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p53 arrests growth and induces differentiation of v-Myb-transformed monoblasts

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Abstract The p53 protein can control cell cycle progression, programmed cell death, and differentiation of many cell types. Ectopic expression of p53 can resume capability of cell cycle arrest, differentiation, and apoptosis in various leukemic cell lines. In this work, we expressed human p53 protein in v-Myb-transformed chicken monoblasts. We found that even this protein possessing only 53% amino acid homology to its avian counterpart can significantly alter morphology and physiology of these cells causing the G2-phase cell cycle arrest and early monocytic differentiation. Our results document that the species-specific differences of the p53 molecules, promoters/enhancers, and co-factors in avian and human cells do not interfere with differentiation- and cell cycle arrest promoting capabilities of the p53 tumor suppressor even in the presence of functional v-Myb oncoprotein. The p53-induced differentiation and cell cycle arrest of v-Myb-transformed monoblasts are not associated with apoptosis suggesting that the

p53-driven pathways controlling apoptosis and differentiation/proliferation are independent.

Key words p53 · differentiation · cell cycle · apoptosis · monoblast

Introduction

The p53 protein plays a central role in the regulation of cell fate (Hussain and Harris, 1999). Its tumor-suppressing activity usually prevents expansion of potentially malignant cells by either induction of apoptosis, an arrest of the cell cycle or cell differentiation (Levine, 1997). Therefore, loss or mutation of p53 occurs frequently in carcinogenesis. p53 has been shown to participate in control of differentiation in a number of tissues such as pancreatic carcinoma cells, muscle cells, keratinocytes, neurons, and thyroid cells (Soddu et al., 1996; Almog and Rotter, 1997; Lang et al., 1998). Interestingly, although loss of p53 activity is less common in hematopoietic malignancies than in solid tumors (Shiohara et al., 1994), introduction or overexpression of p53 induces differentiation of leukemic L12-pre-B cells, erythroleukemic K562 cells, Friend virus transformed erythroleukemic cells, promyelocytic HL-60 cells, monoblastic U937 cells and myeloblastic M1/2 cells (Shaulsky et al., 1991; Feinstein et al., 1992; Johnson et al., 1993; Banerjee et al., 1995; Chylicki et al., 2000; Matas et al., 2004).

p53 is a transcription factor that can induce cell cycle exit or apoptosis through regulation of expression of its target genes such as *bax*, *bcl-2*, or *p21* (Miyashita et al., 1994; Ko and Prives, 1996). It appears that the involvement of p53 in cell differentiation is also mediated through its transcriptional activity, but the mechanism that underlies this effect is poorly understood (Matas et al., 2004). The *v-myb* oncogene of avian myeloblas-

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tosis virus (AMV) induces acute myeloblastic leukemia in chickens and transforms myelomonocytic cells *in vitro* (Baluda and Reddy, 1994). *v-myb* is derived from the *c-myb* protooncogene that controls proliferation, differentiation, and survival of various cell types in vertebrates (Ness, 2003). Therefore, deregulation of the Myb protein often results in tumorigenesis. The Myb proteins function as transcription factors. For example, to control cell cycle progression, the Myb protein can activate transcription of cyclin A1, *cdc2*, and *myc* genes (Ku et al., 1993; Muller et al., 1999; Schmidt et al., 2000); to control programmed cell death, Myb can activate transcription of *bcl-2* (Frampton et al., 1996; Taylor et al., 1996). The line of *v-myb*-transformed monoblasts BM2 that was generated from a culture of AMV-transformed primary bone marrow cells (Moscovici et al., 1982) has been repeatedly used to test tumor-suppressive effects of various genes (Šmarda et al., 1995, 1999; Sevcikova et al., 2002; Bryja et al., 2003; Zahradnicková et al., 2003; Šmardová et al., 2005). The aim of this study was to ectopically express human p53 in BM2 cells and evaluate its effects on proliferation, differentiation, and apoptosis. We demonstrate that p53 induces monocytic differentiation and the G2-phase cell cycle arrest, but not apoptosis of BM2 monoblasts implying independence of the p53-driven apoptotic- and differentiation/proliferation pathways. Our results also document that the human p53 protein retains its tumor-suppressing function in avian cells even in the presence of functional v-Myb oncoprotein.

Materials and methods

Plasmid construction

The cDNA coding for human p53 was excised from the plasmid pcmv-p53-neo-bam (cmvp53) (Baker et al., 1990) with *Bam*HI. The *Bam*HI-resistant fragment was end-filled and cloned into the plasmid pMT-IRES-CD4 (Šmarda and Lipsick, 1993) that had been linearized with *Xba*I and end-filled with Klenow enzyme to produce the plasmid pMT-p53-CD4. All restriction enzymes, T4 DNA ligase, and DNA polymerase were purchased from New England Biolabs (Beverly, MA).

Cell cultivation and transfection

BM2 cells (Moscovici et al., 1982) and their derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM) as described elsewhere (Šmardová et al., 2005). BM2p53 cells were prepared by stable transfection of BM2 cells with the pMT-p53-CD4 (3.5 µg) and pSV2Neo (0.5 µg) (Southern and Berg, 1982) plasmids by lipofection using Fugene 6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Forty-eight hours after the transfection, G418 (GIBCO BRL/Life Technologies, Gaithersburg, MD) was added to a final concentration of 500 µg/ml. The pool of G418-resistant clones was selected by negative and positive selection by anti-CD4-coated paramagnetic beads (Dyna, Oslo, Norway) in a magnetic field as described previously (Šmarda and Lipsick, 1993), and cloned by limiting dilution. BM2CD4 cells are derived from BM2 cells by transfection

with the empty vector backbone pMT-IRES-CD4 (Šmarda and Lipsick, 1993).

Transient transfection of BM2CD4 and BM2p53 cells was performed by electroporation using 10 µg of internal control plasmid cmv-βgal, 10 µg of either EW5luc reporter plasmid containing multiple Myb-binding sites from the *mim-1* gene (Fu and Lipsick, 1996), *pmdm2luc* plasmid containing p53-binding sites from the *mdm2* gene (Zauberger et al., 1993), or *baxluc* reporter plasmid containing p53-binding sites from the *bax* gene upstream of the cDNA coding for luciferase (Friedlander et al., 1996), in a volume of 450 µl as described elsewhere (Šmarda et al., 1999). The *pmdm2luc* and *baxluc* plasmids were kindly provided by Moshe Oren. Twelve hours after transfection, the medium was exchanged for standard cultivation medium and cultured for another 24 hr. Thirty-six hours after the electroporation, harvested cells were suspended in 1 ml of phosphate-buffered saline (PBS) and centrifuged (600 × g, 10 min), the pellet resuspended in 100 µl of 0.25 M Tris-Cl (pH 7.5), and lysed by three cycles of freezing and thawing. The lysates were assayed for luciferase and β-galactosidase activities. Relative light units were normalized for transfection efficiency using β-galactosidase activity as an internal control. For control of p53 expression and comparison of p53 protein level in either zinc-treated or electroporated BM2p53 cells, 6 hr after addition of zinc chloride or electroporation the cells were harvested, suspended in PBS, and centrifuged (600 × g, 10 min). The pellets were dissolved in sodium dodecyl sulfate (SDS)-loading buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Transient transfection of QT6 cells was performed by a modified calcium phosphate precipitation (Chen and Okayama, 1987). The exponentially growing cells were co-transfected with the plasmids coding for p53 (cmvp53) (1 µg), v-Myb (NdGE) (3 µg), and the control plasmid backbone NClα, equalizing the dose of DNA in transfection mixtures to 4 µg (Baker et al., 1990; Grasser et al., 1991; Dini and Lipsick, 1993). In addition, the transfection mixture included either the *baxluc* reporter plasmid (1 µg) (Friedlander et al., 1996) or EW5luc reporter plasmid (Fu and Lipsick, 1996), and the internal control plasmid cmv-βgal (1 µg). Twelve hours later, the medium was exchanged and 24 hr after the transfection the cells were washed with PBS, suspended in 100 µl of 0.25 M Tris-Cl (pH 7.5), and lysed by three cycles of freezing and thawing. The lysates were assayed for luciferase and β-galactosidase activities. Relative light units were normalized for transfection efficiency using β-galactosidase activity as an internal control (Sambrook et al., 1989).

