Nuclear expression of E2F4 induces cell death via multiple pathways in normal human intestinal epithelial crypt cells but not in colon cancer cells

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Garneau H, Alvarez L, Paquin M-C, Lussier C, Rancourt C, Tremblay É, Beaulieu J-F, Rivard N. Nuclear expression of E2F4 induces cell death via multiple pathways in normal human intestinal epithelial crypt cells but not in colon cancer cells. *Am J Physiol Gastrointest Liver Physiol* 293: G758–G772, 2007. First published July 26, 2007; doi:10.1152/ajpgi.00050.2007.—E2F transcription factors control cell cycle progression. The localization of E2F4 in intestinal epithelial cells is cell cycle dependent, being cytoplasmic in quiescent differentiated cells but nuclear in proliferative cells. However, whether nuclear translocation of E2F4 alone is sufficient to trigger intestinal epithelial cell proliferation remains to be established. Adenoviruses expressing fusion proteins between green fluorescent protein (GFP) and wild-type (wt)E2F4 or GFP and nuclear localization signal (NLS)-tagged E2F4 were used to infect normal human intestinal epithelial crypt cells (HIEC). In contrast to expression of wtE2F4, persistent expression of E2F4 into the nucleus of HIEC triggered phosphorylation of serine, cytoplasmic shrinkage, z-isoform, formation of apoptotic bodies, and activation of caspase 9 and caspase 3. Inhibition of caspase activities by zVAD-fmk partially inhibited cell death induced by E2F4-NLS. An induction of p53, phosphorylated Ser15-p53, PUMA, FAS, BAX, RIP, and phosphorylated JNK1 was also observed in HIEC expressing E2F4-NLS compared with wtE2F4-expressing cells. E2F1 and p14ARF expression remained unaltered. Downregulation of p53 expression by RNA interference attenuated cell death induced by E2F4-NLS. By contrast, the level of cell death was negligible in colon cancer cells despite the strong expression of E2F4 into the nucleus. In conclusion, deregulated nuclear E2F4 expression induces apoptosis via multiple pathways in normal intestinal epithelial crypt cells but not in colon cancer cells. Hence, mutations that deregulate E2F4 localization may provide an initial proliferative advantage but at the same time accelerate cell death. However, intestinal cells acquiring mutations (e.g., p53, Bax loci, etc.) may escape apoptosis, thereby revealing the full mitogenic potential of the E2F4 transcription factor.

E2F; intestinal epithelial crypt apoptosis; p53; colon cancer

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The majority of E2F signal transduction and transformation studies have been performed in immortalized rodent fibroblasts. Previous work has established that expression of SV40 large T antigen, which disrupts E2F-Rb complexes, leads to reentry of intestinal epithelial cells into the cell cycle and intestinal hyperplasia, indicating a role of the Rb pathway in regulating intestinal homeostasis (35). Our recent analysis of E2F4 and E2F1 expression in the intact intestinal epithelium and in cultured crypt cells suggests that nuclear E2F4 may be a determinant in the promotion of G1/S phase transition of human intestinal epithelial crypt cells. Indeed, double-staining experiments in vivo and in vitro revealed that crypt epithelial cells, which expressed high levels of nuclear E2F4, were all positive for Ki67. In addition, intestinal cell culture experiments demonstrated that serum growth factors promote nuclear translocation of E2F4. By contrast, whereas E2F1 remained in the nucleus, E2F4 protein was sequestered in the cytoplasm during G0 arrest associated with serum deprivation, confluence, and terminal differentiation of intestinal cells (17). These observations are in agreement with Dagnino et al. (14), who demonstrated that E2F4 is highly and preferentially expressed in the proliferative zone of the murine embryonic intestine. Moreover, the generation of knockout mice revealed an important role for E2F4 in establishing and/or maintaining the intestinal crypt compartment (64). Indeed, in E2F4−/− mice, the number of crypt regions was reduced or completely absent with the villi slimmer and shorter than in control (64).

Virtually all studies examining E2F signaling have utilized rodent immortalized or human tumor-derived cell lines harboring unknown genetic alterations. Hence, the present study was undertaken to analyze whether nuclear E2F4 activation alone is sufficient to trigger human intestinal cell proliferation and transformation, taking advantage of the generation of nonimmortalized human normal intestinal epithelial crypt cell cultures. The HIEC-6 human diploid cells, derived by Perreault and Beaulieu (55) from normal fetal (14–18 wk of gestation) intestinal tissue, have a finite lifetime and thus provide a new model system for investigating the role of nuclear E2F4 activation in intestinal crypt cell proliferation and transformation.

**MATERIALS AND METHODS**

**Materials and Antibodies**

The enhanced chemiluminescence (ECL) immunodetection system was obtained from Amersham-Pharmacia Biotech (Baie d’Urfé, QC, Canada). Antibodies for the detection of E2F4 (C-20), Fas (C-20), Bcl-2 (N-19), Bax (N-20), JNK1/2, PUMA, p53, cleaved caspase 3, and cleaved caspase 7 were from Cell Signaling (Beverly, MA). Antibodies for the detection of β-actin and integrin α5 were from Chemicon (Temecula, CA). Antibodies for the detection of FLIP were from Alexis Biochemicals (Lauzen, Switzerland). Antibodies for the detection of Bid, for phosphoSer15-p53, for caspase 3, and for phosphorylated JNK1/2 were from Cell Signaling (Piercing, ON, Canada). Antibodies for the detection of RIP and Bruce (Birc6, apollon) were from BD Biosciences (Mississauga, ON, Canada). Antibodies for the detection of peroxiredoxin 6 were from ABCam (Cambridge, MA). Antibodies for the detection of Apg4b were purchased from Abgent (San Diego, CA). Antibodies for the detection of caveolin 1 and Claudin 1 were from Zymed Laboratories (San Francisco, CA). Antibodies against PUMA and the broad spectrum caspase inhibitor, z-Val-Ala-Asp-(Ome)-CH2F (zVAD.fmk) were purchased from Calbiochem (Mississauga, ON, Canada). The purified mouse anti-human caspase 7 monoclonal antibody and the fluorogenic substrates of caspases 3, 8, and 9 were from Pharmingen (Mississauga, ON, Canada). Insulin was from Connaught Nova Laboratories (Willowdale, ON, Canada). The Vibrant Apoptosis Assay kit (number 6) was purchased from Invitrogen (Burlington, ON, Canada). All other materials were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless stated otherwise.

**Cell Culture**

Nonimmortalized human intestinal epithelial cells (HIEC for HIEC-6) were cultured as described previously (17, 55) in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 4 mM glutamine, 20 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin (all obtained from Invitrogen), 0.2 IU/ml insulin, and 5% fetal calf serum (FCS). These undifferentiated intestinal epithelial crypt cells block their proliferation upon reaching confluence but never initiate differentiation (16, 55). The colon carcinoma cell line HCT116 was obtained from ATCC (CCL-247) and cultured in McCoy’s medium containing 10% FCS. The colon adenocarcinoma cell line LoVo (CCL 229, ATCC) was cultured in Ham’s F-12 medium containing 20% FCS. The colon adenocarcinoma cell line DLD-1 was obtained from Dr. F. Boudreau (University of Sherbrooke, QC, Canada) and cultured in Ham’s F-12 medium containing 20% FCS.

