHRL1 for 16 h and next transferred to coverslips on 12-well plates. In some cases, the cells were serum-starved for 24 h followed by the addition of 2 ng/ml TGFβ for 30 min. Fixation and staining were performed as described in MATERIALS AND METHODS. Fluorescence intensities associated with anti-HA fluorescein and Hoechst 33258 were measured as described in the legend of Figure 2A.

We next investigated whether addition of exogenous TGFβ could rescue apoptosis induced by serum starvation or forced expression of FKHRL1. Removal of FCS for 72 h increased the proportion of apoptotic cells from 7.98 to 46.78%. The latter was reduced to 24.07% by the addition of 2 ng/ml TGFβ (Figure 6A). Transfection of 5 μg/ml WT- or TM-FKHRL1 increased the proportion of apoptotic cells from 8.60% to 25.66 or 46.86%, respectively (Figure 6B). Notably, expression of FKHRL1 mutant constructs with a deletion of the transactivating Forkhead domain (FKHRL1 ΔTA and TM-FKHRL1 ΔTA) did not induce apoptosis above baseline, implying that the transactivating function of FKHRL1 was required for the induction of cell death. Addition of TGFβ markedly inhibited the apoptosis induced by WT-FKHRL1 but not by TM-FKHRL1 (Figure 6B), further suggesting that the protective effect of TGFβ depended on Akt-mediated phosphorylation of FKHRL1.

Dominant-Negative Akt Abolishes Survival Effect of TGFβ

To obtain direct evidence that Akt may play a role in TGFβ-induced cell survival, we used HaCaT cells in which dn Akt was conditionally expressed. As measured by Apo-BrdU assay, a large proportion of mock-transfected HaCaT cells (>20%) became apoptotic upon withdrawal of serum for 72 h. In the presence or absence of Tet, exogenous TGFβ