Gel electrophoresis and immunoblotting

The same number of BM2CD4 cells and their derivatives was lysed in SDS-loading buffer containing 0.1 M Tris (pH 6.8), 16% v/v glycerol, 3.2% w/v SDS, 10% v/v β-mercaptoethanol, and 0.005% w/v bromophenol blue. Then, the lysates were boiled for 5 min, the proteins were resolved by 10% SDS-PAGE and electroblotted to Immobilon-P membrane (Millipore Corporation, Billerica, MA). The blots were probed with anti-p53 monoclonal antibody Do-1, kindly provided by Vojtesek et al. (1992), polyclonal anti-actin antibody (A5060, Sigma-Aldrich, Prague, Czech Republic), anti-GAS41 antibody (ab15859, Abcam, Cambridge, U.K.), or a mixture of the Myb-specific monoclonal antibodies 4E3-E11, 5E11-E11, 10A3-F11 kindly provided by Sleeman (1993). The blots were developed with either goat anti-rabbit (for anti-actin), goat anti-mouse (for anti-Myb, anti-p53) secondary antibody (1:15,000) conjugated to alkaline phosphatase, or anti-chicken (for anti-GAS41) secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, 1:15,000).

Cyto centrifugation

BM2CD4 and BM2p53 cells were treated with ZnCl₂ (1.2 × 10⁻⁴ M) for 72 hr, washed with PBS, and spread on glass

slides by cytocentrifugation ($500 \times g/7$ min). The cells were then fixed and stained using a Diff-Quik (DADE AG, Dürdingen, Switzerland).

Proliferation, viability, and cell cycle analyses

To assess growth of BM2CD4, BM2p53 pool, and BM2p53 A4 cells, a total of 1×10^6 cells was treated with $ZnCl_2$ (1.2×10^{-4} M) for 4 days. Viable cells were enumerated daily by eosin dye exclusion using a hemocytometer. For cell cycle analysis, the cells were cultured in the presence of $ZnCl_2$ for 48 hr, then washed twice with PBS, resuspended in 0.5 ml PBS, fixed in 4 ml of 70% ethanol, and stored at $4^\circ C$ for 24 hr. Fixed cells were centrifuged, washed with PBS, and stained in 0.5 ml of Vindelov solution (Vindelov, 1977) for 30 min at $37^\circ C$. DNA content in at least 1.5×10^4 cells was measured by a Fluorescence Activated Cell Sorter (FACS) Calibur™ system using 488 nm argon laser for excitation (Becton Dickinson, San Jose, CA). Frequency of the cells in distinct phases of the cell cycle was determined using ModFit 3.0 software (Verity Software House, Topsham, ME).

Annexin V assay

To assess apoptosis, we tested the presence of externalized phosphatidylserine in BM2CD4, BM2p53 pool, and BM2p53 A4 cells as described (Verhove et al., 1995). Briefly, 1×10^6 cells were treated with $ZnCl_2$ (1.2×10^{-4} M) for either 6 or 48 hr. 5×10^5 cells were washed with the Annexin buffer (10 mM HEPES, 140 mM NaCl, 10 mM $CaCl_2$, pH 7.4), resuspended in 100 μ l of the same buffer containing 0.5 μ l of Annexin-V-Fluos (Roche Diagnostics) and 4.5 μ g of propidium iodide, and incubated for 5 min at $37^\circ C$ in the dark. FITC fluorescence was determined by FACS Calibur™ system using 488 nm argon laser for excitation (Becton Dickinson). At least 1×10^4 cells were analyzed in each sample. As a positive control, BM2CD4 cells were treated with cycloheximide (0.5 μ g/ml) for 6 hr.

Detection of apoptotic bodies

A total of 2×10^6 cells were treated with zinc chloride (1.2×10^{-4} M) for 6, 12, 24, and 48 hr. At indicated intervals, 2×10^6 cells were washed with PBS, pelleted by centrifugation ($200 \times g/5$ min), suspended in 500 μ l PBS, fixed in 5 ml of the methanol:acetic acid (3:1) fixative, and stored at $-20^\circ C$ overnight. Then, the cells were pelleted by centrifugation ($200 \times g/8$ min), and resuspended in 100 μ l of the methanol:acetic acid fixative solution. Twenty microliters of the cell suspension was dropped on a slide, allowed to get dry, and stained with 10 μ l of propidium iodide (10 μ g/ml). Apoptotic bodies were enumerated by visual inspection of at least 200 cells by fluorescence microscopy. As a positive control, BM2CD4 cells were treated with cycloheximide (0.5 μ g/ml) for 6 hr.

DNA fragmentation

Genomic DNA was isolated from both floating and attached cells using Invisorb Apoptosis Detection Kit II (Invitex GmbH, Berlin-Buch, Germany) as described by the manufacturer. Fifty microliter samples of the eluted DNA were separated by 1.2% agarose gel electrophoresis. The gel was examined and photographed with an ultraviolet gel documentation system.

Nonspecific esterase assay

BM2CD4, BM2p53 pool, and BM2p53 A4 cells (5×10^5) were treated with $ZnCl_2$ for 48 hr, pelleted by centrifugation ($200 \times g/$

5 min) and washed twice with HEPES/NaCl buffer (150 mM NaCl, 25 mM HEPES, pH 7.6). The cells were resuspended in 750 μ l HEPES/NaCl buffer containing 0.2 mg α -naphthyl acetate (α -NA) and incubated at $37^\circ C$ for 30 min. The reaction was terminated by 500 μ l SDS buffer (4% SDS, 10 mM sodium-barbital, 100 mM sodium acetate, pH 8.0) with 0.2% Fast Blue BB salt. The samples were incubated at laboratory temperature for 10 min. The absorbance was measured by spectrophotometry at 492 and 620 nm wavelengths (Sonne et al., 1991).

Nitro-blue-tetrazolium (NBT) assay

The same number of viable BM2CD4, BM2p53 pool, and BM2p53 A4 cells (1×10^6) was treated with $ZnCl_2$ for 48 hr and centrifuged ($200 \times g/5$ min). The pellets were suspended in 400 μ l pre-warmed DMEM ($37^\circ C$) without sera and 200 μ l NBT/TPA/PBS solution (1 mg/ml NBT, 200 ng/ml TPA in PBS) was added. The samples were incubated at $37^\circ C$ in humidified 10% CO_2 atmosphere for 1 hr. The cells were then lysed by 2.5% Triton \times 100 at laboratory temperature for 20–30 min. The absorbance was assessed by spectrophotometry at wavelengths 570 and 620 nm (Baehner et al., 1975).

Chemiluminescence assay

Production of reactive oxygen species was assessed by luminol-enhanced chemiluminescence using an LM-01 T Luminometer (Immunotech, Prague, Czech Republic) (Pavelkova and Kubala, 2004). The same number of BM2CD4 and BM2p53 cells was treated with $ZnCl_2$ for 48 hr. Each sample containing 1.5×10^5 cells in 150 μ l of PBS was mixed with 50 μ l of 10 mM luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione), and either 50 μ l of opsonized zymosan (2.5 mg/ml) or 50 μ l of TPA (1 μ g/ml). Hank's balanced salt solution (HBSS, pH 7.4) was added to the final volume of 250 μ l. All chemicals were purchased from Sigma-Aldrich. Each sample was measured in duplicate. The kinetics of chemiluminescence (i.e., the oxidative burst of cells) were measured for a period of 60 min, recorded values included the time and intensity of chemiluminescence expressed as relative light units (Lilius and Waris, 1984). Spontaneous chemiluminescence in the absence of any activator was also controlled in each sample. All solutions were free of phagocyte-activating or inhibiting activity.