**Plasmids, Adenoviral Constructs, and Infection**

The GFP, GFP-wtE2F4, and GFP-NLS-E2F4 [which contains NLS from SV40 large T antigen (MVPKKRRKVK)] adenoviral constructs were a generous gift from Dr. Paul Hamel (Department of Laboratory Medicine, University of Toronto, ON, Canada) and have been described and characterized previously (25). Briefly, the HA-tagged version of human E2F4 cDNA was first subcloned in frame with GFP in pEGFP-C1. For GFP-E2F4-4 fusion proteins containing the simian virus 40 (SV40) NLS, oligos encoding the SV40 large T antigen (Lgt) NLS (MVPKKRRKVK) was subcloned into the polylinker between GFP and the E2F4 cDNA maintaining the reading frame. These GFP-tagged recombinant cDNAs were excised from pEGFPC-1 with AgeI (5′ of the GFP tag) and HpaI (in the SV40 polyA tail region) and cloned into the AgeI and HpaI sites of pAdtrack-cytomegalovirus (25). Of note, GFP-tagged wtE2F4 localizes similarly to its endogenous counterpart in uninfected cells; in addition, GFP-wtE2F4 and GFP-NLS-E2F4 fusion proteins are able to complex the pRB family proteins and to bind DNA, indicating that all these additions to the E2F4 protein do not alter protein function. Viruses were amplified as described (27). The titer of virus stock was determined by plaque assay on 293 cells (ATCC; CRL-1573). HIEC cells were infected in 35-mm petri dishes at 70–80% confluence with 100 μl of DMEM, 2% FBS containing 1 × 10^5 PFU of corresponding viruses (MOI 10) for 1 h with agitation. Thereafter, virus-containing medium was removed and replaced by 2 ml of complete medium.

**Protein Expression and Immunoblotting**

Cells were lysed in SDS (sodium dodecyl sulfate) sample buffer [62.5 mM Tris·HCl (pH 6.8), 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Proteins from whole cell lysates were separated by SDS-PAGE in 7.5% or 10% gels. Proteins were detected immunologically following electrophoresis onto nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 1 h at 25°C in 5% milk in Tris buffered saline-Tween 20 (TBST) and incubated overnight with primary antibodies. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence was detected and captured using Kodak BioMax MR film. Membrane bands of interest were quantified by densitometry with the ImageQuantTL software (Amersham Biosciences).
PBS containing 5% powdered milk in PBS and then incubated for 2–4 h at 25°C with primary antibodies in blocking solution followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1,000) IgG (both from Sigma-Aldrich) in blocking solution for 1 h. The blots were visualized by the Amersham ECL system. Protein concentrations were measured using a modified Lowry procedure with bovine serum albumin (BSA) as standard [56].

**Growth Assays**

HIEC cells infected with AdGFP, AdE2F4, or AdE2F4-NLS were plated for growth assay in 12-well plates at a concentration of 15 × 10^3 cells/well. Cell growth was measured after 1–6 days with a cell growth assay kit with bovine serum albumin (BSA) as standard [56].

**Cell Viability Assays**

Viability of infected cells was monitored with Trypan blue exclusion. Floating cells from virus-infected cells were collected and attached cells were trypsinized, resuspended in DMEM-5% FCS, and pooled with the floating cells. The cells were centrifuged at 500 × g for 2 min and the pellets were resuspended in DMEM without serum. An aliquot of the suspension was mixed with Trypan blue (0.1% final concentration) and incubated for 5 min at room temperature. Cells were then counted with a hemocytometer. Viable cells were defined as those excluding the Trypan blue dye. More than 1,000 cells were counted for each variable.

**Flow Cytometry**

Serum-starved HIEC cells on 60-mm diameter culture plates were infected with the adenoviruses as described above. At 72 h postinfection, cells were trypsinized, combined with any floating cells, pelletted, washed with PBS, repelleted, and resuspended in 400 µl of PBS. All centrifugations were at 500 × g for 10 min at 4°C. Cells were subsequently fixed in cold methanol (70%, final concentration) and stored at −20°C for at least 1 h at 4°C. The fixed cells were centrifuged, washed twice with PBS, and resuspended in 0.5 ml of PBS containing propidium iodide (40 µg/ml) and RNase A (0.5 mg/ml). Samples were incubated for 30 min at 37°C prior to flow cytometry analysis with a Becton Dickinson FACScan.

**Annexin V Staining Assays**

The Vybrant Apoptosis Assay Kit was used. The kit contains a recombinant annexin V conjugated to biotin-X, as well as an Alexa Fluor 350 streptavidin conjugate for the secondary detection of biotin-X-annexin V. In addition, the kit includes a ready-to-use solution of the red-fluorescent propidium iodide nuclei acid-binding dye, which is impermeant to live cells and apoptotic cells, but stains necrotic cells and late apoptotic cells with red fluorescence, binding tightly to the nucleic acids in the cells. Briefly, infected cells were incubated with primary antibody (Annexin V: 1 mg/ml in PBS with 1.5% normal blocking serum) for 60 min, followed by a brief wash and incubation with the secondary Alexa Fluor 350 streptavidin conjugated antibody. Slides were mounted with coverslips using aqueous mounting medium, and each slide was examined with a fluorescence microscope with the appropriate filters.

**Electron Microscopy**

Cell cultures were rinsed with PBS, prefixed for 15 min with a 1:1 mixture of culture medium (DMEM), and freshly prepared 2.8% glutaraldehyde in cacodylate buffer (0.1 M cacodylate and 7.5% sucrose) and then fixed for 30 min with 2.8% glutaraldehyde at room temperature. After two rinses, specimens were postfixed for 60 min with 2% osmium tetroxide in cacodylate buffer. Samples were then dehydrated by using incremental ethanol concentrations (40, 70, 90, 95, 100%; three times each) and covered twice with 3 h with a thin layer of Araldite 502 resin (for ethanol substitution). Thereafter, the resin was allowed to polymerize at 60°C for 48 h. Thin sections were prepared with an LKB ultramicrotome, contrasted with lead citrate and uranyl acetate, and observed on a JEOL 100CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, ON).

**Labeling of Active Mitochondria**

MitoTracker red CMXRs (Molecular Probes, Invitrogen) was used to label active mitochondria. Cell preparation and staining were performed according to the Invitrogen protocol. This reduced probe does not fluoresce until it enters an actively respiring cell where it is oxidized to the corresponding fluorescent mitochondrial-selective probe and then sequestered into the mitochondria.