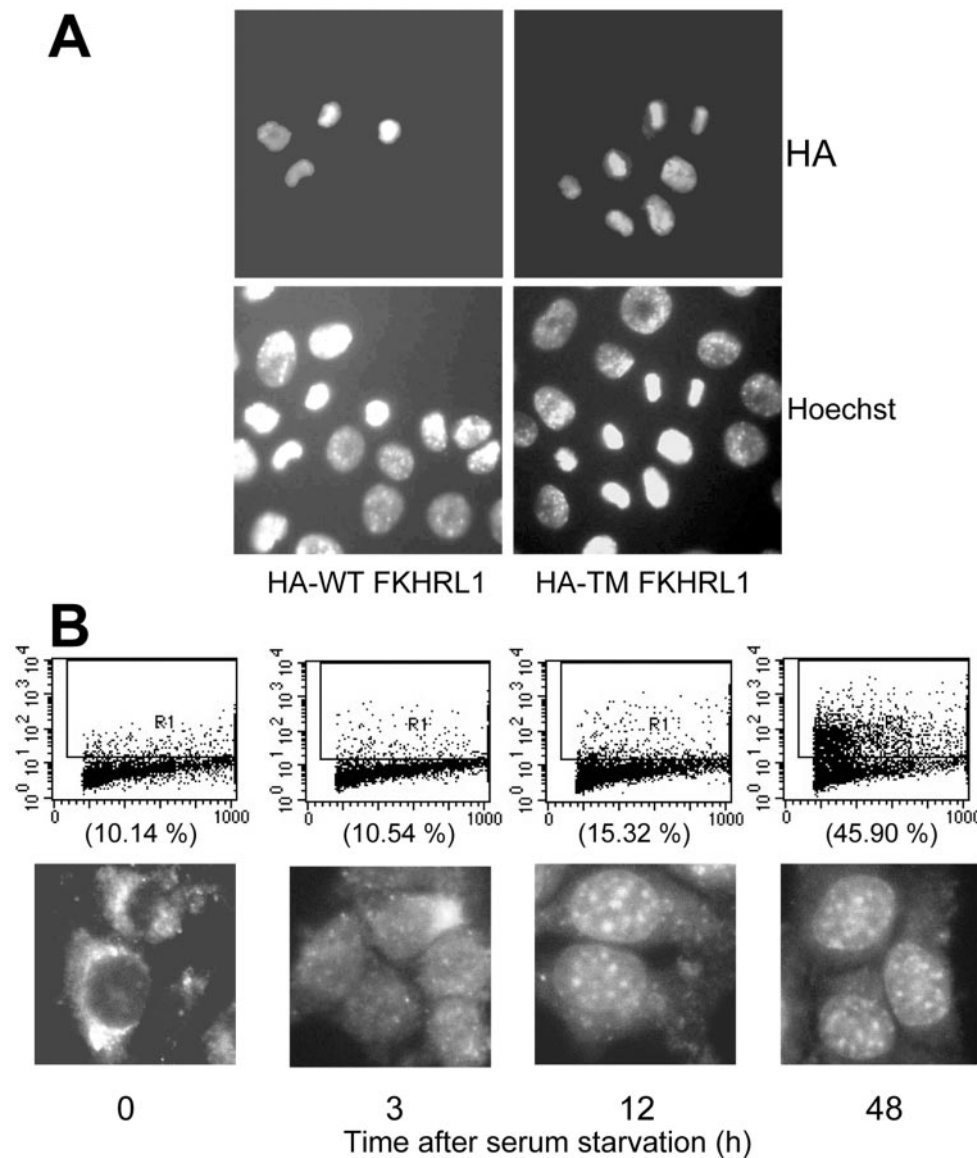


Figure 5. Forced expression of FKHL1 or serum starvation induce apoptosis of NMuMG cells. (A) NMuMG cells in 60-mm dishes (10^6 cells/dish) were transfected with $10 \mu\text{g}/\text{dish}$ of either WT- or TM-FKHL1-HA for 16 h and transferred to coverslips on 12-well plates. After 24-h incubation, the cells were stained with anti-HA fluorescein and Hoechst 33258. The samples were examined in a Zeiss Axiophot microscope. (B) NMuMG cells in 60-mm dishes (10^6 cells/dish) were transfected with $0.2 \mu\text{g}/\text{ml}$ WT-FKHL1-HA for 16 h and transferred to coverslips on 12-well plates. After 24 h, the medium was changed with serum-free medium. At the indicated times, the cells were harvested and assayed for evidence of apoptosis by Apo-BrdU analysis (top row) or stained with anti-Ha fluorescein and observed with a Zeiss Axiophot microscope. The percentages of fluorescein isothiocyanate-positive (apoptotic) cells are shown in parentheses.

completely prevented apoptosis in control (mock) HaCaT cells and in dn Akt cells treated with Tet (Figure 7A, second column). On the other hand, in dn Akt HaCaT cells, removal of Tet and hence induction of kinase-dead Akt, blocked the ability of TGF β to prevent the apoptosis induced by serum starvation. Similar data were obtained in DNA fragmentation assays. Serum starvation induced internucleosomal DNA fragmentation in both control and dn Akt cells. TGF β abolished DNA fragmentation except in dn Akt cells in the absence of Tet (Fig. 7B), implying Akt is causal to the protection from cell death mediated by TGF β .

Survival Effects of TGF β Are Independent of Antiproliferative Effects

Addition of TGF β to proliferating NMuMG cells induces growth arrest. These studies have been done with exponen-

tially growing NMuMG cells in serum-containing medium (Miettinen *et al.*, 1994; Piek *et al.*, 1999), conditions under which FKHL1 localizes mainly in the cytosol and FKHL1-mediated transcription is low (Figure 2). In serum-containing medium, NMuMG displayed a robust S phase (24.3%) and a low level of apoptosis. A 24-h treatment with TGF β resulted in G1 arrest and marked reduction in S phase without a significant effect on the low level of apoptosis (6.9 vs. 11.2%; Figure 8A). Under these serum-containing conditions, TGF β induced a 44% reduction in cell number after 72 h (Figure 8B), consistent with the delay in cell cycle progression. On the other hand, serum withdrawal (for 24 h) *per se* resulted in G1 arrest (89.2%), whereas 21.9% of cells exhibited evidence of apoptosis. Addition of TGF β reduced in half the apoptosis induced by serum deprivation (21.9 vs. 10.8%) but had no detectable effect on NMuMG cell cycle