Determination of changes of intracellular pH (pHi)

BM2CD4 and BM2p53 cells (5×10^5) were treated with $ZnCl_2$ for 48 hr. Then, the cells were pelleted by centrifugation ($200 \times g/5$ min), resuspended in 0.5 ml PBS, and stained with 2.5 μ M carboxy SNARF-1 AM (SemiNaphthoRhodaFluor-1 acetoxymethyl ester; Molecular Probes, Eugene, OR) at $37^\circ C$ in humidified 5% CO_2 atmosphere for 30 min. The stained cells were washed, resuspended in 0.5 ml PBS, and analyzed by flow cytometry using 488 nm argon laser for excitation (FACS Calibur™, Becton Dickinson). The single parameter histograms of SNARF-1 fluorescence at 580 and 640 nm wavelengths were gated according to the forward light-scatter versus fluorescence at 580 nm wavelength to exclude both the cells with too low SNARF-1 fluorescence and the cells with fluorescence in the overflow (uppermost) channel. The changes of the pHi values were calculated from the ratio of fluorescence emission at the 640 and 580 nm wavelengths using the off-line software FCS Assistant 1.1a. Increasing pHi is indicated by increase of the SNARF-1 emission at 640 nm and decrease at 580 nm. To determine the changes of pHi using the SNARF-1 probe, we used a calibration curve that was constructed by incubating the SNARF-1-loaded cells in high-potassium calibration buffers in a pH ranging from 6.4 to 7.4, in the presence of the K^+ ionophore nigericin (Boyer and Hedley, 1994).

Indirect immunofluorescence analysis

To assess the relative amount of the cell surface CD11b antigen, BM2CD4 and BM2p53 cells (5×10^5) treated with $ZnCl_2$ for 48 hr were centrifuged ($200 \times g/5$ min) and washed with PBS. The cells were incubated with undiluted Mo-1-specific monoclonal antibody supernatant (30 μ l) (Liu et al., 1992) on ice for 30 min. Then, 170 μ l of ice-cold PBSA (1% bovine serum albumin in PBS) was added and the cells were centrifuged again ($400 \times g/5$ min). The primary antibody was washed out with another 200 μ l of PBSA and following centrifugation, the cells were resuspended in FITC-conjugated goat anti-mouse IgG (50 μ l) (Sigma-Aldrich, dilution 1:100), and kept light-protected on ice for 30 min. Then, the cells were washed twice with PBSA, suspended in 500 μ l of PBSA and relative fluorescence was determined by FACS Calibur™ system using 488 nm argon laser for excitation (Becton Dickinson). At least 2×10^4 cells were analyzed in each sample. Frequency of CD11b-positive versus CD11b-negative cells was determined by the CellQuest Pro software (Becton Dickinson). As a negative control, the same samples were processed in the absence of primary antibody.

Results

Inducible expression of human p53 in avian cells

In order to express the human p53 protein in avian cells, we cotransfected AMV-transformed chicken monoblasts BM2 (Moscovici et al., 1982) with the plasmids pMT-p53-CD4 and pSV2Neo, and selected a pool and 16 independent clones of G418-resistant BM2p53 cells. Inducible p53 expression from the metallothionein (MT) promoter in BM2p53 cells treated with zinc chloride was verified by SDS-PAGE and immunoblotting. The p53 protein was clearly produced in a zinc-dependent manner in the BM2p53 pool as well as the individual clones (Fig. 1). The same procedure was used to isolate a control cell line BM2CD4, which had integrated the pMT-IRES-CD4 plasmid vector and synthesized CD4 but not p53 protein (Šmarda and Lipsick, 1993). These data demonstrate that human p53 was successfully expressed in v-Myb-transformed avian cells.

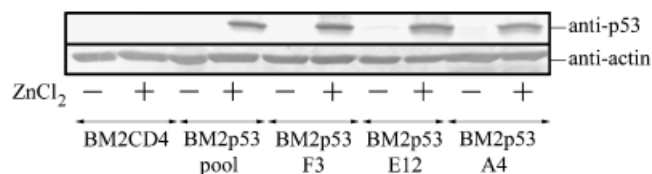


Fig. 1 Expression of human p53 in BM2 monoblasts. BM2CD4 and BM2p53 cells (pooled clones of G418-resistant BM2p53 cells and three independent clones) were treated with $ZnCl_2$ for 6 hr. Protein extracts from harvested cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using the p53-specific- (upper box) and actin-specific antibody as a loading control (lower box).

Human p53 protein activates transcription from the *mdm-2* gene promoter in avian cells

In order to determine whether the human p53 protein retains its transcriptional activation function in avian cells, we transiently transfected BM2CD4 and BM2p53 cells with either *pmdmluc* reporter plasmid containing the p53-binding site of the *mdm-2* gene or *baxluc* reporter plasmid containing p53-binding site of the *bax* gene promoter placed upstream of the luciferase cDNA (Zauberman et al., 1993; Friedlander et al., 1996). As expression of MTs is up-regulated in stressed cells, we used electroporation to both perform transfection, and to induce expression of p53 from the MT promoter in transfected cells. The level of p53 production in electroporated BM2p53 cells was controlled by SDS-PAGE and immunoblotting (Fig. 2). The luciferase activity found in extracts of the p53-expressing BM2p53 cells transfected with the *pmdmluc* plasmid was significantly higher than in control BM2CD4 cells or in the BM2p53 cells transfected with the *baxluc* reporter plasmid (Fig. 2). This suggests that human p53 can transactivate the *mdm-2*- but not the *bax* gene expression in avian BM2p53 cells.

BM2 as well as BM2p53 cells constitutively express v-*myb*. In order to determine whether the v-Myb oncoprotein can suppress transactivation of the *bax* gene by human p53, we transiently transfected avian QT6 cells with the *baxluc* reporter plasmid, the expression plasmid coding for p53 (cmvp53), the v-Myb-coding plasmid (NdGE), or the *myb*-less control plasmid (NCl α) and measured luciferase activity in cell extracts. We found that the v-Myb protein did not affect p53-driven transactivation of the *bax* gene promoter (Fig. 3, left panel), although it was clearly expressed and capable of transactivating the luciferase reporter gene from the promoter equipped with multiple Myb-binding sites in the plasmid *Ew5luc* (Fig. 3, right panel). This suggests that the v-Myb protein does not interfere with *bax* transactivation by p53 in avian cells.

p53 arrests growth of BM2 cells in G₂-phase of the cell cycle

In order to assess the effects of p53 on growth of BM2 cells, we first compared the growth rates of BM2CD4 and BM2p53 cells. The same number of BM2CD4 and BM2p53 cells (both the A4 clone and pooled clones) was treated with zinc chloride or left untreated for 4 days and number of viable cells was counted daily. Proliferation rates of untreated BM2CD4 and BM2p53 cells were similar (Fig. 4A). However, when exposed to inducer, BM2CD4 and BM2p53 cells behaved differently. While the zinc-treated BM2CD4 cells grew at a similar rate as untreated controls, the growth of BM2p53 cells was efficiently arrested. This was

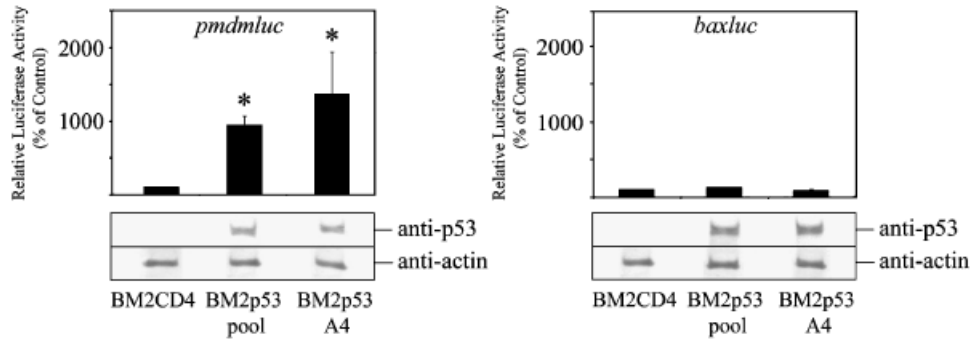


Fig. 2 p53 transactivates *mdm-2* but not the *bax* gene reporter expression in avian BM2p53 cells. BM2CD4 cells, BM2p53 cells of pooled clones, and the clone A4 were transiently transfected with either *mdmluc* or *baxluc* reporter plasmid and *cmv-βgal* internal control plasmid by electroporation and cultured for 36 hr. Luciferase and β-galactosidase activities in harvested cells were analyzed as described in Materials and methods. Data show the mean values of relative luciferase activity (as percentage of BM2CD4 control) normalized according to the transfection efficiency from three

independent experiments. Error bars indicate standard deviations. *Significant difference ($p < 0.05$) from BM2CD4 cells as determined by Student's *t*-test. The level of the p53 protein in electroporated cells was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting using the p53-specific antibody Do-1 (upper boxes). To control for sample loading, the same blots were probed with an actin-specific antibody (lower boxes).