**Caspase Protease Assays**

Caspase activities were assayed as described by Pharmingen. Briefly, cells were harvested in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 20 mM MgCl2, 5 mM EGTA, 0.1 mM PMSF, 1 µg/ml leupeptin, 1 µM pepstatin A, 1% Triton X-100 for 15 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 5 min at 4°C. Proteins from lysates (30 µg) were incubated with 20 µM of the corresponding fluorogenic substrates for caspases 3, 8, and 9 for 2 h at 37°C. The AMC released from the substrates was measured with a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for caspase 3 and an excitation wavelength of 400 nm and an emission of 505 nm for caspases 8 and 9.

**RNA Extraction and Gene Expression Analysis**

Total RNA was isolated and processed by using the Totally RNA extraction kit (Ambion). Reverse-transcription PCR (RT-PCR) analysis was performed by using AMV-RT (Roche Diagnostics) according to the manufacturer.

**E2F1.** Quantitative real-time PCR was performed using the Quantitech SYBRgreen kit from Qiagen with the Roche Diagnostic Light Cycler. To determine conditions for quantitative analysis, cDNA samples were serially diluted and each primer pair was tested for exponential amplification. The relative levels of each RNA were calculated by the standard curve method and normalized to the corresponding TATA-binding protein RNA level. Primer sequences are available on request.

**FAF1.** Aliquots of the diluted cDNA preparations were then used as templates for PCR reactions with the following primers: FAF1 forward, ATG AGA CTC ATG GCT GCA ATG G; FAF1 backward, ACG CTT ACC AGG ACG TAT 30; FAF1 forward, AGA AAA GGG TTT CTT GAG GGA A. Reactions were performed using 2.5 units of Taq polymerase (Qiagen, Mississauga, ON, Canada). Parameters for DNA amplification were 94°C for 45 s, annealing temperature (50°C for 45 s, and 72°C for 60 s. Oligonucleotide primers used for DNA amplification were synthesized by Invitrogen. DNA amplification products were analyzed by gel electrophoresis on a polyacrylamide gel stained with ethidium bromide.

**Generation of shRNAs Against p53**

The lentiviral short hairpin RNA (shRNA) expression vector (pLent6-U6) was constructed by cloning the U6 promoter from...
pSilencer 2.0-U6 (Ambion) into pLentiv5/V5-D-TOPO (Invitrogen). Briefly, the U6 promoter was amplified by PCR from pSilencer 2.0-U6 using the forward primer cca tgc atg att acg aat tgc aac g (insertion of a ClaI site) and the reverse primer ggc cag tgc caa gct tg. The PCR product was digested by BamHI and ClaI and cloned into reconstituted pLentiv6-V5-D-TOPO between BamHI and ClaI sites that replaced the cytomegalovirus promoter. shRNA oligonucleotides were designed according to Ambion guidelines (technical bulletin no. 506) using the small interfering RNA sequences GCG CAC AGA GGA AGA GAA TCT (no. 1), GCC TGA CTC AGA CTG ACA TTC (no. 2), or GCT GGT GGG TTG GTA GTT TCT (no. 3) and TTCAAGAGA as the loop sequence. The oligonucleotide-anneled product was subcloned into the pLentiv6-U6 between BamHI and XhoI sites, giving rise to pLentiv6-sh(p53)1, pLentiv6-sh(p53)2, and pLentiv6-sh(p53)3. The control scrambled sequence was TAA GGA CTG ACG AAT CTG CAG AAT. Lentiviruses were produced and used for cell infection according to Invitrogen recommendations (ViraPower Lentiviral Expression System, instructions manual).

Immunofluorescence

HIEC cells and colon cancer cell lines grown on LabTecks were serum-deprived during 48 h. Thereafter, cells were washed twice with ice-cold PBS and subsequently fixed in paraformaldehyde (3%) for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with PBS-BSA 2% (15 min at room temperature). Cells were immunostained following an 1-h incubation with primary antibody for the detection of E2F4 followed by 30 min with FITC-conjugated secondary antibody. Negative controls (no primary antibody) were included in all experiments.

Isolation of Nuclear Proteins

The cells were washed twice with ice-cold PBS and resuspended in 1 ml buffer A (20 mM HEPES pH 7.9, 10 mM KC1, 0.1 mM EDTA, 1 mM DTT, and 1.5 mM MgCl2 with 1× cocktail protein inhibitor) and kept on ice for 30 min. 10% NP40 was added to a final concentration of 0.25% by gentle pipetting, and cells were incubated on ice for 5 min. The nuclei were pelleted at 12,000 g for 30 s and the supernatant (cytoplasmic proteins) was transferred into a new tube. The nuclei were washed once with 1 ml buffer A without NP40 and resuspended in 200 μl extraction buffer (20 mM HEPES pH 7.9, 10 mM KC1, 0.1 mM EDTA, 1 mM DTT, 1.5 mM MgCl2, 0.5 M NaCl, 25% glycerol with 1× cocktail protein inhibitor). The lysates were incubated on ice for 30 min and centrifuged at 12,000 g for 10 min, after which the nuclear protein was concentrated and stored at −80°C.

Analysis of Gene Expression by Microarray

RNA extraction, probe preparation, and hybridization and image processing were performed as recently reported by our group (77). cDNA microarrays were obtained from the University Health Network of Toronto (ON, Canada). A total of 12 slides (3 independent biological samples from each of the two cell models: HIEC-expressing GFP and HIEC-expressing E2F4-NLS; two slides per biological sample, comprised of two different slide sets, SS-H19k6 and SS-H11021) were used for the experiments. Arrays were scanned with a ScanArray Express dual-color confocal laser scanner (Perkin-Elmer). Data were collected in Cy3 and Cy5 channels and stored as paired TIFF images.

Data analysis. Spots were identified and local background subtracted using the TIGR Spotfinder 2.2 software (68). A quality control (QC) filter was used to remove questionable array features. Two criteria for spot rejection were a spot shape that deviated from a circle and a low signal-to-noise ratio. Hybridization intensity data were normalized using iterative mean-log2(ratio)-centering (data range for mean centering ± 3 SD) and Lowess procedures (smoothing parameter was set to 33%) using the native Java function of the TIGR MIDAS 4.0 software (Microarray Data Analysis System). Statistical significance was assessed by one-class t-test (P < 0.05) using the TMEV 3.0 software (TIGR MultiExperiment Viewer) (68). All softwares are available at The Institute for Genomic Research (TIGR) (website, http://www.TIGR.org).

Data Presentation and Statistical Analysis

Typical Western blots shown are representative of at least 3 independent experiments. Densitometric analyses were performed using the Scion Image 4.02 software (Scion, Frederick, MD). Cell viability assays were performed in duplicate or triplicate in three independent experiments and results were analyzed by the Student’s t-test and were considered statistically significant at P < 0.05. Representative results of in situ fluorescence from at least three independent experiments are shown. Caspase assays were performed in triplicate, in three independent experiments, and results were analyzed by the Student’s t-test and were considered statistically significant at P < 0.05.