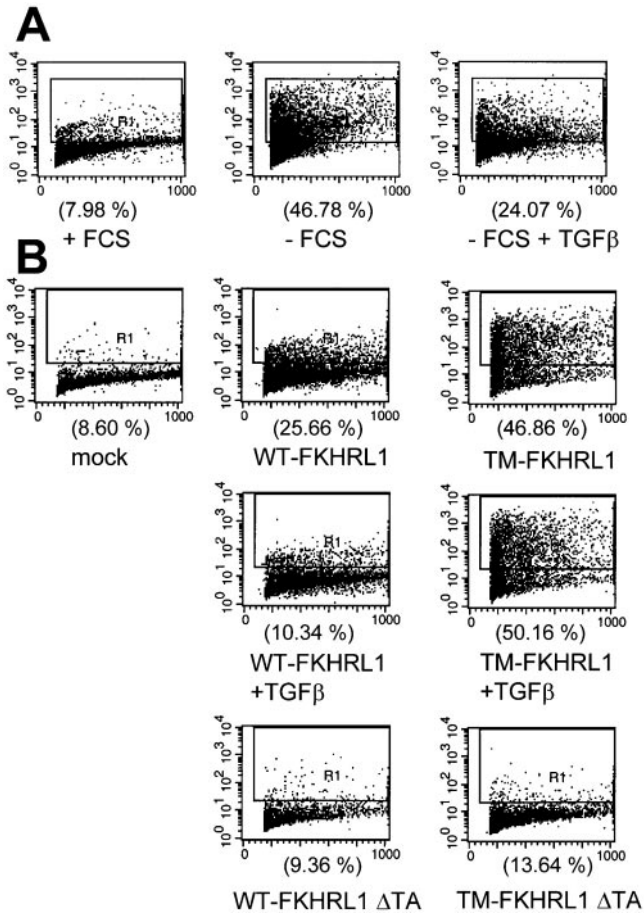


Figure 6. TGFβ inhibits NMuMG cells apoptosis induced by serum starvation or by forced expression of FKHL1. (A) NMuMG cells seeded in serum-free medium at the density of 5×10^5 cells/well in six-well dishes were treated with or without 2 ng/ml TGFβ. Seventy-two hours later, the cells were harvested and evaluated for apoptosis by Apo-BrdU analysis as described in MATERIALS AND METHODS. (B) NMuMG cells seeded at the density of 5×10^5 cells/well in six-well dishes were transfected with 5 μg/ml the indicated WT- and TM-FKHRL1 constructs for 16 h. After transfection, cells were incubated for 72 h in the presence or absence of 2 ng/ml TGFβ and finally subjected to Apo-BrdU analysis.

distribution as measured by flow cytometry (Figure 8A). Consistent with its antiapoptotic effect, these results, addition of TGFβ to serum-deprived cells increased cell number 90% above untreated control cells after 72 h (Figure 8B). These data suggest that TGFβ-mediated signals that result in growth inhibition may be independent from those involved in the blockade of apoptosis and enhanced survival.

DISCUSSION

Results presented herein with mammary epithelial cells and skin keratinocytes indicate that TGFβ induced phosphorylation and nuclear exclusion of both the endogenous and transfected Forkhead transcription factor FKHL1.