observed in all independent BM2p53 clones that we tested. To determine the cytotoxicity of overexpressed p53 protein, we measured the viability of BM2p53 cells treated with zinc chloride for 1, 2, 3, and 4 days by eosin dye exclusion. Although viability of the p53-expressing BM2p53 cells decreased by 20% during 4 days of cultivation in the presence of zinc chloride due to cell death (Fig. 4B), the cytotoxicity of the p53 protein was not sufficient to explain the low number of BM2p53 cells detected in Figure 4A. Therefore, we tested the effects of the p53 protein on cell cycle of BM2p53 cells. BM2CD4 and pooled clones of BM2p53 cells were

treated with zinc chloride or left untreated for 48 hr. Then, the cells were fixed, stained with propidium iodide and DNA content in individual cells was determined by flow cytometry. The frequency of S-phase cells decreased from 22.3% in untreated BM2p53 cells and 29.7% in zinc-treated BM2CD4 cells to 6.7% in zinc-treated BM2p53 cells with a compensatory accumulation of cells in G2/M (Fig. 4C). Frequency of the cells in G2/M-phase of the cell cycle increased from 12.7% in untreated BM2p53 cells and 13.3% in zinc-treated BM2CD4 cells to 25.0% in zinc-treated BM2p53 cells (Fig. 4C). Microscopic enumeration of mitoses revealed

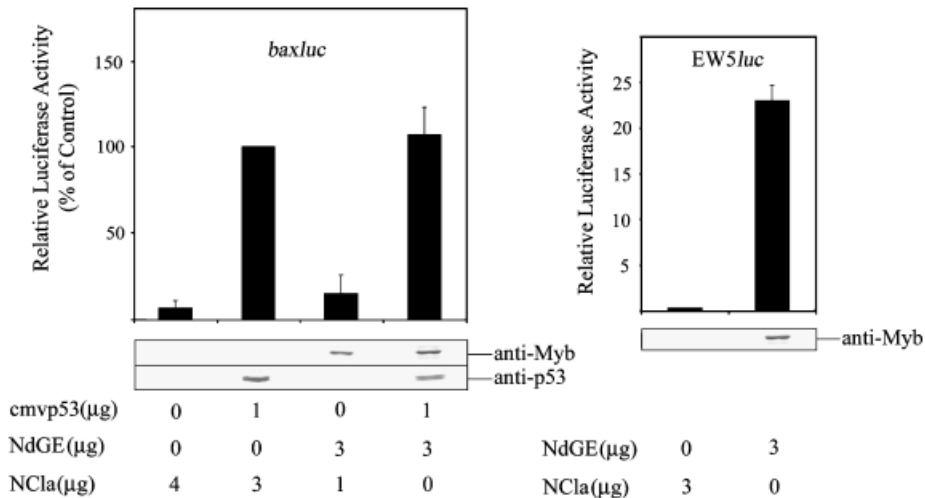


Fig. 3 v-Myb does not inhibit transactivation from the *bax* gene promoter by p53. QT6 cells were transiently transfected with p53- (*cmvp53*), v-Myb (*NdGE*) expression plasmids, and the *baxluc* reporter plasmid and cultured for 36 hr. The *myb*-less plasmid backbone *NClA* was used to equilibrate the total amount of transfected plasmid DNA. Mean relative luciferase activity present in cell extracts, which was normalized according to the transfection efficiency, is indicated as percentage of activity found in *cmvp53*-transfected cells from at least three independent experiments

(left panel). The v-Myb transactivation rate in similarly transfected cells was tested using the *EW5luc* reporter plasmid. Data show the mean values of relative luciferase activity in arbitrary light units from at least three independent experiments (right panel). Error bars indicate standard deviations. The levels of p53 and v-Myb in transfected cells were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting using the p53- and Myb-specific antibodies.

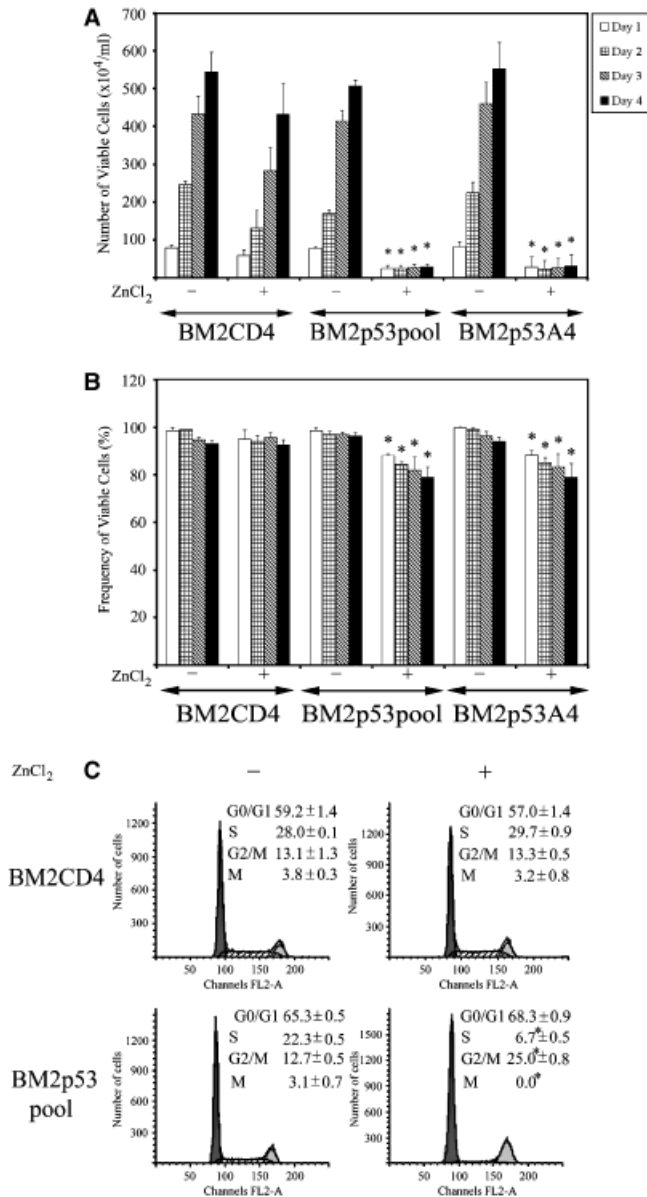


Fig. 4 p53 affects proliferation, viability, and cell cycle progression of BM2p53 cells. BM2CD4, BM2p53pool, and BM2p53A4 cells were treated (+) or untreated (-) with ZnCl₂ for 4 days. The bars indicate total number of viable cells determined daily by eosin dye exclusion using hemocytometer (A). Frequency of viable cells was also determined by eosin dye exclusion using hemocytometer (B). Cell cycle analysis was performed in BM2CD4 and BM2p53 pool cells treated with ZnCl₂ (+) or left untreated (-) for 2 days (C). The cells were fixed and stained with propidium iodide in the presence of RNase. To determine frequency of cells in G0/G1, S, and G2/M phases of the cell cycle, the DNA content of at least 15,000 individual cells was determined by Fluorescence Activated Cell Sorter. Cells in M phase were enumerated by visual microscopic inspection of at least 400 cytocentrifuged and stained cells. Numbers represent the frequency of cells in the phases of the cell cycle (%) \pm standard deviations. Data obtained from three independent experiments were processed using Student's *t*-test. *Significant difference ($p < 0.05$) from BM2CD4 cells and untreated BM2p53 cells.

that p53 reduced the frequency of cells in M-phase from about 3% in zinc-treated BM2CD4 cells to 0% in similarly treated BM2p53 cells. Therefore, all of the p53-expressing BM2p53 cells detected in the G2/M window by FACS analysis were in fact in G2. These results suggest that the p53 protein induces cell death and arrests growth of BM2p53 cells in the G2 phase of the cell cycle.