RESULTS

Expression, Localization, and Impact of GFP, GFP-WTE2F4, and GFP-NLS-E2F4 on Infection With A Recombinant Adenovirus

The cellular localization of E2F4 in intestinal epithelial crypt cells is cell cycle dependent: although E2F4 is found in the nucleus of proliferative intestinal cells, it is mostly distributed in the cytoplasm of quiescent intestinal crypt cells and differentiated enterocytes (17). In light of these data, we first verified whether forced persistent nuclear translocation of E2F4 alone was sufficient to trigger intestinal epithelial cell proliferation. Although E2F1, E2F2, and E2F3 contain a nuclear localization signal (NLS), E2F4 and E2F5 on the other hand do not (62). However, cytoplasmic sequestering of E2F4 could be overridden using the strong NLS from SV40 LgT (31, 84). Hence, adenoviruses expressing fusion proteins between GFP (AdGFP) and wild-type E2F4 (AdGFP-wtE2F4) and between GFP and NLS-tagged E2F4 (AdGFP-NLS-E2F4) were used to infect HIEC. At 16 to 72 h after infection, GFP was mostly localized in the nucleus (Fig. 1A, I–4) whereas GFP-wtE2F4 staining was mostly cytoplasmic, although some nuclear staining was also observed in a few cells depending of their cell cycle (Fig. 1A, 5–8), hence reflecting immunostaining results of the endogenous protein in HIEC cells (17). The addition of NLS to GFP-tagged E2F4 resulted in their exclusive nuclear localization (Fig. 1A, 9–12). Phase-contrast microscopy reveals that GFP-wtE2F4 expressing cells were healthy, exhibiting an epithelial shape, whereas GFP-NLS-E2F4-expressing cells appeared more elongated after 48 h and were detached from the culture plates after 72 h (Fig. 1B). GFP, GFP-wtE2F4, and GFP-NLS-E2F4 proteins were detected as early as 3 h after infection (not shown), with increased expression at 16–72 h after infection (Fig. 1C). Equal protein loading for each lane was confirmed by using an anti-actin antibody.

Persistent Nuclear E2F4 Expression Inhibits Cell Proliferation and Induces Cell Death Associated With Apoptosis

To provide additional information regarding the possible long-term effects of nuclear expression of E2F4, the prolif-
cells were infected with adenoviruses expressing GFP, GFP-Trypan blue exclusion assay. Asynchronously growing HIEC led to a progressive reduction in cell number, previously demonstrated (17). By contrast, expression of GFP-NLS-E2F4 in HIEC led to a progressive reduction in cell number, whereas necrotic cells stain with both markers while healthy cells remain unstained. Forty-eight hours after viral infection, no staining with annexin V or propidium iodide was detected in GFP-expressing (data not shown) or wtE2F4-expressing cells (Fig. 2C, top). By contrast, numerous GFP-NLS-E2F4-expressing cells displayed bright staining with annexin V (Fig. 2C, bottom), but not with propidium iodide (L. Alvarez, unpublished observations) after 48 h, indicating that these cells were in the early stages of apoptosis.

Flow cytometry was subsequently used to measure DNA content in HIEC infected with AdGFP, AdGFP-wtE2F4, or AdGFP-NLS-E2F4. At 72 h after infection, cells infected with AdGFP-wtE2F4 exhibited a normal distribution into the cell cycle with 64% of cells in G0/G1, 15% of cells in S phase and 18% of cells in G2/M (Fig. 2D, top). By contrast, the profile of AdGFP-NLS-E2F4-infected cells had changed, defining a new population (31% of cells) with DNA content less than that of G1 cells (sub-G0 phase) (Fig. 2D, bottom), suggesting that these cells underwent cell death. Of note, FACS analysis revealed no difference between AdGFP-infected cells and AdGFP-wtE2F4-infected cells (not shown).

The red fluorescent Mitotracker dye was also used to label active mitochondria. This reduced probe does not fluoresce until it enters an actively respiring cell where it is oxidized to its corresponding fluorescent mitochondrion-selective probe and subsequently sequestered into the mitochondria. As shown in Fig. 2E (right), a marked reduction in labeling was observed in AdGFP-NLS-E2F4 infected cells compared with cells infected with AdGFP. Altogether, these results suggest that persistent nuclear E2F4 engaged in a programmed cell death (PCD) process associated with apoptosis.

**Forced Nuclear E2F4 Expression Induces Morphological and Cellular Changes Associated With Apoptosis**

As an extension to the above results, time-lapse microscopy was used to monitor the events associated with HIEC cell death (Fig. 3A). AdGFP-infected and AdGFP-wtE2F4-infected cells demonstrated an epithelial shape and cell-cell contacts typical of HIEC throughout the experiments (Fig. 3A, 1–7). In contrast, 60 h after infection by AdGFP-NLS-E2F4, many of the cells exhibited a thin and more elongated appearance (Fig. 3A, 8, arrows and arrows with double heads). Thereafter, cells expressing GFP-NLS-E2F4 still exhibited some cell-cell contacts that were progressively lost (Fig. 3A, 9–12, arrows). By 72 h postinfection, most cells had detached from the culture.
plates (Fig. 3A, 13). This cell death was accompanied by extensive plasma membrane blebbing characteristic of apoptotic cells (Fig. 3A, 10 and 11, arrowheads), although there were no observable traits such as fragmentation of the nucleus (Fig. 3A, 10–12, see asterisks), ruptured cells, or flocculent densities in organelles or cytosol that would signal necrosis. In addition, there was no evidence of nuclear lamina breakdown and apoptosis-associated internucleosomal DNA laddering could not be detected in GFP-NLS-E2F4-expressing cells (L. Alvarez, unpublished observations). Electron microscopy analysis revealed that infected cells showed no signs of altered ultrastructure after 36 h. Similar to AdGFP-infected and AdGFP-wtE2F4-infected cells (Fig. 3B, 1–2), AdGFP-NLS-E2F4-infected cells maintained their nuclear membrane integrity (Fig. 3B, 3). However, chromatin margination was consistently observed in GFP-NLS-E2F4-expressing cells (Fig. 3B, 3, arrows).

