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Regulation of ES cell differentiation by functional and conformational modulation of p53

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Embryonic stem (ES) cell lines were used to examine the role of p53 during *in vitro* differentiation. Undifferentiated ES cells express high levels of p53 exclusively in the wild-type conformation, immunoprecipitable by monoclonal antibody PAb246, and p53 was found to be functionally active as determined by its ability to bind DNA specifically and to activate transcription of target genes. Differentiation *in vitro* resulted in a decrease in the levels of p53 and in a shift in its conformational status to the mutant form, detectable by monoclonal antibody PAb240, with a concomitant loss of functional activity. The presence of functional p53 in the undifferentiated ES cells renders them hypersensitive to UV irradiation, whereas the differentiated cells were resistant to UV treatment. ES cells lacking p53 exhibit enhanced proliferation in both the undifferentiated and differentiated state, and apoptosis accompanying differentiation was found to be reduced. Furthermore, wild-type ES cells undergoing apoptosis expressed functional p53. Expression of the temperature-sensitive p53val135 mutant in wild-type ES cells resulted in a reduction of apoptosis accompanying differentiation when it adopted a mutant conformation at 39°C. These data demonstrate that functional inactivation of p53 allows differentiating cells to escape from apoptosis, and suggest that the conformational switch could regulate the inactivation process.

Keywords: apoptosis/cell differentiation/conformation/embryonic stem cells/p53

Introduction

Embryonic development is the result of a concerted integration of a multitude of proliferation and differentiation cues. Proliferation signals have to be compromised in order for differentiation to proceed. A vast spectrum of growth regulatory gene products are involved in this complex processing of signals, and many are tissue and cell-type specific. Upon induction of differentiation, stem cells begin to lose their capacity for self-renewal, and their unrestricted developmental potential is progressively lost. The transition from a proliferating stem cell to a quiescent differentiated cell is often governed by an intricate arrangement of multifactorial complexes such as cyclins, cyclin-dependent kinases (cdks), their inhibitors (Hunter, 1993), transcription factors, such as E2F (Nevins, 1992), and additional proteins, such as p53 and Rb (Perry and Levine, 1993; Wilman, 1993). Thus, factors that promote G_1–S transition, such as the G_1-specific cyclins D2 and D3, cyclin A and cdk 4 have been shown to be down-regulated during differentiation, whereas those that slow down the cell cycle, such as the cdk inhibitors p21 and p27, are up-regulated (Parker *et al.*, 1995; Wang *et al.*, 1996). Furthermore, the levels of tumor suppressors p53 and Rb are modulated (Savatier *et al.*, 1994; Weinberg *et al.*, 1995; Lutzker and Levine, 1996). Therefore, a fine balance between positive and negative regulators of the cell cycle is critical in maintaining the transition towards differentiation, which if disturbed could lead to deleterious effects as manifested in many tumors of stem cell origin (for review, see Pierce and Speers, 1988; Knudson, 1992).

The tumor suppressor gene product, p53, encodes a phosphoprotein which has been shown to play a critical role in controlling cell proliferation, growth arrest and apoptosis (for review, see Gottlieb and Oren, 1996; Ko and Prives, 1996). Its inactivation has thus been shown to be a key event in the formation of a variety of tumors, and overexpression of wild-type p53 in several cell lines often leads to reversion of the malignant phenotype or induction of apoptosis (Gottlieb and Oren, 1996; Ko and Prives, 1996). p53 acts as a tumor suppressor by preventing the propagation of DNA damage to daughter cells (Purdie *et al.*, 1994). The levels and activities of wild-type p53 have been shown to increase in response to irradiation and other DNA-damaging agents (Kastan *et al.*, 1991a; Lu and Lane, 1993). Wild-type p53 enters the nucleus and transcriptionally activates target genes, and regulates the onset of DNA replication at the G_1/S boundary (Vogelstein and Kinzler, 1992; Dulic *et al.*, 1994). Thus, in the absence of a wild-type p53 or the presence of a mutated p53 gene product, cells fail to arrest in G_1 or undergo apoptosis in response to DNA damage, and mutations are propagated leading to tumorigenesis (Kemp *et al.*, 1994; Symonds *et al.*, 1994). Functional modulation of p53 activity has also been shown to inactivate p53 in the absence of mutations (Ueda *et al.*, 1995; Knippschild *et al.*, 1996). Several studies using the temperature-sensitive p53 mutant, tsp53val135 (ts mutant), have indicated that p53 functional activity is associated with its conformational flexibility (Michalovitz *et al.*, 1990; Gottlieb *et al.*, 1994; Milner, 1995). Thus, p53 is functionally active (i.e. able to bind DNA specifically and activate transcription) at 32°C when the ts mutant adopts a 'wild-type' conformation, detectable by monoclonal antibody PAb246. At 39°C, the conformation of the ts mutant changes to the mutant form, detectable by monoclonal antibody PAb240, and the functional activity is lost. Similarly, the wild-type p53 protein (unmutated gene product) has also been proposed to exist in either the

wild-type or mutant conformation, depending on the physiological status of the cell, thus allowing proliferation to proceed when in a mutant conformation (Milner, 1984; Milner and Watson, 1990).

Cell cycle arrest and apoptosis induction by p53 seem to be differentially regulated functions. Recent reports suggest that p53 levels may determine the decision between apoptosis or cell cycle arrest. High levels of p53 have been shown to induce apoptosis and lower levels cause cell cycle arrest (Chen *et al.*, 1996; Ronen *et al.*, 1996). Moreover, it has been proposed that different classes of p53-responsive promoters may exist, whose ability to be activated by p53 can be differentially regulated (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996), resulting in distinct effects of p53 on cell fate.

Besides playing an important role in controlling proliferation and apoptosis, p53 has also been implicated in regulating cellular differentiation (for review, see Wolkowicz and Rotter, 1997). Introduction of wild-type p53 in a pre-B cell line leads to a differentiated phenotype (Shaulsky *et al.*, 1991). Similarly, overexpression of wild-type p53 in leukemic K562 cells, HL-60 cells, Friend virus-transformed erythroleukemic cells or squamous carcinoma cells induces limited differentiation (Feinstein *et al.*, 1992; Brenner *et al.*, 1993; Soddu *et al.*, 1994; Bannerjee *et al.*, 1995; Ehinger *et al.*, 1995). In addition, studies using non-transformed cells of the myeloid and muscle lineages indicated that enforced expression of p53 results in a differentiated phenotype, and dominant-negative versions of p53 inhibit differentiation (Soddu *et al.*, 1996). Though these results suggest a role for p53 in regulating differentiation, it cannot be excluded that the observed effects were a consequence of proliferation inhibition by p53 in some cell lines. In addition, several other reports showed that endogenous p53 levels and activities are modulated upon differentiation. p53 expression was shown to be up-regulated during maturation of human hematopoietic cells (Kastan *et al.*, 1991b). Moreover, it has been reported that p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein (Weinberg *et al.*, 1995). p53 has also been reported to be down-regulated at the protein and RNA levels during embryogenesis (Louis *et al.*, 1988). These findings suggest a cell-type-specific and stage-specific role for p53 in differentiation. Furthermore, whereas p53-null mice are viable (Donehower *et al.*, 1992; Jacks *et al.*, 1994), mice lacking mdm2, a negative regulator of p53, die at an early embryonic stage, and this lethality could be rescued in a p53-null background (de Oca Luna *et al.*, 1995; Jones *et al.*, 1995). This implies that p53 is functional at an early embryonic stage, and its functional activity has to be controlled for survival of embryos and subsequent development.