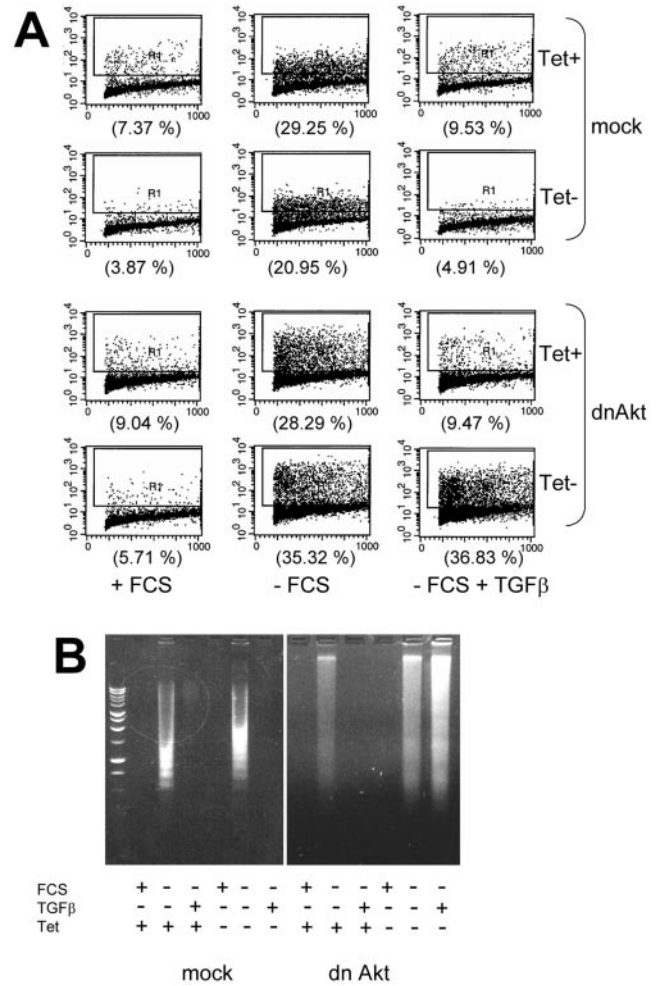


Figure 7. Expression of dominant-negative Akt blocks the antiapoptotic effect of TGFβ1. (A) Control (mock) and dn Akt HaCaT keratinocytes were incubated in serum-containing or serum-free medium ± Tet for 72 h in the presence or absence of 2 ng/ml TGFβ. The proportion of apoptotic cells was assessed by Apo-BrdU analysis and is indicated in parentheses. (B) Mock and dn Akt HaCaT cells (10^6 cells/dish) on 60-mm dishes were incubated in medium containing FCS or in serum-free medium ± 2 ng/ml TGFβ for 72 h. To suppress dn Akt, Tet was added during the incubation period where indicated. Adherent and floating cells were pooled, their DNA was collected, and next evaluated for evidence of internucleosomal fragmentation in 1.5% agarose gels as described in MATERIALS AND METHODS.

LY294002, a small molecule inhibitor of the p110 catalytic subunit of PI3K, blocked these effects, suggesting that TGFβ-mediated regulation of FKHL1 required PI3K function. LY294002 did not inhibit TGFβ-induced PAI-1 luciferase reporter activity in Mink lung epithelial cells (our unpublished data). This suggests that the blockade of TGFβ effects on FKHL1 by LY294002 was not due to inhibition of TGFβ type I receptor (TβRI) kinase activity. Moreover, dn Akt did not prevent TGFβ-induced C-terminal phosphorylation of Smad2 in HaCaT cells (Figure 3C) nor in NMuMG cells