**Persistent Nuclear E2F4 Expression Induces Expression of p53 and Its Transcriptional Targets**

To gain further insight into the molecular mechanisms involved in GFP-NLS-E2F4-induced intestinal epithelial crypt cell apoptosis, an assessment of p53 protein expression levels was first performed since p53 has been shown to mediate the immediate apoptotic response to various forms of DNA damage in intestinal crypts, including ionizing radiation, alkylation agents, and 5FU (13, 61, 76). As shown in Fig. 4A, p53 expression levels were markedly enhanced in AdGFP-NLS-E2F4-infected cells compared with AdGFP-wtE2F4-infected cells, 24–72 h after infection. This prompted the verification of expression levels of the proapoptotic molecules Bax, PUMA, and FAS (73). Interestingly, the expression of all these p53 targets was significantly induced in AdGFP-NLS-E2F4-infected cells compared with AdGFP-wtE2F4-infected cells.
were examined by Q-PCR analysis. As shown in Fig. 4**C** through transactivation of the ARF promoter (15, 69). That E2F1 induces apoptosis by increasing expression levels of anti-actin antibody. Secondly, previous studies have shown that p53 through transactivation of the ARF promoter (15, 69). These phosphorylation events stabilize p53 by blocking Mdm2 binding. A phospho-specific antibody was therefore used herein to verify whether GFP-NLS-E2F4 expression induces a phosphorylation of p53 on Ser15. As shown in Fig. 4D, expression of GFP-NLS-E2F4 led to p53 accumulation and phosphorylation at Ser\(^{15}\). Expression of GFP-wtE2F4 (Fig. 4D) or GFP (not shown) did not induce any p53 accumulation or phosphorylation. Equal protein loading for each lane was confirmed by using an anti-actin antibody.

**p53 Knockdown Delays GFP-NLS-E2F4-Induced Cell Death**

In fibroblasts, the ability of E2F1 to induce apoptosis is mostly dependent on functional p53 (59). To further investigate the importance of p53 in E2F4-NLS-induced apoptosis of HIEC, recombinant lentiviruses encoding anti-p53 shRNA were developed to stably suppress p53 mRNA levels. Several lentiviral constructs were generated and tested for their ability to knock down p53 protein. Three of these viral shRNAs were selected and designated as sh(p53)1–3. HIEC were henceforth infected with sh(p53)1 lentiviruses. In addition, a shRNA with scrambled p53 RNA sequence was also generated leading to the production of an HIEC cell population stably expressing this shRNA as a control. The pLentiV5-U6 lentiviral vector, which coexpresses a blasticidin S resistance gene, allowed the selection of pure populations of transduced cells within 10 days. Cells infected with sh(p53)1 exhibited strongly reduced p53 protein synthesis, in contrast to cells transfected with shRNA targeting the scrambled sequence (H. Garneau, unpublished observations). Endogenous p53 protein expression levels were next analyzed in these populations following AdGFP-NLS-E2F4 infection. As shown in Fig. 5A, the scrambled shRNA did not alter the inducing effect of GFP-NLS-E2F4 expression on endogenous p53 protein levels. By contrast, induction of p53 levels by expression of GFP-NLS-E2F4 was markedly reduced by 80% in HIEC cells stably expressing sh(p53)1. Analysis of GFP-NLS-E2F4 protein expression confirmed the comparable efficiency of adenoviruses to infect HIEC expressing scrambled shRNA and HIEC-expressing sh(p53)1. Therefore, coinfection with an adenovirus and lentivirus does not result in decreased multiplicity of infection for the adenovirus. To determine whether p53 is essential for GFP-NLS-E2F4-induced HIEC apoptosis, the capability of GFP-NLS-E2F4 to induce cell death was verified in sh(p53)1-expressing HIEC cells and in sh(scrambled)-expressing HIEC cells. AdGFP-wtE2F4 infection was also performed as control. As illustrated in Fig. 5B, expression of GFP-NLS-E2F4, but not GFP-wtE2F4, in cells expressing the scrambled shRNA resulted in a significant loss in viability 48, 72, and 120 h after viral infection. By contrast, no significant cell death was observed 48 and 72 h after GFP-NLS-E2F4 expression in sh(p53)1-expressing cells. However, 120 h after infection, significant cell death was observed in sh(p53)1 cells infected by AdGFP-NLS-E2F4 although at a reduced level compared with sh(scrambled) cells infected by AdGFP-NLS-E2F4. Of note, Western blot experiments shown in Fig. 5A demonstrate that p53 expression levels remained strongly downregulated during the entire analysis period (5 days) in sh(p53)1-expressing HIEC cells.

Equal protein loading for each lane was confirmed by using an anti-actin antibody. Secondly, previous studies have shown that E2F1 induces apoptosis by increasing expression levels of p53 through transactivation of the ARF promoter (15, 69). Hence, the effects of GFP-NLS-E2F4 on E2F1 expression were examined by Q-PCR analysis. As shown in Fig. 4B, neither GFP nor GFP-NLS-E2F4 had any effect on the expression of E2F1 gene expression, indicating that GFP-NLS-E2F4 may induce p53 and apoptosis independently of E2F1. In addition, there was no modulation of p14ARF protein expression detected in GFP-NLS-E2F4-expressing cells (Fig. 4C).

Finally, E2F1 has recently been reported to utilize the ATM signaling pathway to induce p53 and apoptosis, independently of ARF, in normal human fibroblast cultures (60). Following irradiation, ATM and ATR directly phosphorylate p53 on Ser\(^{15}\) (73) and mediate the phosphorylation of additional residues through the activation of other kinases (3, 66). These phosphorylation events stabilize p53 by blocking Mdm2 binding. Western blots were probed with a phospho-Ser\(^{15}\) p53-specific antibody. As shown in Fig. 4D, expression of GFP-NLS-E2F4 led to p53 accumulation and phosphorylation at Ser\(^{15}\). Expression of GFP-wtE2F4 (Fig. 4D) or GFP (not shown) did not induce any p53 accumulation or phosphorylation. Equal protein loading for each lane was confirmed by using an anti-actin antibody.

**Fig. 3. Forced nuclear E2F4 expression induces morphological and cellular changes associated with apoptosis.** A: exponentially growing HIEC cells were infected with AdGFP, AdGFP-wtE2F4, or AdGFP-NLS-E2F4. Cells were monitored by videomicroscopy and images were acquired (for GFP fluorescence) 60 h after viral infection. The arrows indicate cell-cell contacts; arrows with double heads indicate the loss of epithelial morphology and the acquisition of fibroblastic morphology; arrowheads indicate membrane blebbing. See text for details. B: exponentially growing cells were infected with AdGFP, AdGFP-wtE2F4, or AdGFP-NLS-E2F4. After 36 h, cells were fixed in glutaraldehyde and osmium tetroxide prior to epoxy embedding for electron microscopy analysis. Arrows indicate chromatin margination. Bars: 500 nm.
ing cells infected by AdGFP-NLS-E2F4. Equal protein loading for each lane was confirmed by using an anti-actin antibody. After 5 days, all sh(p53)1 cells that expressed GFP-NLS-E2F4 had died, indicating that p53 knockdown markedly delays but does not prevent intestinal epithelial cell death induced by persistent nuclear E2F4 expression.