Previous studies using embryonal carcinoma (EC) cells demonstrated the presence in F9 cells of high levels of p53 that was non-functional (Lutzker and Levine, 1996). Differentiation with retinoic acid (RA) resulted in an increase in functional activity although the levels of p53 protein were decreased (Lutzker and Levine, 1996). In an attempt to determine the role of p53 in cell proliferation and in early differentiation processes, we have analysed p53 activity in embryonic stem (ES) cell lines *in vitro*. ES cells, derived from the inner cell mass of mouse blastocysts, have high proliferative capacity and can be induced to differentiate both *in vivo* and *in vitro* (Evans *et al.*, 1979). These cells have the capacity to give rise to all lineages of the embryo *in vitro*, thus recapitulating the *in vivo* differentiation process, including the occurrence of programmed cell death accompanying differentiation. Here, we present evidence for the functional modulation of p53 activity during ES cell differentiation *in vitro*, concurrent with a conformational change of the protein which seems to be necessary for differentiation to proceed.

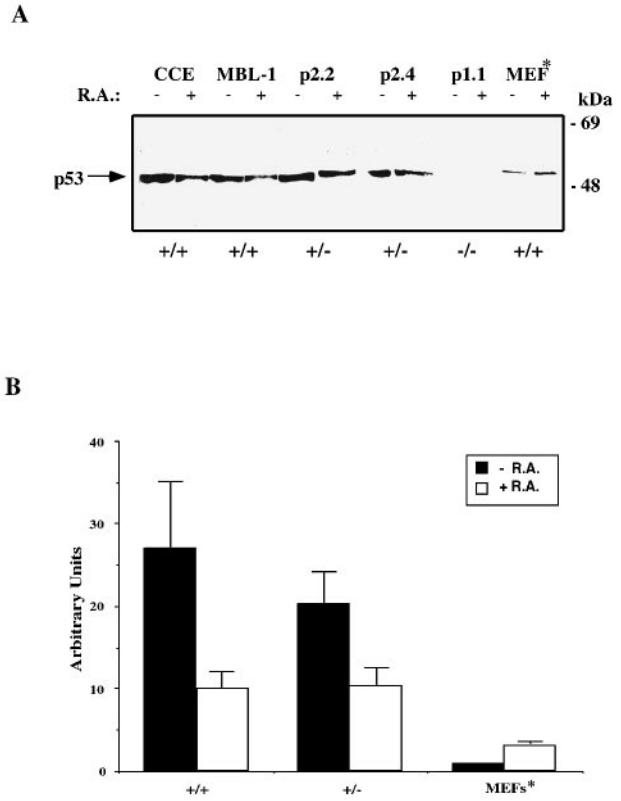

Fig. 1. Undifferentiated ES cells express higher levels of p53 than their differentiated derivatives. (**A**) Extracts from 5×10^5 undifferentiated (–) or differentiated cells (+) (following RA treatment) (except MEF) were analysed by Western blotting using rabbit polyclonal antibody (CM5). * indicates either untreated (–) or UV-irradiated (+) MEF extracts which were used as reference. The p53 genotypes of the cell lines are indicated below the gel. (**B**) Quantitative analysis of levels of p53, in arbitrary units, using primary MEFs as reference. Each column represents the average of two cell lines of the same genotype as follows: +/+, CCE and MBL-1; +/–, p2.2 and p2.4.

Results

The conformation of p53 changes upon ES cell differentiation

To determine the status of p53 in undifferentiated ES cell lines, the steady-state levels of p53 were assessed by Western blotting using CCE and MBL-1 (p53+/+), p2.2 and p2.4 (p53+/–) and p1.1 (p53–/–) cell lines (Figure 1). All p53-positive (+/+ and +/–) ES cell lines examined expressed high levels of p53 as compared with primary mouse embryonic fibroblasts (MEFs), which express extremely low levels (Figure 1A). CCE and MBL-1 ES cells expressed ~27 times more and p2.2 and p2.4 ES cells expressed ~20 times more p53 protein than primary

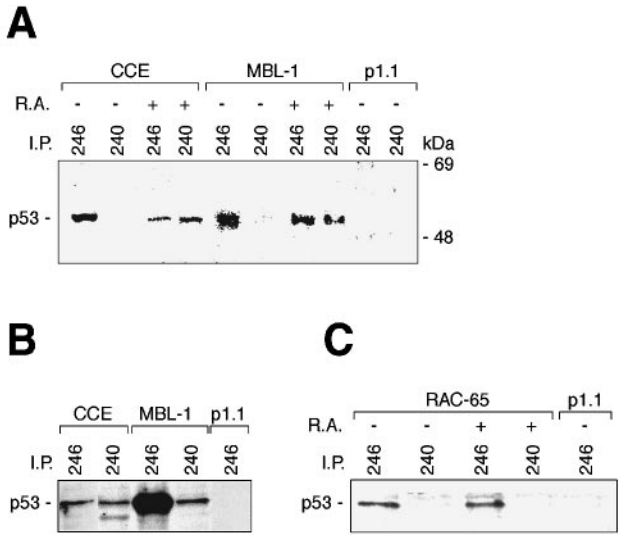

Fig. 2. Changes in conformational status of p53 upon differentiation of ES cells with RA (**A**), or by embryoid body formation (**B**). Equal amounts of whole cell extracts from undifferentiated (–) or differentiated (+) cells were immunoprecipitated with either wild-type (PAb246) or mutant-specific (PAb240) antibodies and visualized by Western blot analysis with antibody CM5. (**C**) RAC-65, an EC cell line which is resistant to RA-induced differentiation was used as a control to ensure that RA treatment does not result in the denaturation of p53 independent of differentiation.

MEFs (Figure 1B). No p53 protein was detected in the p1.1 ES cells.