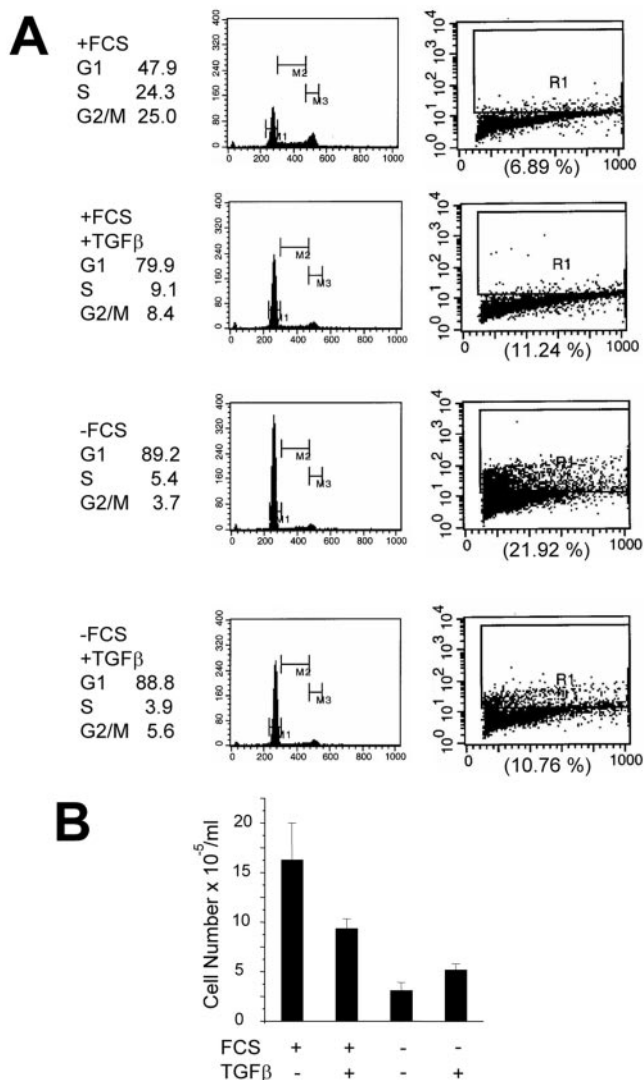


Figure 8. TGF β inhibits cell cycle progression of proliferating NMuMG cells but protects serum-starved NMuMG cells from apoptosis. (A) NMuMG cells seeded at the density of 10^6 cells/dish in 60-mm dishes were incubated in DMEM/10% FCS or serum-free DMEM, each \pm 2 ng/ml TGF β . After 24 h, cells were harvested and analyzed for cell cycle and apoptosis as described in MATERIALS AND METHODS. (B) NMuMG cells (10^6 cells/dish) in 60-mm dishes were incubated in DMEM/10% FCS or serum-free DMEM, each \pm 2 ng/ml TGF β for 72 h. At this time, the monolayers were washed with PBS and the adherent cells were trypsinized and counted with the use of a Coulter counter. Each bar represents the mean \pm SD of four dishes.

(Bakin *et al.*, 2000), implying further that blockade of TGF β action on FKHRL1 by dn Akt did not involve an effect on T β RI kinase activity.

Several results implicated activation of Akt as an effector mechanisms for FKHRL1 regulation by TGF β . First, a mutant of Forkhead in which all three Akt phosphorylation sites have been replaced with Ala localized exclusively in

the nucleus of three cell lines (NMuMG, 4T1, and HaCaT) and was insensitive to TGF β -mediated retention in the cytosol. Second, in HaCaT cells, inducible kinase-inactive Akt blocked the effects of TGF β on FKHRL1 Ser253 phosphorylation, nuclear exclusion, and transcriptional activity. Third, in serum-starved NMuMG and 4T1 cells, the transcriptional activity of TM-FKHRL1 was higher than that of WT-FKHRL1. However, the TM-FKHRL1-mediated transcription was still inhibited by TGF β , suggesting that the mutant construct was unable to override endogenous Forkhead factors potentially regulated by the addition of TGF β . Thus, we speculate that the reduction in reporter activity mediated by TGF β in TM-FKHRL1-expressing cells (Figure 2B) might be conferred by the endogenous FKHRL1, which is still subjected to Akt-mediated phosphorylation. Finally, the apoptotic effect of WT-but not TM-FKHRL1 was abrogated by TGF β as long as Akt was functional. Nonetheless, transfection of a FKHRL1 constructs in which the DNA-binding domain was mutated (H212R) did not prevent TGF β -induced protection from cell death (our unpublished data). The inability of this ectopic protein with intact Akt phosphorylation sites to reduce the antiapoptotic effect of TGF β implied that Akt-mediated phosphorylation of FKHRL1 may not be a saturable process. Although the induced dn Akt was effective in blocking TGF β -induced nuclear exclusion and cytosolic retention of FKHRL1, it only partially induced nuclear localization of FKHRL1 in the presence of serum without added TGF β (Figure 4). It is conceivable that other activities not affected by dn Akt, such as serum- and glucocorticoid-induced kinases (SGKs; Brunet *et al.* 2001), may also regulate FKHRL1 localization and/or function in these cells, potentially explaining the cytosolic retention of FKHRL1 despite expression of dn Akt shown in Figure 4. This possibility will require further experiments beyond the scope of this report.