Fig. 5. p53 knockdown delays GFP-NLS-E2F4-induced apoptosis. A: HIEC were stably infected with lentiviruses encoding for a control short hairpin RNA (shRNA; scrambled sequence) or encoding a p53-specific shRNA as described under MATERIALS AND METHODS. These stable cell populations were thereafter infected with AdGFP-NLS-E2F4. At 24, 48, 72, and 120 h following viral infections, cells were lysed and proteins were analyzed by Western blotting for expression of p53, GFP-NLS-E2F4, and actin with specific antibodies. B: HIEC stably infected with lentiviruses encoding for a control shRNA (scrambled sequence) or encoding a p53-specific shRNA (no. 1) were infected with AdGFP-wtE2F4 or AdGFP-NLS-E2F4. At 48, 72, and 120 h following viral infections, viable cells were counted by trypan blue exclusion. *Significantly different from sh(scrambled)/GFP-wtE2F4 for the indicated times at P < 0.05, Student’s t-test. **Significantly different from sh(p53)/GFP-wtE2F4 for the indicated times at P < 0.05, Student’s t-test. **Significantly different from sh(scrambled)/GFP-NLS-E2F4 for the indicated times, at P < 0.05, Student’s t-test.
Persistent Nuclear E2F4 Overexpression Leads to the Activation of Caspases and Fas Signaling Pathway

To analyze the possible relationship between GFP-NLS-E2F4 expression and the molecular events regulating apoptosis, activities of caspases 3, 8, and 9 were measured by fluorogenic substrate assays. As shown in Fig. 6A, enhanced activities of caspases 3, 8, and 9 were observed, reaching significance 48–72 h after AdGFP-NLS-E2F4 infection with maximal activation for caspase 3 at 72 h. Western blot analyses confirmed the expression of the large fragments of activated caspase 3 (17/19 kDa) and caspase 7 (20 kDa) resulting from their cleavage by initiator activated caspases in cells expressing GFP-NLS-E2F4 but not GFP-wtE2F4 (Fig. 6B). The induction of Fas protein (Fig. 4A) and the significant activation of caspase 8 (Fig. 6A) prompted us to verify the implication of the FAS-dependent signaling pathway in GFP-NLS-E2F4-induced apoptosis. The apoptotic machinery components activated by this death receptor following expression of GFP-NLS-E2F4 were therefore characterized. As shown in Fig. 6C, GFP-NLS-E2F4-expressing cells had lower long FLICE-inhibitory protein (FLIP) expression and higher cleaved BCL-2 interacting domain (Bid) than GFP-expressing cells. The next step was to assess the possible involvement of RIP and JNK signaling in cell death induced by nuclear E2F4. In addition to their implication in Fas-induced apoptosis (42), RIP and JNK could play an essential role in reactive oxygen species-induced caspase-independent cell death (28, 33, 46). Moreover, we found a significant reduction in genes regulating intracellular redox environment in cells expressing GFP-NLS-E2F4 (Fig. 7A). As shown in Fig. 6C, GFP-NLS-E2F4-expressing cells exhibited enhanced RIP expression and JNK1 phosphorylation levels. All these data suggest that the proapoptotic signaling downstream of the Fas is activated in GFP-NLS-E2F4-expressing cells. The implication of Fas signaling pathway was ultimately confirmed by the use of Fas-ligand NOK1 neutralizing antibodies. As shown in Fig. 6D, treatments with either NOK1
antibodies or the broad-spectrum cell-permeable irreversible caspase inhibitors zVAD-fmk both prevented GFP-NLS-E2F4-induced apoptosis during the first 48 h. However, after 72 h, significant cell death was observed in presence of both inhibitors although at a reduced level compared with untreated cells. After 5 days, all GFP-NLS-E2F4-expressing cells had died despite the persistent inhibition of Fas-ligand or caspase activities during treatment (data not shown). To investigate whether p53, caspases, and Fas act in common or rather through parallel pathways, the effect of GFP-NLS-E2F4 expression was assessed in sh(p53)1 cells in which p53 protein expression was inhibited. The combination of sh(p53) with zVAD or NOK1 did not result in a better inhibition of cell death following GFP-NLS-E2F4 expression, suggesting that p53, Fas, and caspases reside in a common pathway to induce apoptosis in response to persistent nuclear E2F4 expression. Finally, these results also suggest that p53 and caspase activation are dispensable for final execution of intestinal epithelial cell apoptosis induced by nuclear E2F4.

**Expression of Genes Regulating Survival and Cell Death in GFP-NLS-E2F4-Expressing Cells**

To understand how GFP-NLS-E2F4 regulates apoptosis in intestinal epithelial cells, the pattern of gene expression in HIEC cells overexpressing GFP-NLS-E2F4 was analyzed by microarray. Results from microarrays comparing GFP-expressing cells with GFP-NLS-E2F4-expressing HIEC cells identified many potential targets of nuclear E2F4. A total of 335 genes were observed with at least a twofold difference in expression (data not shown). From these results, 17 genes have previously been implicated in survival or apoptosis. The differential expression of these genes was verified by Western blot and/or RT-PCR analysis (Fig. 7). These include genes directly involved in signal transduction (caveolin-1, Akt1, M-Ras, protein kinase Cδ), in cell adhesion (integrin α5, claudin-1), in stress response (Hsp90), in regulation of mitochondrial outer membrane permeabilization (Bcl-2, caspase 8), in actin organization (M-Ras, claudin-1, integrin α5), in the regulation of p53 (Mdm2, pCAF, Birc6), in autophagy (APG4B), in Fas signaling (caspase 8, FAF1), and in regulation of the intracellular redox environment (thioredoxin reductase 1, peroxiredoxin 6), and in the regulation of sister chromatid cohesion during mitosis (Rad21). Equal protein loading for each lane was confirmed by using an anti-actin antibody.

**Overexpression and Nuclear Localization of E2F4 in Colorectal Cancer Cells**

Our previous data demonstrated that nuclear E2F4 may be determinant in the promotion of cell cycle progression of human normal intestinal epithelial crypt cells. However, we demonstrate herein that persistent nuclear expression of E2F4 induces intestinal epithelial cell apoptosis. Therefore, E2F4 expression and localization were investigated in various colon adenocarcinoma and carcinoma cell lines, which either exhibit...
or do not exhibit mutations in p53 gene. These cell lines included HCT116 and LoVo cell lines (nonmutated p53 gene) as well as the DLD-1 cell line (mutated p53 gene). As shown in Fig. 8, A and B, colon cancer cells exhibited much higher levels of E2F4 protein than normal HIEC, with immunostaining mostly detected in the nucleus for colon cancer cells, despite the absence of exogenous serum growth factors. However, Western blot analysis on nuclear and cytoplasmic extracts revealed that E2F4 was overexpressed in both compartments of HCT116 and DLD-1 cells compared with exponentially growing HIEC. By contrast, in LoVo cells, the nuclear-to-cytoplasmic ratio of E2F4 expression was much more elevated (Fig. 8C). Interestingly, despite the strong expression of E2F4 into the nucleus, the level of cell death was negligible in colon cancer cells compared with normal HIEC cells expressing GFP-NLS-E2F4 (Fig. 8D and Fig. 2B). These results suggest that colon cancer cells have acquired resistance against cell death induced by persistent nuclear E2F4.