The wild-type p53 protein has been shown to have a very short half-life in various cell types, which is stabilized by mutations or in response to stress stimuli (Oren *et al.*, 1981; Maltzman and Czyzyk, 1984; Rogel *et al.*, 1985). We found that the half-life of p53 in the ES cell lines (CCE, MBL-1, p2.2 and p2.4) is ~4 h (data not shown), which is comparable with the half-life in EC cells (Oren *et al.*, 1982). Sequencing of the p53 gene did not reveal any mutations in the ES cell lines (data not shown), thus excluding the possibility of mutations that would stabilize the p53 protein. Therefore, we tested the possibility that highly proliferating undifferentiated ES cells might express p53 in the mutant conformation. Immunoprecipitation with conformation-specific antibodies revealed that undifferentiated ES cells expressed p53 exclusively in the wild-type conformation, immunoprecipitable by wild-type conformation-specific monoclonal antibody PAb246 (Figure 2A). No p53 protein was immunoprecipitated by the mutant conformation-specific monoclonal antibody PAb240 (Figure 2A). The same amount of p53 was immunoprecipitated by a pan-p53 monoclonal antibody PAb421 (conformation non-specific) as was immunoprecipitated by PAb246 (data not shown), indicating that all of the p53 is in the wild-type conformation.

We next investigated whether the induction of differentiation alters the p53 status. RA-treated cells were analysed for the absence of ECMA-7 antigen, which is expressed only on undifferentiated ES cells and not in the differentiated derivatives (Kemler, 1980), and only cultures with >90% ECMA-7-negative cells were used. Differentiation with RA resulted in a marked decrease in p53 steady-state levels to ~35–45% of undifferentiated cells, which were still higher than both untreated and UV-irradiated primary MEFs, where the p53 protein is stabilized (Figure 1A and B). There was no significant difference in the half-life of the p53 protein after differentiation (data not shown). Immunoprecipitation with conformation-specific antibodies revealed that differentiation led to a shift in conformation of the p53 protein to the mutant form (Figure 2A). Both the wild-type and mutant forms of p53 were now detected by conformation-specific antibodies PAb246 and PAb240, respectively (Figure 2A). Undifferentiated ES cells maintained the wild-type conformation in the same experiments, ruling out denaturation of p53 during processing.

In order to exclude the possibility that the observed conformation change of p53 occurred in response to RA treatment rather than due to the differentiation process, we differentiated ES cells into embryoid bodies in the absence of leukemia inhibiting factor LIF and RA. Both the wild-type and mutant forms of p53 were again detected in these differentiating ES cell cultures (Figure 2B), suggesting that the conformation change was a direct result of differentiation of ES cells. We further tested an EC cell line, RAC 65, which is resistant to RA-induced differentiation (Pratt *et al.*, 1990) for the status of p53 conformation upon RA treatment. These cells expressed p53 in the wild-type conformation, and RA treatment did not alter the conformational status of p53 (Figure 2C). Taken together, these results indicate that undifferentiated ES cells express high levels of p53 protein in the wild-type conformation, and differentiation results in both a decrease in the levels of p53 and a change in its conformational status.

Functional activity of p53 in ES cells and their differentiated derivatives

Expression of high levels of p53 has been shown to negatively regulate cell proliferation (for review, see Gottlieb and Oren, 1996). Since ES cells continue to proliferate in the presence of high levels of wild-type p53, we investigated whether p53 in undifferentiated ES cells is non-functional, as was previously reported for EC cells (Lutzker and Levine, 1996). Electrophoretic mobility shift assays (EMSAs) using consensus p53 binding sequence and random oligonucleotides were performed to determine whether p53 in undifferentiated ES cells could specifically bind DNA (El-Deiry *et al.*, 1992). Both untreated and UV-irradiated primary MEFs were used as controls. As shown in Figure 3A, nuclear extracts from both CCE and p2.2 cells exhibited very strong DNA-binding activity (Figure 3A, lanes 2 and 4), and no binding was observed when random oligonucleotides were used (Figure 3A, lanes 3 and 5). p1.1 ES cell extracts did not exhibit any DNA-binding activity (Figure 3A, lane 6). There was also no observable DNA-binding activity in extracts from untreated MEFs, and UV irradiation resulted in DNA-binding (Figure 3A, compare lanes 8 and 9). This DNA-binding activity observed in undifferentiated ES cell extracts was markedly reduced after differentiation with RA (Figure 3B, compare lane 2 with lane 3), and p59 ES cell (p53–/–) extracts did not exhibit any DNA-binding activity (Figure 3B, lanes 4 and 5). The specificity of the binding was further confirmed by shifting the complex with monoclonal anti-p53 antibody, PAb 421 (Figure 3C, lane 3). In the presence of PAb 421, there was a shift of

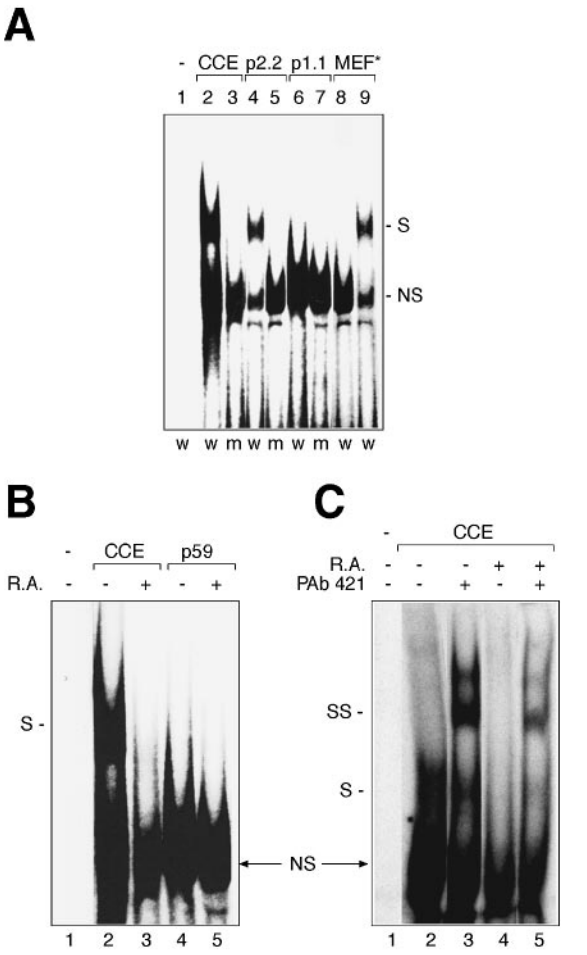

Fig. 3. Specific DNA binding activity of p53 in ES cells before and after differentiation with RA. (**A**) Electrophoretic-mobility shift assay was performed using oligonucleotides corresponding to the p53 consensus binding sequence (w) or random binding sequence (m) with nuclear extracts from undifferentiated ES cells. * indicates either untreated (lane 8) or UV-irradiated (lane 9) MEF extracts which were used as controls. EMSA of (**B**) undifferentiated and differentiated cell nuclear extracts; (**C**) in the absence (–) or presence (+) of PAb421, using oligonucleotides corresponding to the p53 consensus binding sequence (indicated w in A). S represents specific binding, NS non-specific binding and SS shifted specific complexes in the presence of PAb421.

the specific complex resulting in slower-migrating bands (Figure 3C). Extracts from differentiated ES cells were also bound to the oligonucleotide probe in the presence of PAb 421 (Figure 3C, lane 5), which has been shown to activate the DNA-binding activity of latent inactive p53 (Hupp *et al.*, 1995).