p53 does not induce apoptosis of BM2p53 cells

In addition to cell cycle progression, p53 can also control programmed cell death. In order to test whether human p53 can induce apoptosis of BM2p53 cells, we exposed them to zinc inducer for 6 hr. Then, we measured exposure of phosphatidylserine (PS) on the cell surface by flow cytometry using Annexin V assay. The Annexin V staining measures the presence of an externalized PS, a phospholipid normally found in the inner leaflet of the plasma membrane, and externalized during the apoptotic process. Frequency of Annexin V positivity in population of zinc-treated BM2p53 cells was the same as in zinc-treated BM2CD4 cells (1.3%), and no significant difference was also found in BM2CD4 (2.3%), and BM2p53 cells (0.9%) cultured in the absence of zinc inducer (Fig. 5). In contrast, frequency of Annexin V-positive BM2CD4 cells increased to 10.6% upon treatment with apoptosis-inducing cycloheximide and this increase was statistically significant. Similar low frequency of apoptosis was detected also in BM2CD4 and BM2p53 cells treated and untreated with zinc chloride for 48 hr (not shown). These results document that p53-expressing BM2p53 cells do not expose PS on the cell surface. We also failed to detect the other hallmarks of apoptosis, such as formation of apoptotic bodies and DNA fragmentation in p53-expressing BM2p53 cells, although both these features were clearly detectable in cycloheximide-treated positive controls (Fig. 6). These results document that p53 expression is not sufficient to initiate apoptosis of BM2p53 cells. The lack of p53-induced apoptosis correlates with the low capacity of the p53 protein to activate transcription from the pro-apoptotic *bax* gene promoter in BM2p53 cells (Fig. 2).

p53 induces differentiation of BM2p53 cells

In order to further address the effects of p53 on BM2 cells, we examined the morphology and physiology of BM2p53 cells. First, the morphology of BM2CD4 and BM2p53 cells treated with zinc chloride for 72 hr was analyzed by phase-contrast microscopy. At the same time, the cytocentrifuged, fixed, and stained cells were examined by light microscopy. The size of zinc-treated BM2p53 cells increased, the cells lost regular spheric shape of monoblasts and partially developed adgency

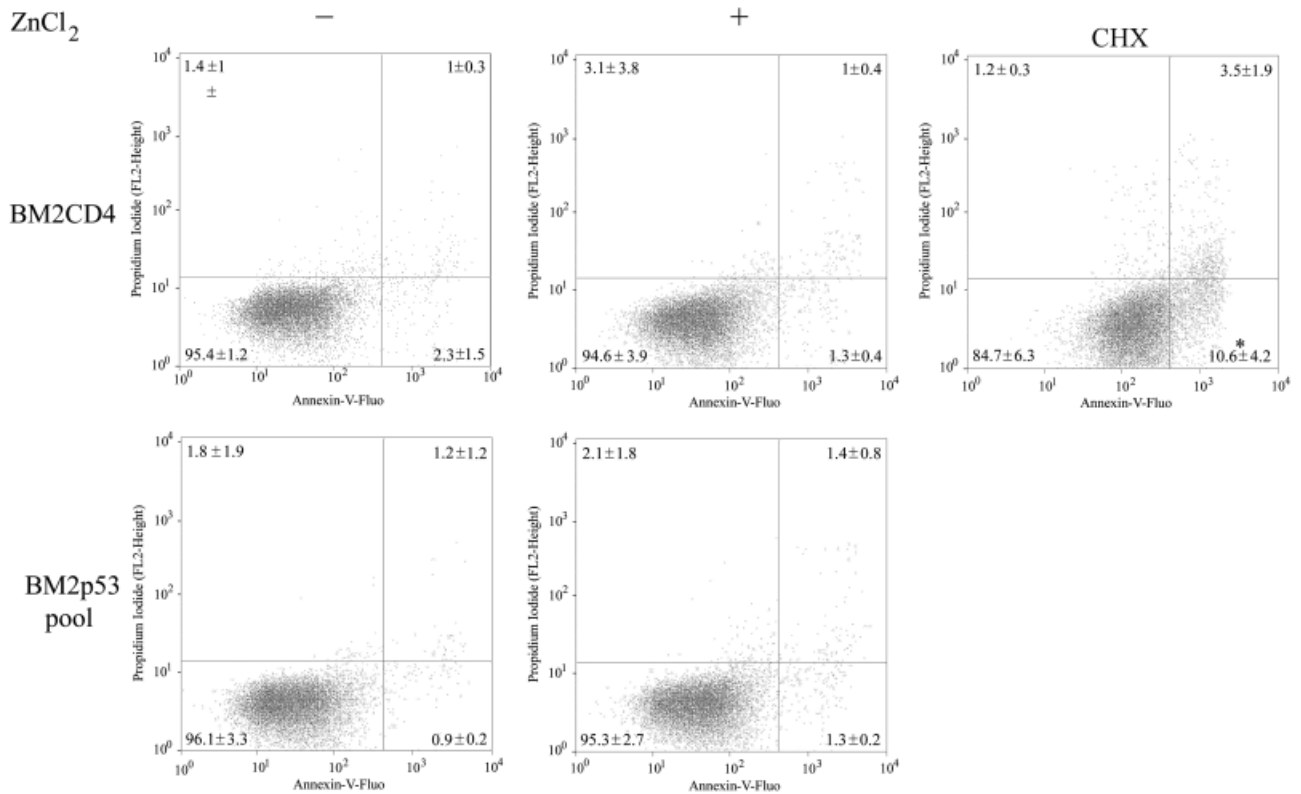


Fig. 5 p53 does not induce externalization of phosphatidylserine (PS) in BM2p53 cells. BM2CD4 and BM2p53 cells were cultured in the presence (+) or absence of ZnCl₂ (-) for 6 hr. The exposure of PS on the surface of at least 10,000 individual cells was assessed by Fluorescence Activated Cell Sorter using FITC-conjugated Annexin V probe. The numbers indicate the averages from three inde-

pendent experiments ± standard deviations. As a control, BM2CD4 cells were cultured in the presence of cycloheximide (CHX) for 6 hr. Increases in the Annexin V positive- and PI negative quadrants indicate apoptosis. *Significant difference ($p < 0.05$) from untreated and zinc-treated BM2CD4 and BM2p53 cells as determined by Student's *t*-test.

(Fig. 7). These features are typical of the monoblasts maturing along macrophage differentiation pathway.

To further explore the ability of p53 to induce the differentiation of v-Myb-transformed monoblasts, we tested for the expression of several differentiation-specific markers in BM2p53 cells. First, we assayed nonspecific esterase activity, which increases upon differentiation of monoblasts into monocytes (Sonne et al., 1991). We detected an approximately 2.5-fold increase of the nonspecific esterase activity in the pool of BM2p53 clones and in the clone BM2p53 A4 in comparison with similarly treated BM2CD4 control (Fig. 8A). This difference was statistically significant. Second, we examined generation of reactive oxygen radicals that increases in cells active in phagocytosis. The pool of BM2p53 clones and the clone BM2p53 A4 were about twofold more active in the production of reactive oxygen radicals than BM2CD4 control cells as determined by NBT assay (Fig. 8B). This difference was also statistically significant. Following phagocytosis of opsonized bacteria or zymosan particles, the monocytes display chemiluminescence resulting from myeloperoxidase activity (McNally and Bell, 1996). In contrast to monocytes, the monoblasts do not possess the myeloperoxidase activity (Glasser, 1981). Therefore, we meas-

ured the luminol-enhanced chemiluminescence of the BM2p53 and BM2CD4 cells upon induction with either opsonized zymosan or phorbol ester TPA. Both TPA- and opsonized zymosan-treated BM2p53 cells exposed to zinc inducer emitted significantly higher levels of chemiluminescence than similarly treated BM2CD4 or untreated BM2p53 cells (Fig. 8C).

In order to characterize the p53-induced maturation of BM2p53 cells in more detail, we measured expression of the CD11b protein. Production of this myeloid-specific protein is specifically increased in monocytes but it declines again as the cells differentiate to macrophages (Cheng et al., 1996; Park et al., 2003). BM2CD4 and BM2p53 cells were treated with zinc chloride for 48 hr, and the level of the CD11b in these cells was determined by FACS analysis. The level of CD11b increased more than twofold in zinc-treated BM2p53 cells in comparison with the similarly treated BM2CD4 cells (Fig. 9). In contrast, the level of CD11b in TPA-treated BM2CD4 cells undergoing terminal differentiation to macrophage-like cells (Šmarda and Lipsick, 1994) was significantly down-regulated. These results suggest that the p53 protein can induce differentiation of BM2 monoblasts rather to monocytes than to macrophages.