**DISCUSSION**

Intestinal tissue homeostasis is maintained by a tight balance between cellular proliferation and cell death processes such as apoptosis. We have previously reported that nuclear translocation of E2F4 may be determinant in the promotion of G1/S phase transition of human intestinal epithelial crypt cells. Indeed, the localization of E2F4 in intestinal epithelial cells is cell cycle dependent, being cytoplasmic in quiescent differentiated cells but nuclear in proliferative cells stimulated by growth factors (17). Herein, by using an adenoviral vector system to transfer the NLS-tagged E2F4 gene to intestinal epithelial crypt cells, we demonstrate that persistent nuclear E2F4 expression is not sufficient to trigger intestinal crypt cell proliferation. By contrast, persistent expression of E2F4 into the nucleus inhibits proliferation by inducing cell death.

A growing body of evidence indicates that oncogenes and growth factors can induce proliferation and promote cellular survival but when overexpressed in nonimmortalized cells can cause apoptosis and growth arrest (20, 72). This mechanistic duality appears to be true for E2F transcription factors. Early studies with E2F1 suggested that this protein can function as an oncogene when overexpressed because of the transactivation of a series of genes, which facilitate transition of the cell from G1 to S phase (18). On the other hand, in vivo experiments using homozygous E2F-1 null mice demonstrated increased cell proliferation and neoplasia in several tissues, thus supporting the putative tumor suppressor activity of E2F1 (21, 83). Furthermore, overexpression of E2F1 has been shown to induce apoptosis in several cell types, indicating that E2F1 plays a role not only in stimulating cellular proliferation but also in coordinating PCD (6). The ability of other E2F family members to induce apoptosis is less documented, aside from some evidences for E2F4 (9, 54, and this study).

Aptoptosis is vital for normal crypt homeostasis and its impairment may be an early event in the neoplastic process (49). Indeed, death of abnormal cells in the crypts is an important process for preventing clonal expansion and tumor formation. Herein, we demonstrate that persistent expression of E2F4 into the nucleus of human normal intestinal crypt cells triggers some typical features of classical apoptosis (42) including 1) phosphatidylserine exposure, 2) cytoplasmic shrinkage, 3) zoeisis, 4) formation of apoptotic bodies and 5) activation of initiator caspases 8 and 9 and executioner caspases 3 and 7. However, classical apoptosis is usually defined by stage II chromatin condensation with compact figures and internu-

![Fig. 8. E2F4 is overexpressed in colorectal cancer cells. A: subconfluent HIEC, HCT116, DLD-1, and LoVo cells were lysed and proteins were analyzed by Western blotting for total expression of E2F4 and actin with specific antibodies. B: HIEC and the colon cancer cells HCT116, DLD-1, and LoVo were serum deprived during 36 h and were thereafter fixed with 3% paraformaldehyde and permeabilized with a solution of 0.1% Triton X-100 before immunofluorescence staining for E2F4. Bars: 50 μm. C: nuclear (N) and cytoplasmic (C) extracts from exponentially growing HIEC, HCT116, DLD-1, and LoVo cells were prepared as described in MATERIALS AND METHODS. Protein expression levels of E2F4, lamin B (nuclear marker), and calpain 2 (cytoplasmic marker) were analyzed by Western blotting. D: exponentially growing HIEC cells were infected with AdGFP-wtE2F4 or AdGFP-NLS-E2F4 and viable cells were determined by Trypan blue exclusion 72 h later. Cell viability of exponentially growing HIEC, HCT116, DLD-1, and LoVo cells was also evaluated by Trypan blue exclusion, 72 h after seeding.](http://ajpgi.physiology.org/)
cleosomal DNA fragments (65). In the present instance, there was no evidence of nuclear lamina breakdown in GFP-NLS-E2F4-expressing cells. In addition, we could not detect the generation of internucleosomal DNA fragments or cleavage of PARP in cells expressing GFP-NLS-E2F4, suggesting a lack of nuclear caspase involvement since lamin, DNA, and PARP are subjected to caspase-mediated proteolysis during apoptosis (7). Nevertheless, we did observe chromatin margination in cells expressing NLS-E2F4 and FACS analysis revealed the presence of a population of cells with a DNA content inferior to that of G₁ cells. Leist and Ja¨a¨ttela ¨ (42) proposed a model which classifies death into four subclasses, according to their nuclear morphology. In this regard, there are many forms of “apoptosis-like PCD,” in which the chromatin condenses into less geometric shapes (so-called stage I chromatin condensation), phagocytosis is displayed before cell lysis and cytosolic hallmarks of apoptosis predominate (e.g., shrunken morphology, deformations of the plasma membrane, blebs). Apoptosis-like PCD is often caused by caspase-independent factors such as apoptosis-inducing factor (AIF), endonuclease G, cathepsins, or other proteases. Taken together, our results suggest that nuclear E2F4 expression triggers some typical features of classical apoptosis but also induces an apoptosis-like PCD in intestinal epithelial cells, as shown herein by 1) peripheral chromatin condensation; 2) observed translocation of AIF from a mitochondrial to a perinuclear location (H. Garneau, unpublished observations); 3) the demonstration of DNA loss, possibly caused by high molecular weight (50 kb) loss typically induced by AIF/endoG (50); and finally 4) the observed cell death in absence of caspase activation. Indeed, there is an array of well-characterized cell death models occurring in the absence of caspase activation which also fall into this category (7). For example, such suicide programs can be driven by the growth suppressor PML (63), the c-myc-interacting protein Bin1 (19), the protease cathepsin B (22), the Fas-binding protein Daxx (10), and the adenoviral death factor E4orf4 (41). Regardless of the biochemical pathways involved, study of the cytoplasmic apoptotic events (the extranuclear phase of apoptosis) has lagged, and it is still unclear how cell shape and apoptosis signaling are integrated.

We also observed that forced and persistent nuclear E2F4 expression induces p53 and some of its transcriptional targets PUMA, FAS, and Bax (39), which have been shown to be directly involved in execution of cell apoptosis. It has been widely demonstrated that p14ARF, a negative regulator of Mdm2, participates in the induction of p53 expression in response to deregulated expression of E2F1 (69), Myc (52), and E1A (47). However, we did not detect any variation of E2F1 and p14ARF expression levels in cells expressing NLS-E2F4, indicating the existence of an alternative mechanism for p53 activation in response to deregulated E2F4 activity. Accordingly, it has been recently demonstrated that E2F1 uses the ATM signaling pathway to induce p53, chk2 phosphorylation, and apoptosis in normal human fibroblasts (60). A variety of stress stimuli, including DNA damage, stimulate p53 activity by inducing phosphorylation of p53. In agreement with these results, we demonstrate herein that deregulated nuclear E2F4 expression stimulates phosphorylation of Ser15. However, how nuclear E2F4 activates ATM signaling pathway is unclear. A recent report suggests that it is not DNA damage per se but rather widespread chromatin remodeling that activates ATM (2). It is therefore possible that deregulated E2F4 results in aberrant DNA synthesis or in inducing large-scale chromatin remodeling as the mechanism of ATM activation, perhaps through several transcriptionally activating genes (3). In this regard, we found that HAT1 and PCAF, two transcriptional coactivators containing histone acetylase transferase activity (32), were both induced in cells expressing NLS-E2F4. Such a mechanism has been suggested for E2F1 (60). However, it has also been recently reported that overexpression of various oncogenes (c-myc, β-catenin, and HPV-18 E7) leads to the formation of phosphorylated H2AX foci, a common indicator for DNA damage (53).