In order to determine whether the observed DNA binding resulted in p53-mediated transcriptional activation in undifferentiated ES cells, we investigated the expression of several endogenous p53 target genes. Northern blot analysis revealed that p53–/– cell lines expressed p21 mRNA at marginally higher levels than p53+/– ES cell lines (Figure 4A). However, mdm2 mRNA levels were higher in both p53+/– undifferentiated ES cell lines compared with p53–/– cell lines (Figure 4A). We next investigated the expression of other target genes like cyclin G, cyclin D1, gadd45 and bax-1a. RT–PCR analysis revealed that cyclin G levels were much higher in the undifferentiated p53+/– ES cell lines compared with p53–/– cell lines (Figure 4B, compare with β-tubulin controls). However, there were no significant differences in the levels of cyclin D1, gadd45 and bax-1a between p53+/– and –/– cell lines (Figure 4B). Upon differentiation, the levels of cyclin G were relatively lower in differentiated p53+/– cells than their undifferentiated counterparts (Figure 4B, compare with β-tubulin controls), indicating that the loss of transcriptional activity of p53 resulted in reduced target gene expression. There were no significant changes in the levels of most other target genes between p53+/– and –/– differentiated cells, as was for undifferentiated cells (Figure 4A and B). Although p21 mRNA levels were not significantly altered upon differentiation in both p53+/– and –/– cell lines, the levels of p21 protein increased upon differentiation (Figure 4A), as has been previously reported (Parker *et al.*, 1995). Taken together, these data indicate that there is preferential activation of p53 target genes in the p53+/– ES cell lines compared with p53–/– cell lines, suggesting that p53 is able to activate transcription in these cells.

To further confirm the transcription activating potential of p53, we stably transfected p53+/+ and –/– ES cells with either a p53-responsive promoter construct expressing a β-galactosidase reporter gene (pRGCΔfos lacZ) or the negative control construct (pΔfos lacZ) to examine p53-dependent gene expression. Individual stable clones (two per cell line, per transfection) were derived and the β-galactosidase activity was determined. Both clones from each group gave similar results. Representative results from one clone each are shown in Figure 5. Undifferentiated p53+/+ ES cell clones carrying pRGCΔfos lacZ stained positive for β-galactosidase activity in contrast to p53–/– ES cell clones (Figure 5A and C). We have ruled out the possibility that selection pressure (i.e. addition of hygromycin B) induces p53 transcriptional activity, as undifferentiated p53+/+ ES cells cultured in medium free of hygromycin B for several passages also stained positive for β-galactosidase activity (data not shown). Both p53+/+ and p53–/– ES cell clones carrying the control construct were negative for β-galactosidase activity (data not shown). Differentiation with RA resulted in a dramatic decrease in the expression of β-galactosidase in p53+/+ cells with only occasional blue cells being detectable (Figure 5B). These data indicate that high levels of p53 in undifferentiated ES cells results in p53-dependent gene expression which is drastically reduced in differentiating cells.

Undifferentiated ES cells are hypersensitive to UV irradiation

Since undifferentiated ES cells express high levels of functional p53, we next sought to examine its physiological role in these highly proliferating cells. We investigated whether the functionally active p53 of undifferentiated ES cells is involved in the response to DNA damage. Undifferentiated and differentiated ES cells were subjected to UV irradiation and their viability determined by lactate dehydrogenase (LDH) release assays. Between 50 and 85% of all undifferentiated p53+/+ and +/– ES cells were killed within 9 h after 40 J/m^2 of UVC treatment (Figure 6). In contrast, their differentiated counterparts were much more resistant to UVC treatment, and there

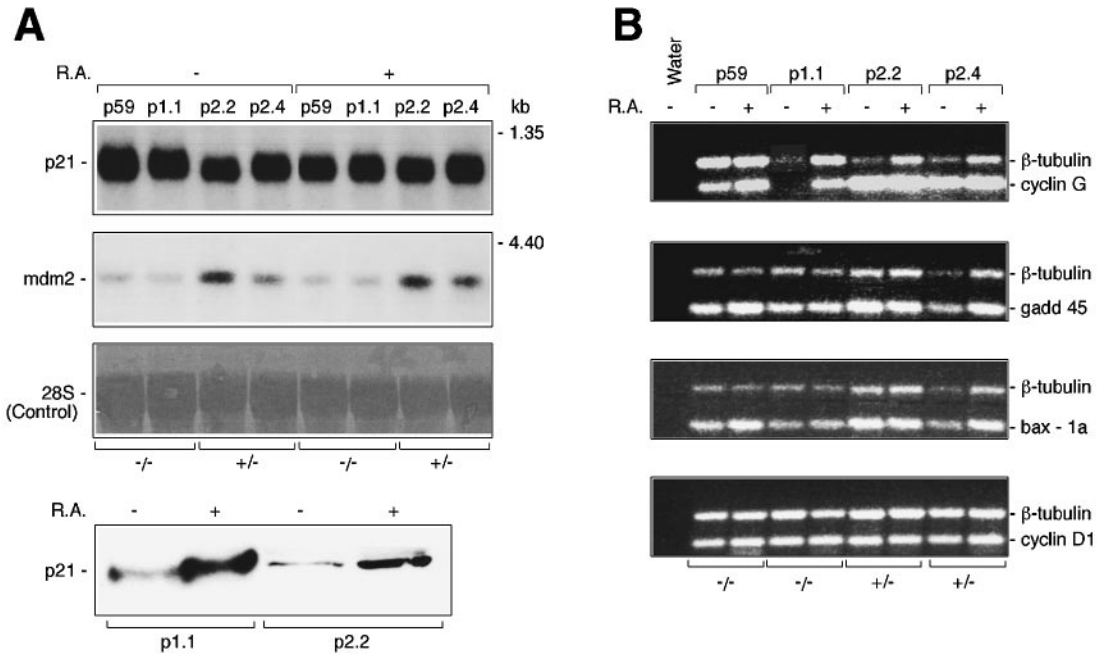

Fig. 4. Expression of p53 target genes. (**A**) Northern blot analysis of p21 and mdm2 levels and Western blot analysis of p21 levels in undifferentiated (–) and differentiated (+) p53+/– and –/– cells. 20 μg of total RNA were loaded per lane. Whole cell extracts from 5×10^5 cells were used for analysis of p21 protein levels using rabbit polyclonal antibody, M19. (**B**) RT–PCR analysis of cyclin G, gadd 45, bax-1a and cyclin D1 levels in undifferentiated (–) and differentiated (+) cells. Control for loading was determined by β-tubulin expression levels in each reaction. First lane indicates water control in the absence of RNA.