The potent antiapoptotic effect of Akt and its disabling effect of FKHRL1 function suggested that FKHRL1 may induce apoptosis in the epithelial cells used in our studies. Indeed, overexpression of both WT- and TM-FKHRL1 induced NMuMG cell death as implied by nuclear fragmentation in cells also expressing either the ectopic WT- or TM-FKHRL1 construct (Figure 5A). Apoptosis was not observed with transient transfection of a mutant FKHRL1 that lacked DNA-transactivating function. Notably, transient transfection efficiencies were consistently low (\approx 3%) with the WT and TM constructs but much higher (\approx 15%) with FKHRL1 Δ TA, further supporting a causal association between FKHRL1 expression and apoptosis. Moreover, the nuclear localization of FKHRL1 in serum-starved NMuMG cells preceded the onset of apoptosis, implying that cell death is subsequent to the nuclear localization of FKHRL1 and that the latter was not due to an indirect effect of the apoptotic program on nuclear export. Supporting this possibility is the fact that, despite inducing high levels of apoptosis, TM-FKHRL1 was consistently localized in the nucleus (Figures 2A, 4, and 5A). Consistent with the transcriptional reporter activity driven by an FHRE (Figure 2B), the proportion of apoptotic cells was higher in NMuMG cells transfected with TM- than with WT-FKHRL1 (Figure 5A; see RESULTS), implying that a low level of basal Akt activity is able to ameliorate the cell death induced by WT-FKHRL1. It is conceivable that inhibition of other proapop-

otic molecules beyond the scope of this report, such as BAD, caspase 9, or I κ Ks (Datta *et al.*, 1999), are involved in Akt-mediated protection from cell death. However, in preliminary experiments we have been unable to detect increased caspase 9 activity in serum-starved NMuMG cells.

Our data concur with those in other reports. Tang *et al.* (1999) showed that another Forkhead ortholog (FKHR), in which all three Akt phosphorylation sites have been mutated to Ala (TM-FKHR), induced features of apoptosis as membrane blebbing and DNA fragmentation 48 h after transfection into 293T cells. Cells transfected with WT-FKHR showed minimal evidence of apoptosis despite expressing higher level of WT-FKHR than TM-FKHR. In addition, a mutation in the DNA-binding domain of FKHR reduced the ability of this expression vector to induce apoptosis (Tang *et al.*, 1999). In this report (Figure 6B), a mutant FKHRL1 lacking its transactivation domain failed to elicit apoptosis, implying that both DNA binding and activation of transcription are required for the occurrence of Forkhead-induced apoptosis. Brunet *et al.* (1999) reported induction of apoptotic cell death by a triple-Akt-sites-mutant of FKHRL1 in cerebellar neurons, CCL39 fibroblasts, and Jurkat T cells. They also provided some evidence that FKHRL1-induced apoptosis was mediated in part by its ability to induce transcription of the FasL gene. Recently, it was reported that TGF β could decrease apoptosis of human T cells while inhibiting the expression of FasL (Genestier *et al.*, 1999). In PC12 cells, removal of nerve growth factor results in increased JNK activation, enhanced FasL expression, and neuronal cell death (Le-Niculescu *et al.*, 1999). In addition, studies in T lymphocytes have shown that forced expression of FKHRL1 up-regulates the anti-Bcl-2 molecule Bim concomitant with the induction of apoptosis (Dijkers *et al.*, 2000b). Both Forkhead factors AFX and FKHRL1 also have been shown to induce transcription of the cyclin-dependent kinase inhibitor p27^{Kip1} (Dijkers *et al.*, 2000a). This allows for effectors of PI3K, via phosphorylation of Forkhead factors and inhibition of p27 gene transcription, to regulate cell proliferation in addition to cell survival. However, because we did not observe changes in cell cycle distribution in TGF β -protected cells, which would have been expected from down-regulation of p27, we did not pursue the role of this cyclin-dependent kinase inhibitor on TGF β -mediated enhanced survival.