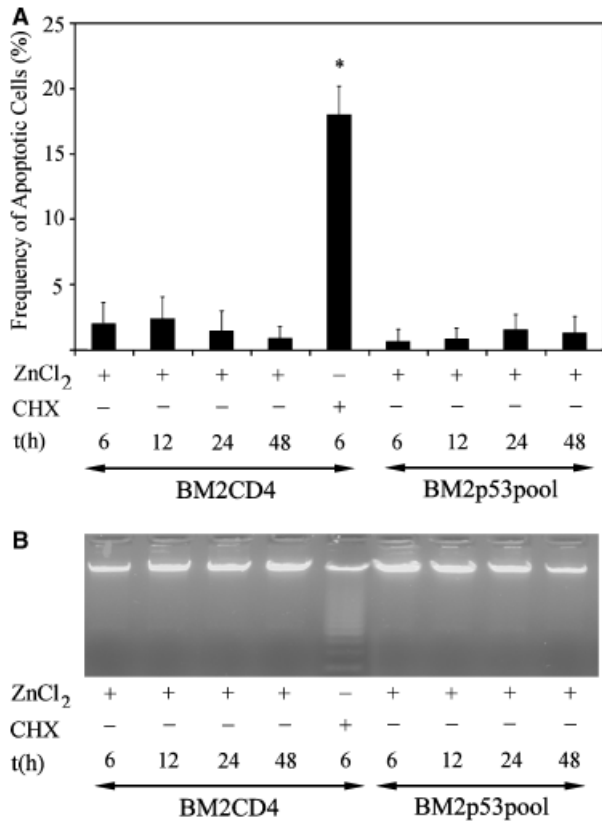


Fig. 6 p53 does not induce formation of apoptotic bodies and DNA fragmentation in BM2p53 cells. BM2CD4 and BM2p53pool cells were cultured in the presence of ZnCl₂ or cycloheximide (CHX) for indicated periods. Then, the cells were fixed and stained with propidium iodide for fluorescence microscopy (A) or lysed and used for purification and gel electrophoresis of genomic DNA (B). Apoptotic morphology of at least 200 cells was assessed using fluorescence microscopy. Bars indicate the averages from three independent experiments \pm standard deviations. *Significant difference ($p < 0.05$) from zinc-treated BM2CD4 and BM2p53 cells as determined by Student's *t*-test.

Next, we measured pHi of BM2p53 cells, because alkalization generally correlates with cell transformation (Gillies et al., 1990), and suppression of cytoplasmic alkalization prevents the development of transformation-associated phenotype (Reshkin et al., 2000). To investigate the effects of p53 on pHi, the BM2p53 and control BM2CD4 cells were treated with zinc chloride for 48 hr. As a positive control, BM2CD4 cells were treated with TPA (Šmarda and Lipsick, 1994). The changes of pHi were determined using the pH-sensitive fluorescent probe SNARF-1 by FACS. The pHi of TPA-treated BM2CD4 cells was about 0.2 units lower than in untreated controls. Similar statistically significant decrease of pHi was found also in p53-expressing BM2p53 cells (Fig. 10). These results document that human p53 induces significant changes in morphology and physiology of BM2 monoblasts that are typical of maturation along macrophage differentiation pathway.

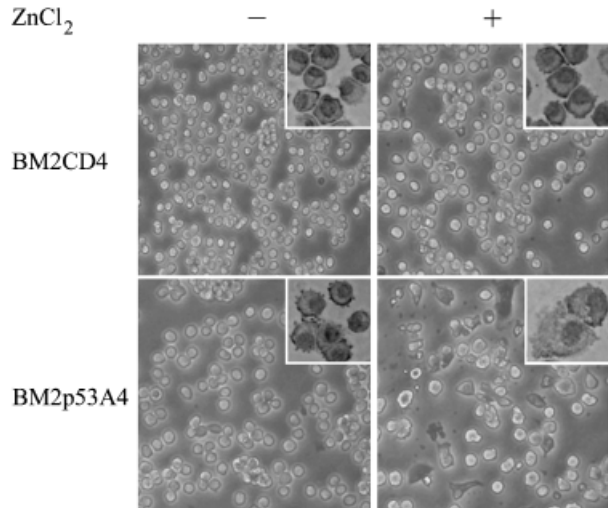


Fig. 7 p53 induces maturation of BM2p53 cells. BM2CD4 and BM2p53A4 cells were cultured in the presence (+) or absence of ZnCl₂ (-) for 3 days. Morphology of cells was observed by either phase-contrast microscopy or light microscopy of cytocentrifuged, fixed, and stained samples (right upper corners). The same magnification was used for each set of photomicrographs.

Activity of the v-Myb oncoprotein increases in p53-expressing BM2p53 cells

The p53 protein directly interacts with c-Myb and inhibits the c-Myb-dependent transactivation of model reporter genes (Tanikawa et al., 2004). In order to investigate whether p53 can affect transactivation function of the v-Myb oncoprotein, we transiently transfected BM2 and BM2p53 cells with the Myb-specific reporter *EW5luc* (Fu and Lipsick, 1996) by electroporation. Transcriptional activation by the Myb protein increased sixfold in the clone A4 of BM2p53 cells, and more than fourfold in the pool of BM2p53 clones in comparison with BM2CD4 control cells (Fig. 11A). In addition, expression of endogenous Myb-target gene, the *GAS41*, was also up-regulated in p53-expressing BM2p53 cells as documented by SDS-PAGE and immunoblotting (Fig. 11B). No effects of the p53 protein on the levels of v-Myb in BM2p53 cells were detected (not shown). These results suggest that the presence of exogenous p53 enhances capability of the v-Myb protein to activate expression of both model reporter gene and endogenous Myb-target gene in BM2p53 cells.

Discussion

v-myb oncogene of AMV encodes a transcription factor that transforms myelomonocytic cells *in vitro* and *in vivo*. The line of *v-myb*-transformed monoblasts BM2 that was generated from a culture of AMV-transformed primary bone marrow cells (Moscovici et al., 1982) has been repeatedly used to investigate mechanisms of