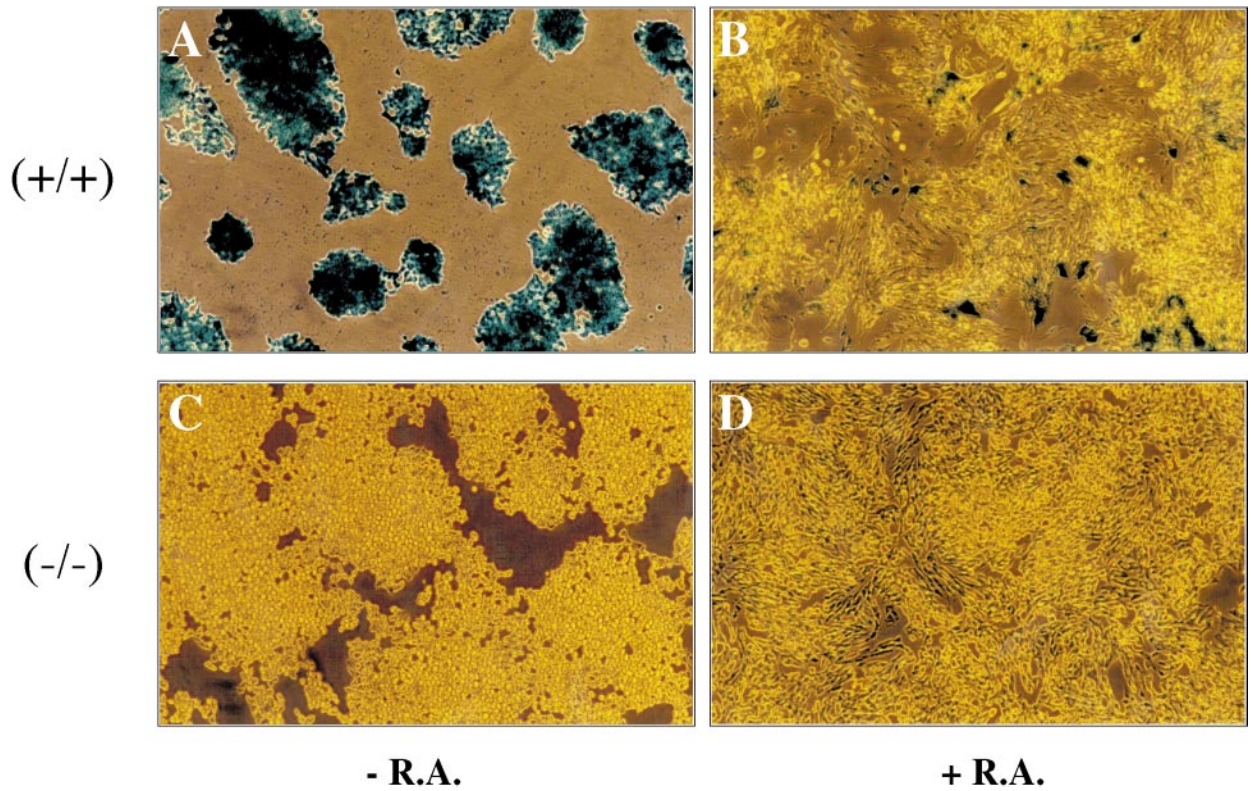

Fig. 5. p53 is transcriptionally active in undifferentiated ES cells and this activity is lost upon differentiation with RA. Both p53+/+ (CCE) and p53–/– (p59) ES cell lines stably transfected with the p53-responsive element–β-galactosidase gene construct (pRGCΔfos lacZ) were stained for β-galactosidase activity before (–) and after (+) differentiation with retinoic acid for 96 h.

was no observable cell death at 9 h post-irradiation (Figure 6). Likewise, both undifferentiated and differentiated p53–/– ES cells were highly resistant to UVC treatment and no cell death was observed at 9 h post-irradiation (Figure 6). These data are consistent with the fact that undifferentiated wild-type ES cells would not allow

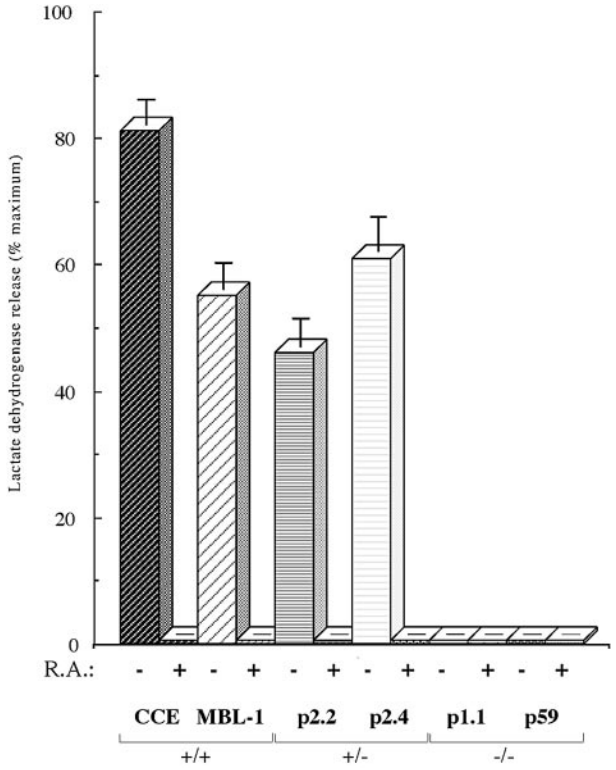

Fig. 6. Undifferentiated ES cells are hypersensitive to UV irradiation. Both undifferentiated and differentiated p53+/+ (CCE and MBL-1), +/– (p2.2 and p2.4) and p53–/– (p1.1 and p59) ES cells were irradiated with 40 J/m^2 of UVC, and the survival rate was determined as a function of LDH release 9 h post-irradiation. Data represent average of triplicates.