The effect of TGF β on apoptosis has been investigated in different cell systems. In some cells, TGF β is a potent inducer of apoptosis (Selvakumaran *et al.*, 1994; Lømo *et al.*, 1995; Sánchez *et al.*, 1996), whereas in others it can effectively inhibit apoptosis (Sachsenmeier *et al.*, 1996; Chin *et al.*, 1999; Saile *et al.*, 1999; Huang *et al.*, 2000). In human keratinocytes, the apoptotic cell death induced by loss of anchorage is attenuated by both endogenous and exogenous TGF β (Sachsenmeier *et al.*, 1996). In this study, TGF β -neutralizing antibodies enhanced DNA fragmentation after cell suspension, indicating that endogenous TGF β , via autocrine signaling, may mediate the enhanced survival. The apoptosis precipitated by serum starvation in macrophages is also prevented by exogenous TGF β via mitogen-activated protein kinase (Chin *et al.*, 1999). In A549 lung adenocarcinoma cells, the antiapoptotic effect of TGF β requires modulation of JNK activity and phosphorylation of c-Jun (Huang *et al.*, 2000). In addition, TGF β was reported to act as a survival

factor to prevent c-Myc induced cell death in Rat-1 fibroblasts and this response was independent of any effect on cell cycle progression. Expression of dominant-negative forms of various components of the JNK signaling pathway, including Rac1, Cdc42, MKK4, and c-Jun abolished TGF β -induced survival (Mazars *et al.*, 2000). In our studies with NMuMG cells, the survival effect of TGF β was clearly dissociated from its antimutagenic effect (Figure 8). Although it is still conceivable that the same Smad-mediated transcriptional responses that induce epithelial cell cytoskeleton mediate the antiapoptosis effect of TGF β , a requirement of Smad signaling for the regulation of FKHRL1 and the prevention of cell death in epithelial cells requires further investigation. In a recent study, however, overexpression of Smad2 almost completely abrogated the JNK-dependent survival effect of TGF β (Mazars *et al.*, 2000), suggesting that Smad signaling was independent and antagonistic of the latter cellular response.

In summary, our results suggest that FKHRL1-dependent transcription may play a role in inducing apoptotic epithelial cell death and that TGF β partially reverses this effect by mechanism(s) involving Akt-dependent phosphorylation and nuclear exclusion of FKHRL1. We recently reported that PI3K function and its effector kinase Akt are required for TGF β -mediated fibroblastic transition and cell migration in epithelial cells (Bakin *et al.*, 2000), events involved in the metastatic progression of carcinomas. Transfection of dominant-negative T β RII constructs that disable autocrine TGF β have been shown to inhibit this mesenchymal transdifferentiation and reduce tumor cell invasiveness and metastases (Oft *et al.*, 1998; Portella *et al.*, 1998; Yin *et al.*, 1999; McEarchern *et al.*, 2001) suggesting that via subversion of an epithelial phenotype, autocrine/paracrine TGF β can contribute to the metastatic progression of epithelial cancers. Based on the data presented, we propose that down-regulation of Forkhead-dependent transcription and its subsequent positive effect on epithelial cell survival is an integral part of a multisignaling program by which TGF β contributes to epithelial transformation and tumor progression.

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