Using RNA interference to specifically knockdown p53 expression, we were also able to demonstrate that persistent nuclear E2F4 expression induces HIEC apoptosis in both p53-dependent and -independent manners. p53-Dependent apoptosis involves several p53-regulated genes (Bax, Puma, Noxa) that localize to the mitochondria and promote the loss of mitochondrial membrane potential and cytochrome c release, resulting in the formation of the apoptosis complex with Apaf-1 and caspase 9. The subsequent activation of the caspase family of cysteine proteases results in cleavage of cellular substrate and production of the apoptotic phenotype (42). Another class of proapoptotic genes that can be regulated by p53, such as Fas (75) and c-FLIP (4), are components of the death receptor-mediated cell death pathway. In this instance, caspase activation (caspase 8) occurs at the plasma membrane following the clustering of death receptors that occur following their occupation by factors such as TRAIL or the Fas ligand. Herein, we demonstrate that deregulated E2F4 activity significantly modulated the expression and/or activity of many of these proteins (Fas, Puma, Bax, c-Flip, caspase 8) well known to be involved in p53-dependent apoptosis. Interestingly, inhibition of p53 (by RNA interference) or Fas signaling (by NOK neutralizing antibodies) or blockade of caspases (by zVAD) all prevented cell death observed after 48 h following nuclear expression of E2F4. Nevertheless, after 72 h and later, cell death was observed. Furthermore, the combination of shRNA against p53 with zVAD and NOK1 also delayed but did not result in a better inhibition of NLS-E2F4-induced cell death than that observed with the inhibition of individual components (unpublished observations), suggesting that p53, Fas, and caspases reside in a common pathway to induce cell death in response to nuclear E2F4 expression. Therefore, these results suggest that p53 and caspase activation are dispensable for the final execution of intestinal epithelial cell apoptosis induced by persistent nuclear E2F4 and that alternative mechanisms are also induced to mediate cell death in these cells.

Like classic apoptosis, alternative death programs can be induced by altered oxygen metabolism (7, 12, 22). Interestingly, expression of peroxiredoxin 6 and thioredoxin reductase, two constituents of the cellular antioxidant defense system (1, 38), was markedly reduced in cells expressing NLS-E2F4. Microarray results also pointed out other potential candidates that may be involved in induction of alternative death programs by deregulated nuclear E2F4 activity. For example, the strong downregulation of caveolin-1 in NLS-E2F4-expressing cells is quite relevant since it has been recently reported that loss of caveolin 1 causes hyperproliferation of intestinal crypt stem cells with increased sensitivity to whole body γ-radiation (45). Another potential and appealing candidate is FAF1 (Fas-asso-
iated factor 1), which was originally discovered as a member of the Fas death-inducing signaling complex (67) and as a suppressor of NF-kB activity (36). FAF1 has been shown to be involved in apoptosis induced by CK2 (26) and in protein degradation in the ubiquitin-proteasome pathway (71). Another candidate is PKCδ, which has been recently shown to be required for mitochondrial apoptosis induction by genotoxic stress in the absence of p53 (40). Finally, one of the highly induced genes observed in our microarray analysis was the hsp90 gene. Interestingly, Hsp90 controls the stability and function of a number of cell-signaling molecules including RIP (44). Hence, the marked induction of RIP protein expression observed in NLS-E2F4-expressing cells may be attributable to its reduced degradation due the induction of Hsp90 expression. Moreover, disruption of Hsp90 function reverts tumor necrosis factor-induced necrosis to apoptosis (79).

Despite the numerous attempts to characterize PCD induced by deregulated nuclear E2F4, exclusive models are difficult to establish and are probably artifactually owing to the overlap and shared signaling pathways between the various cell death programs. Thus we postulate that the dominant cell death phenotype is determined by the relative speed of the available cell death programs; although characteristics of several such death pathways can be brought forth, only the fastest and most effective pathway is usually evident. Herein, we demonstrate that deregulated nuclear E2F4 activity rapidly (in the first 48 h) triggers classic apoptosis-dependent pathways involving p53, Fas, and caspases. However, if p53, Fas signaling, or caspases are inhibited, slow cell death is still observed, indicating that alternative death routes are also induced following persistent nuclear E2F4 expression. The advantage of the existence of multiple death pathways is obvious: it protects the cell against transformation and carcinogenesis. In this regard, we were able to demonstrate that E2F4 is overexpressed in the nucleus of human colon cancer cells. Despite the persistent presence of E2F4 into the nucleus, a very low level of cell death was observed during culture of these colon cancer cell lines. These results are in agreement with those of Mady et al. (48) in which the expression of E2F4 was greater in colorectal tumor cells than in their corresponding benign epithelium. Aside from demonstrating E2F4 overexpression in colon cancer cells, our study reveals that, in contrast to normal cells (17), there is strong significant accumulation of E2F4 into the nucleus in colon cancer cells in absence of exogenous growth factors. Hence, one could speculate that the growth factor dependency of E2F4 nuclear localization is lost during intestinal cell transformation. Indeed, in the intact intestine, whereas E2F4 staining was generally weak in the nuclei and cytoplasm of the majority of crypt and villus cells, strong staining was observed in the nuclei of several proliferative cells of the crypt (17). Hence, these results suggest that this is not the high levels of nuclear E2F4 expression that cause cell death of human intestinal epithelial crypt cells, but this is its persistent localization into the nucleus that, in fact, induced PCD. Therefore, we speculate that mutations that deregulate E2F4 localization may provide an initial proliferative advantage but also accelerate cell death. Intestinal cells acquiring mutations in p53, Bax, APC, or other loci encoding survival/apoptosis-regulatory genes may escape apoptosis, thereby revealing the full mitogenic potential of the E2F4 transcription factor. Interestingly, E2F4 gene possesses a serine repeat trinucleotide (ACG)n, which is frequently mutated in various tumors including human sporadic colorectal cancers (29). These E2F4 mutations cause elevated transactivation of E2F consensus promoter sites and confer a growth advantage in rodent fibroblasts and HEK293 cells (74). It remains unknown, however, whether these genetic alterations affect E2F4 function in normal human intestinal crypt cells, especially with regard to the regulation of cell proliferation vs. cell survival. It may be reasonable to speculate that E2F4 mutants have lost the capacity to trigger apoptosis, thereby contributing to neoplastic progression in the intestinal epithelium. Experiments are currently ongoing in our laboratory to verify this hypothesis.

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