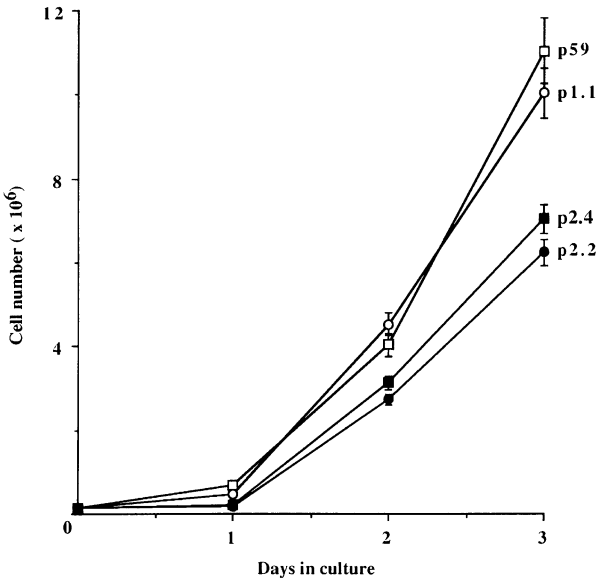

Fig. 7. p53–/– ES cells have higher proliferative potential than p53+/– cells. Cell numbers were determined at the time points indicated. Data represent average of triplicates.

mutations to be propagated and that damaged cells would be aborted, further implying a function for p53 in this process.

ES cells lacking p53 proliferate faster and are less susceptible to apoptosis

As undifferentiated ES cells are able to proliferate in the presence of functional p53 without undergoing apoptosis or cell cycle arrest, we next investigated whether the presence of p53 affects their proliferation rate. Undifferentiated ES cells (p53+/– and –/–) derived from littermate embryos were cultured and their cell numbers were determined over 3 days. p53–/– ES cells were found to have higher proliferative potential than p53+/– ES cells, resulting in a higher number of –/– cells evident by 2 days in culture (Figure 7). The p53–/– ES cells proliferated with a doubling time of ~10 h compared with p53+/– ES cells which were doubling every 14–15 h. Thus, the proliferation rate of p53+/– cells is ~60% of the p53–/– cells (Figure 7), indicating that the presence of functional p53 affects proliferation of undifferentiated ES cells.

We next examined whether the presence of high levels of p53 regulates the rate of apoptosis during differentiation, besides influencing proliferation. Both p53+/– and –/– ES cells were induced to differentiate with RA and the number of cells undergoing apoptosis was determined after 3 days. Differentiation resulted in ~25% of p53+/– cells undergoing apoptosis, indicated by their sub-G_1 DNA content, compared with ~6% of untreated cells (Figure 8A). In contrast to the p53+/– cells, differentiation of p53–/– ES cells resulted in only ~6% of cells undergoing apoptosis compared with ~3% apoptotic cells in undifferentiated cultures (Figure 8A). These data indicate that the lack of p53 leads to a reduction in the apoptotic rate during differentiation.

Since p53–/– ES cells show a reduction in the apoptotic rate during differentiation, we investigated whether this leads to an increase in the number of differentiated cells. Both p53+/– and –/– undifferentiated cells were cultured in the presence of RA and the cell numbers were determined. By 3 days after differentiation, there were more p53–/– differentiated cells than p53+/– cells (Figure 8B), even though the extent of differentiation, as determined by loss of ECMA-7 antigen, was similar (data not shown). The increase in the p53–/– population was not due to the slower differentiation rate of p53–/– ES cells at any given time (data not shown), thus excluding the possibility that varying differentiation rates may contribute to the increase in the p53–/– cell population. The number of p53–/– differentiated cells increased dramatically by day 6 (Figure 8B), which could be due either to a reduction in the apoptotic rate (as shown in Figure 8A), or to increased proliferative potential of differentiated p53–/– cells, or both. Thus, to examine whether this increase in the number of differentiated cells was also due to enhanced proliferation of the differentiated p53–/– cells, both p53+/– and –/– ES cells treated with RA for 3 days were re-plated at equal density and the cell numbers were determined after a further 3 days. The p53–/– differentiated cells proliferated faster than p53+/– differentiated cells (Figure 8C) resulting in ~2-fold more p53–/– cells than p53+/– cells (Figure 8C), consistent with previous observations that fibroblasts lacking p53 have a higher proliferative potential than p53+/+ and +/– fibroblasts (Harvey *et al.*, 1993). This indicates that the differentiated p53–/– cells also proliferate faster than their p53+/–