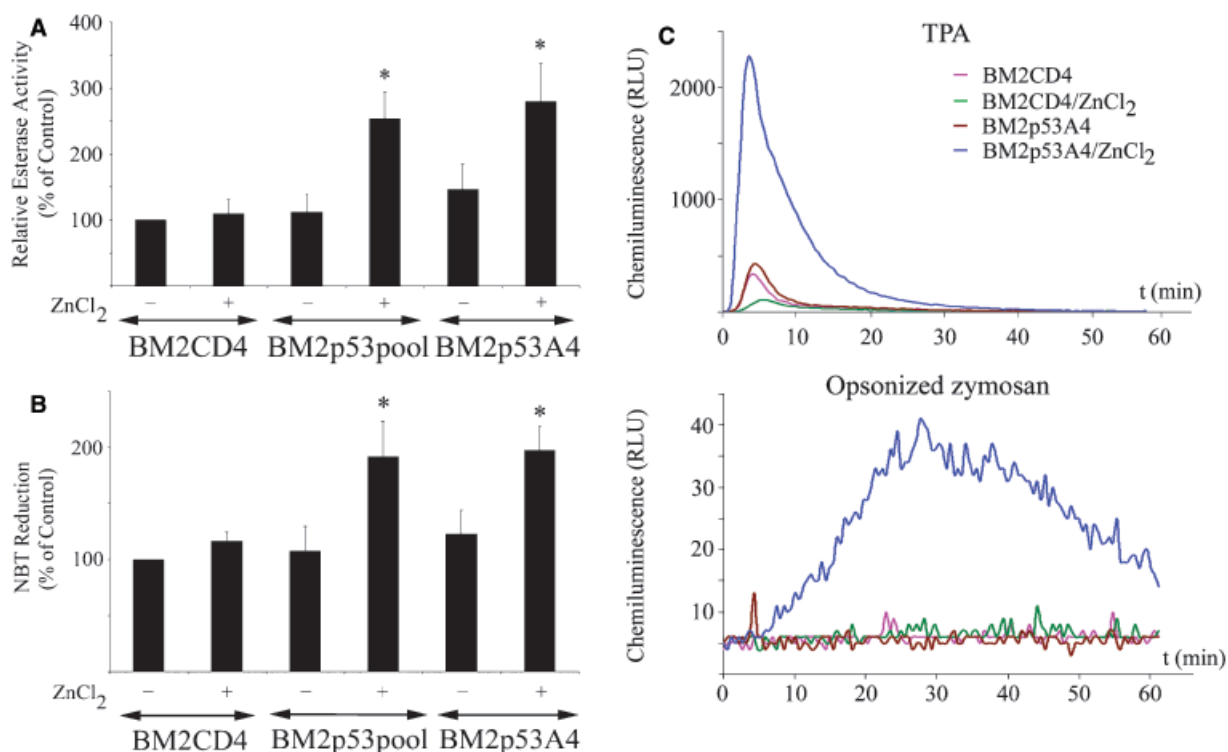


Fig. 8 p53 induces differentiation of BM2 monoblasts. BM2CD4 and BM2p53 cells were cultured in the presence (+) or absence (-) of ZnCl₂ for 48 hr. (A) Nonspecific esterase activity in cell extracts was determined by spectrophotometry using α -naphthyl acetate. (B) Production of oxygen radicals by 1×10^6 viable cells was determined by spectrophotometry upon incubation with nitro-blue-tetrazolium (NBT). Data in (A) and (B) represent the mean of three independent experiments (as percentage of untreated BM2CD4 control cells). Error bars indicate standard deviations. *Significant difference ($p < 0.05$) from both untreated and zinc-treated BM2CD4

and untreated BM2p53 cells as determined by Student's *t*-test. (C) Luminol-enhanced chemiluminescence was measured in 1.5×10^5 cells in two parallels. To induce the production of oxygen radicals, the cells were stimulated with either TPA or opsonized zymosan in the presence of luminol. The kinetics of the chemiluminescence reaction was assessed for 1 hr in 30 sec intervals. The curves represent the light emission (expressed as relative light units [RLU]) produced in the individual time points. The graphs show the representative results of three independent experiments. Two parallel samples of the same cell population were analyzed in each experiment.

v-Myb suppression by ectopically expressed human, mouse, and chicken genes (Šmarda et al., 1995, 1999; Sevcikova et al., 2002; Bryja et al., 2003; Zahradníčková et al., 2003; Šmardová et al., 2005). The aim of this study was to express p53 in BM2 cells and evaluate its effects on proliferation, differentiation, and apoptosis. Endogenous chicken p53 in BM2 cells possesses mutated DNA-binding domain as determined by RT-PCR followed by DNA sequencing (not shown). To avoid risk of dominant negative inhibition of exogenous p53 wt by its mutated endogenous counterpart resulting from formation of defective tetramers (Cho et al., 1994; Chan et al., 2004), we expressed human rather than chicken p53 in BM2 cells. These two variants of the p53 protein are not capable of forming heterodimers (Mateu and Fersht, 1999).

It has been widely accepted that p53 can induce cell cycle arrest, programmed cell death, and/or differentiation of stressed cells (Oren, 2003). Although there is only 53% amino acid homology in human and avian p53 proteins (<http://www.ncbi.nlm.nih.gov/>), we report in this study that human p53 retains some functions in avian cells. It can block proliferation of BM2 cells in the

G2 phase of the cell cycle. This capability of p53 to induce growth arrest in the G2/M checkpoint is well known (Taylor and Stark, 2001). At the same time, the p53 protein induces differentiation of BM2p53 monoblasts along monocytic pathway as demonstrated by monitoring of several differentiation markers: the expression of the CD11b integrin, nonspecific esterase- and myeloperoxidase activities as well as production of reactive oxygen radicals increased and pHi decreased. All of these parameters mark maturing of monoblastic cells (Glasser, 1981; Hagag et al., 1987; Gillies et al., 1990; Sonne et al., 1991; Cheng et al., 1996; Reshkin et al., 2000; Park et al., 2003). The question remains, whether p53 preferentially induces differentiation of BM2 monoblasts to monocytes, as suggested by increased expression of CD11b (Park et al., 2003) or to macrophage-like cells, as suggested by similar decrease of pHi upon p53 expression and TPA treatment. We believe that the first option is correct. Down-regulation of pHi in monocytes differentiating to macrophages was reported indeed but using the dextran-FITC probe that can detect the pHi in cytoplasmic vesicles (Basta et al., 1999). In this work, we used the SNARF-1 probe that

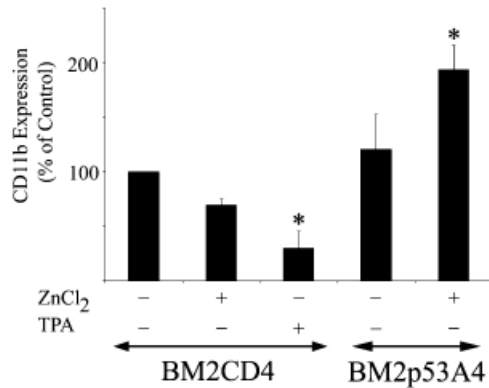


Fig. 9 p53 induces differentiation of BM2 monoblasts to monocytes. BM2CD4 and BM2p53A4 cells were treated with ZnCl₂ for 48 hr (+) or left untreated (-). As a positive control, BM2CD4 cells were treated with TPA for 24 hr. The relative amount of the CD11b surface antigen in each sample was detected using the Mo1-specific antibody and FITC-conjugated secondary antibody by Fluorescence Activated Cell Sorter. The amount of fluorescence was determined as a difference of fluorescence intensities of the cells treated with both primary and secondary antibodies and the same cells treated with fluorescein-conjugated antibody only. The bars indicate the mean fluorescence values obtained from at least three independent experiments (as percentage of BM2CD4 control). Error bars show the standard deviations. *Significant difference ($p < 0.05$) from both untreated and zinc-treated BM2CD4 cells and untreated BM2p53 cells as determined by Student's *t*-test.

undergoes conversion to SNARF-1- free acid by intracellular esterases once internalized within cell. The resulting free acid cannot penetrate the membranes of acidic vesicles that are often present in macrophage-like cells (Slayman et al., 1994). Therefore, the similar decrease of pH_i in p53-expressing and TPA-treated BM2 cells using the SNARF-1 probe can occur in cytosol of

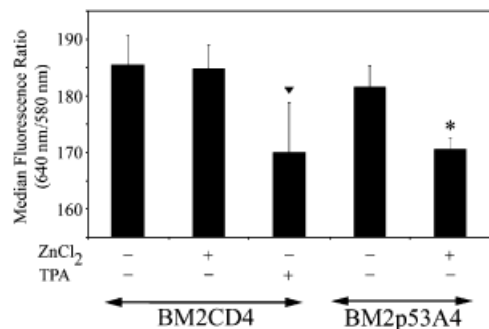


Fig. 10 p53 decreases pH_i in BM2p53 cells. BM2CD4 and BM2p53 cells were cultured in the presence (+) or absence (-) of ZnCl₂ for 48 hr. As a positive control, BM2CD4 cells were treated with TPA for 24 hr. The cells were exposed to SNARF-1 fluorescence probe and the ratio of fluorescence emission at 640 and 580 nm wavelengths was determined by Fluorescence Activated Cell Sorter for each sample. The bars represent the average of at least three independent experiments. The error bars indicate standard deviations. *Significant difference ($p < 0.05$) of zinc-treated BM2p53 cells from similarly treated BM2CD4 and untreated BM2p53A4 cells; ▼significant difference ($p < 0.05$) of TPA-treated BM2CD4 cells from untreated BM2CD4 controls as determined by Student's *t*-test.