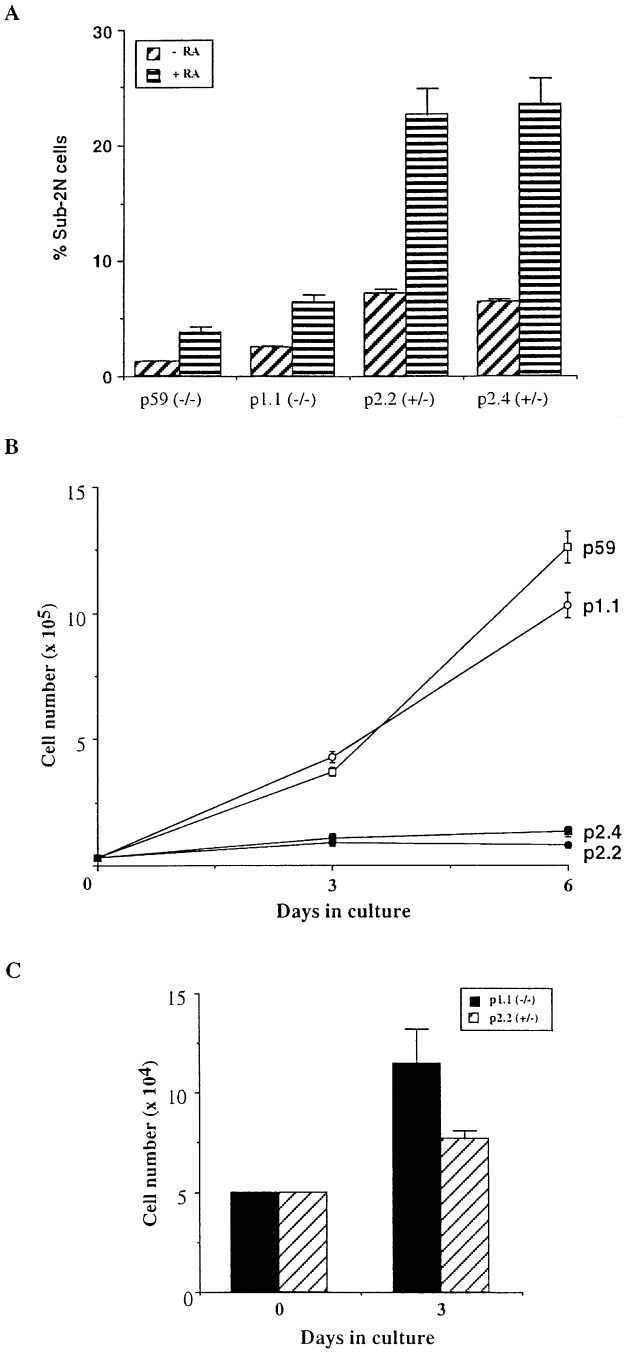

Fig. 8. p53–/– ES cells are less susceptible to apoptosis during differentiation and their differentiated derivatives proliferate faster than p53+/– cells. (**A**) Rate of apoptosis during differentiation with retinoic acid was determined as a function of cells with sub-G_1 DNA content. (**B**) Differentiation results in increased cell numbers in p53–/– cultures in contrast to p53+/– cultures. (**C**) p53–/– differentiated cells proliferate faster than their p53+/– counterparts. Data represent average of triplicates.

counterparts, further contributing to a larger pool of p53–/– cells after differentiation (Figure 8B).

p53 is functionally active in apoptosing cells during differentiation

Lack of p53 results in a reduction in the apoptotic rate accompanying differentiation, implying an active role for p53 in this process. Therefore, we next sought to determine whether p53 is functional in apoptosing cells. Undifferentiated ES cells expressing pRGCΔfos lacZ were treated with RA and were stained with Hoechst-33342 and propidium iodide (PI) to detect early apoptotic cells, which specifically exclude PI but stain positive for Hoechst-33342 (Belloc *et al.*, 1994), together with a FITC-labelled substrate for β-galactosidase [5-chloromethylfluorescein di-β-D-galactopyranidase (CMFDG)], to detect β-galactosidase activity by flow cytometry. There was no significant apoptosis in the undifferentiated wild-type ES cell population [Figure 9A(i)], and these cells exhibited β-galactosidase activity (Figure 9B). The levels of β-galactosidase activity were further analysed in both the non-apoptosing and apoptosing populations in wild-type ES cells treated with RA [Figure 9A(ii), G_1 and G_2 respectively]. There was a reduction in β-galactosidase activity in the non-apoptosing population upon RA treatment [Figure 9A(ii), G_1, and 9B]. In contrast, the early apoptotic cell population exhibited strong β-galactosidase activity [Figure 9A(ii), G_2, and 9B], suggesting the presence of functional p53. Wild-type ES cells expressing the mutant pΔfos lacZ construct and p53–/– ES cells expressing pRGCΔfos lacZ did not exhibit β-galactosidase activity (data not shown). These data indicate that the functional activity of p53 is maintained in apoptosing cells upon differentiation, and that this activity is down-regulated in the differentiating population.

Is the conformational change of p53 essential for differentiation?

To gain further information for a possible causal relationship between the conformational and functional modulation of p53 and the ES cell differentiation process, we transiently expressed the tsp53val135 mutant in p53+/– and +/+ ES cells and analysed the effects during differentiation at either 32 or 39°C. We reasoned that the tsp53 product would alter the conformation of endogenous p53 to that of the mutant form when cultured at 39°C as demonstrated in previous studies (Milner and Metcalf, 1991), and hence might affect the differentiation process. Similarly, endogenous p53 in a mutant conformation in differentiating cells might alter the activity of the transfected tsp53 product at 32°C, and hence, would negate the effects of the exogenous functional wild-type p53 in differentiated cells.

All ES cells were transfected with either vector (pPGK) and plasmid expressing the green fluorescent protein (pPGK-EGFP) (to detect transfected cells) or the pPGKtsp53 and pPGK-EGFP, and were induced to differentiate with RA. Three days later, the rate of differentiation and the rate of apoptosis accompanying differentiation were analysed. There was no significant difference in the rate of differentiation in tsp53-transfected cells (data not shown). The rate of apoptosis in the GFP-positive population was determined by TUNEL reaction, which scores cells with DNA strand breaks (Gottlieb *et al.*, 1994). Undifferentiated CCE cells transfected with vector and cultured at 39°C had 4.0% and 12.5% apoptotic cells in two independent experiments. Upon treatment with RA, there was an increase in the apoptotic rate to 28.9% and 31.0% (Table I). When cultured at 32°C, the basal apoptotic rates for vector-transfected undifferentiated cells were 8.0% and 6.3%, and upon differentiation, these levels

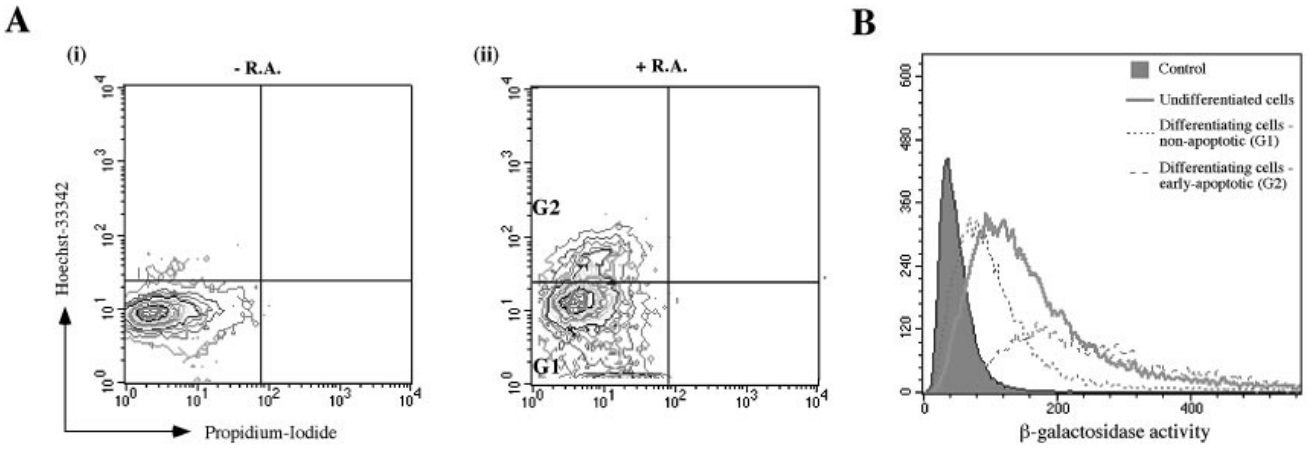

Fig. 9. p53 is functionally active in apoptosing cells during differentiation. (**A**) Detection of early apoptotic cells in undifferentiated (i) and differentiating (ii) CCE ES cells stably transfected with the p53-responsive element–β-galactosidase gene construct (pRGCΔfos lacZ), (CCE-RGC cells) by Hoechst-33342 and PI staining. Gates were set on non-apoptotic cells (G_1) and early apoptotic populations (G_2) of differentiating cells for analysis of β-galactosidase activity. (**B**) The levels of β-galactosidase activity were determined in the undifferentiated cells (——), and the non-apoptotic (·····) and apoptotic cell (- - -) populations of differentiating cells. The filled peak represents background β-galactosidase activity in CCE ES cells expressing the mutant pΔfos lacZ construct (control).