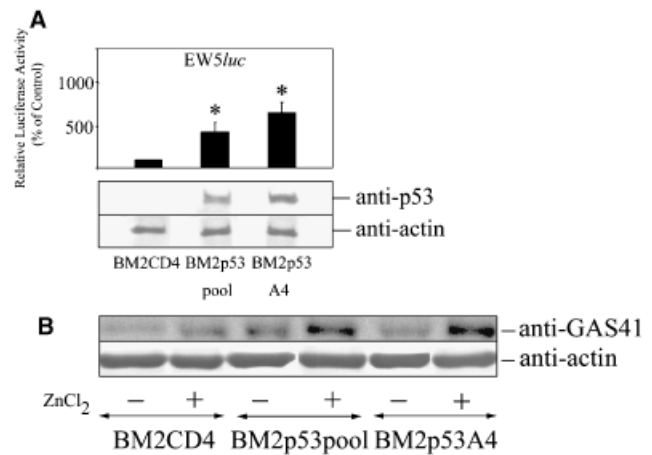


Fig. 11 p53 enhances transactivation by the v-Myb protein. (A) BM2CD4, a pool of BM2p53 clones and BM2p53 clone A4 were transiently transfected with the Myb-specific reporter plasmid EW5luc by electroporation and cultured for 36 hr. Luciferase activity present in extracts of harvested cells was normalized according to the transfection efficiency. Data show the mean values of relative luciferase activity (as percentage of BM2CD4 control) from three independent experiments. Error bars indicate standard deviations. *Significant difference ($p < 0.05$) from BM2CD4 cells as determined by Student's *t*-test. The level of the p53 protein in electroporated cells was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using the p53-specific antibody. To control for sample loading, the same blot was probed with the actin-specific antibody. (B) BM2CD4, pool of BM2p53 clones and BM2p53 clone A4 were treated or left untreated with ZnCl₂ for 15 hr. Protein extracts from harvested cells were resolved by SDS-PAGE and analyzed by immunoblotting using either the GAS41-specific antibody or the actin-specific antibody for control of sample loading.

both monocytes and macrophages. The dextran-FITC probe would presumably reveal more dramatic decrease of pH_i in TPA-treated BM2 cells than the SNARF-1 probe due to the penetration to the multiple vacuoles and other membrane vesicles of macrophages (Plank et al., 1994; Basta et al., 1999).

Human p53 protein clearly affected cell cycle progression and differentiation status but it did not initiate apoptosis of BM2 cells. Although viability of p53-expressing BM2 cells decreased by 20% due to cell death, we did not detect apoptotic DNA fragmentation, formation of apoptotic bodies nor exposure of phosphatidylserine on the surface of BM2p53 cells. It was shown earlier that the level of p53 expression can determine fate of the cell. Low level of p53 induces differentiation, while high level of p53 induces apoptosis of human promyelocytic leukemia cells HL-60 (Ronen et al., 1996). This may result from different sensitivity of the *bax* gene promoter to transactivation by p53 in high- and low-intracellular concentrations (Inga et al., 2002). Therefore, the level of p53 expressed from the MT promoter in BM2p53 cells may not be sufficient to induce apoptosis but it is sufficient to induce the cell cycle arrest and differentiation. Once undergoing growth arrest and differentiation, the cells are usually resistant to

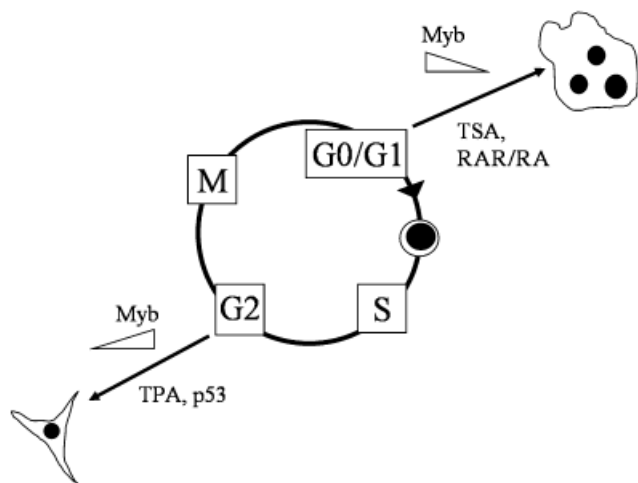


Fig. 12 Transactivation potency of the Myb protein may determine the way of cell cycle exit and differentiation of BM2 monoblasts. Upon induction with trichostatin A (TSA) or liganded retinoic acid receptor α (RAR), the transactivation by the Myb protein declines, the cell cycle is arrested in the G0/G1 phase, and the BM2 monoblasts differentiate into multinucleated macrophage-like cells. In contrast, the phorbol ester TPA or p53-expression up-regulates the transactivation by the Myb protein, thus causing cell cycle exit in the G2 phase and formation of mostly mononuclear monocytes or macrophages.

apoptosis-promoting stimuli (Asada et al., 1999; Samuel et al., 2001).

We also considered hypotheses that human p53 does not induce apoptosis of avian BM2 cells due to species-specific differences in the sequence of p53, promoters/enhancers and/or p53 co-factors that interfere with control of pro-apoptotic genes by p53 or due to interaction with the v-Myb oncoprotein. We found that human p53 did not activate transcription of the reporter gene from the pro-apoptotic *bax* gene promoter in chicken BM2p53 cells, while the transactivation from the *mdm-2* gene promoter by p53 occurred normally. This suggested that human variant of the p53 protein lost capability to activate the *bax* gene expression in avian cells. However, the human p53 protein activated transcription from the *bax* gene promoter in the other avian cell line, the quail fibroblasts QT6. Therefore, the lack of the *bax* gene transactivation by human p53 is not a general feature of avian cells but rather specificity of the BM2 cell line. This result documents that the tissue/cell-context is significantly involved in control of the p53 transactivating function. Similarly, p53 was shown to activate transcription from the *bax* gene promoter in human osteosarcoma cell line Saos-2, but not in human breast carcinoma cell line MDA-MB-453. There are indications that the cell type-specific post-translational modifications may be involved in control of the p53 conformation that makes a decision on binding to the *bax* gene promoter (Thornborrow and Manfredi, 1999). We also investigated whether it is the v-Myb protein that can suppress the *bax* gene transactivating function of p53. However, when co-expressed

in transiently transfected QT6 cells, v-Myb did not affect efficiency of the *bax* gene promoter activation by p53. This documents that the failure of p53 to transactivate *bax* does not result from the presence of v-Myb in BM2p53 cells.

Next, we addressed the opposite question whether overexpressed p53 can modify the function of the v-Myb oncoprotein in BM2p53 cells. Previous studies on control of the Myb protein activity demonstrated that p53 down-regulates transactivation by Myb in 293T, CV-1 cells, and HepG2 cells (Tanikawa et al., 2000, 2004). In contrast, the rate of v-Myb transactivation increased in p53-expressing BM2p53 cells. Similar increase of v-Myb transactivation function was detected in BM2 cells differentiating to macrophage-like cells upon treatment with TPA (Ganter et al., 1998). In addition, BM2 cells treated with TPA or expressing p53 share other features: they exit cell cycle in the G2-phase and differentiate to mostly mononuclear derivatives (Šmarda and Lipsick, 1994; Nemažerova et al., 2003). In contrast, the G1-phase blockers and differentiation inducers, such as trichostatin A or retinoic acid suppress transactivation by the v-Myb oncoprotein and increase formation of multinuclear macrophage-like cells (Šmarda et al., 1995; Zemanova and Šmarda, 1998; Vodicka et al., 2000; Nemažerova et al., 2003; Šmardová et al., 2005). The G1-phase cell cycle arrest and formation of multinucleated giant cells also occur in chicken yolk sac cells transformed with v-Myb-estrogen receptor fusion protein and withdrawn from estrogen (Engelke et al., 1997). These results further support the model of Engelke that there are at least two ways to suppress transformation by the v-Myb oncoprotein in BM2 cells (Engelke et al., 1997). The first one is based on potentiation of the v-Myb transactivation function causing cell cycle exit in G2-phase of the cell cycle and resulting in formation of mononuclear cells (Fig. 12). The second one is based on suppression of transactivation by the v-Myb protein resulting in G1-phase cell cycle arrest and formation of mostly multinuclear macrophage-like cells.

In conclusion, we showed that human p53 can induce monocytic differentiation, and impose cell cycle block in v-Myb-transformed chicken monoblasts BM2 documenting that ability of p53 to control cell cycle progression and differentiation is conserved in spite of obvious differences between the human and avian cells. In contrast, human p53 did not induce apoptosis, presumably due to failure to transactivate the pro-apoptotic *bax* gene in BM2 cells. These results document that the p53-driven pathways controlling apoptosis and differentiation/proliferation are independent, thus supporting and extending the data published previously (Cerone et al., 2000; Chylicki et al., 2000).

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