Table I. Effects of tsp53 expression on apoptosis during ES cell differentiation

Cell line:	Transfected with:	R.A.:	% Apoptotic cells	
			Expt. 1	Expt. 2
CCE (+/+)	pPGK + pEGFP	–	4.0	12.5
	(39°C)	+	28.9	31.0
	(32°C)	–	8.0	6.3
		+	13.2	18.4
	tsp53 +pEGFP	–	7.7	4.8
	(39°C)	+	8.4	6.3
	(32°C)	–	6.3	7.4
		+	12.6	27.0

increased to 13.2% and 18.4%, indicating the occurrence of apoptosis during differentiation (Table I). Undifferentiated CCE cells transfected with tsp53 mutant had 7.7% and 4.8% apoptotic cells when cultured at 39°C, and upon differentiation, there was no significant increase in the rate of apoptosis, the levels being 8.4% and 6.3% (Table I). However, when the tsp53 mutant-transfected undifferentiated cells were grown at 32°C, the basal apoptotic rate was 6.3% and 7.4%, and the levels rose to 12.6% and 27.0% upon differentiation induction, similar to that observed in vector-transfected cells (Table I). Similarly, the rate of apoptosis in vector and tsp53-transfected p2.2 cells induced to differentiate at 32°C was comparable, whereas culturing of tsp53-transfected p2.2 cells at 39°C resulted in a significant reduction in the apoptotic rate compared with vector-transfected p2.2 cells upon differentiation (data not shown). These results indicate that the expression of tsp53 in the mutant conformation reduces the apoptotic rate, suggesting that p53 is indeed functional during differentiation in apoptosing cells, and that the conformational change and functional modulation of p53 activity might be a mechanism allowing survival of differentiated cells.

Discussion

Embryonic stem cell lines were employed as a model system to investigate the role of p53 during *in vitro* differentiation. Undifferentiated ES cells were found to express high levels of functional p53 in a wild-type conformation, and differentiation resulted in a reduction in p53 levels and a conformational change to the mutant form. Functional activity of p53 was lost upon differentiation, concomitant with these cells becoming less sensitive to UV irradiation as compared with undifferentiated ES cells. However, p53 is maintained in a functionally active state in the apoptotic cell population. Expression of tsp53 in the mutant conformation led to a reduction in the apoptotic rate accompanying differentiation in wild-type ES cells. Similarly, p53–/– ES cells were more resistant to differentiation-induced apoptosis, suggesting that the conformational change might be a mechanism regulating p53 functional activity during differentiation.

In response to stress signals, p53 has been shown to be functionally activated and acts as the 'guardian of the genome', inducing either cell cycle arrest to allow repair of damaged DNA, thereby negatively regulating proliferation, or apoptosis, depending on the cell type (for review, see Gottlieb and Oren, 1996; Ko and Prives, 1996). Moreover, overexpression of p53 has also been shown to result in similar effects. However, undifferentiated ES cells are neither arrested nor do they undergo apoptosis in the presence of high levels of functional p53. How ES cells maintain high p53 levels is presently unclear. It is possible that the recently suggested mechanism of regulation of p53 stability by mdm2 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) might not be operational in this system, and hence p53 is maintained at high levels. This hypothesis is currently under investigation. It is however, likely that these cells have evolved to tolerate the negative effects of p53, probably by the expression of other growth-promoting genes, like the expression of phosphorylated pRb in undifferentiated ES cells (Savatier *et al.*, 1994), which may override the effects of p53 as shown for retinal cells (Morgenbesser *et al.*, 1994). In this respect, it is

interesting to note that the concerted expression of oncogenes like *myc* and *ras* in mouse prostate cells has been shown to bypass p53's inhibitory effects (Lu *et al.*, 1992). In addition, proliferating ES cells have a short cell cycle with a very short G_1 phase (Savatier *et al.*, 1995). Hence, it is possible that p53 is not able to execute cell cycle arrest in such a short G_1 phase. Support for this notion was reported by Levine and co-workers who demonstrated that wild-type p53 acts in G_1 to block cells from entering the S phase of the cell cycle (Martinez *et al.*, 1991). Once cells are in the S phase, they are immune to negative regulation by p53 and are able to tolerate p53 (Martinez *et al.*, 1991). It is likely that stem cells with a very short G_1 phase have evolved to tolerate high levels of functional p53 expression, which is probably maintained as a 'ready to go' machinery in response to stress stimuli, although we cannot exclude the possibility that some functional inactivation of p53 has occurred which is not measurable by the assays employed.

High p53 levels resulted in preferential expression of some endogenous p53 target genes in undifferentiated ES cells (i.e. cyclin G and mdm2), as well as the β-galactosidase activity driven by the transfected pRGCΔfos lacZ construct. There were no significant differences in the levels of other target genes. These data support the notion that p53 in ES cells has specificity for some promoters containing the p53 binding site, as has been described in other *in vitro* systems by transient transfection experiments (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996), although the mechanism for this differential regulation is at present unclear.

UV treatment of undifferentiated ES cells resulted in immediate cell death, in contrast to their differentiated derivatives. ES cells appear to be unable to repair their DNA when damaged, and damaged cells are being aborted (MacQueen, 1979). Thus, it is conceivable that stem cells have a greater need to prevent propagation of mutations and hence maintain an immediate stress response machinery to combat adverse conditions, in which p53 plays a central role. Upon differentiation, this need is drastically reduced and hence, p53 is functionally inactivated, as has also been suggested by recent work using transgenic mice harbouring the β-galactosidase gene under a p53-responsive promoter (MacCallum *et al.*, 1996; Gottlieb *et al.*, 1997). In this respect, it is worth noting that treatment of pregnant mice with teratogens during early pregnancy resulted in resorption, whereas treatment at later times led to fetuses with malformations (Generoso *et al.*, 1990; Rutledge *et al.*, 1994). p53–/– ES cells are also resistant to UV irradiation, consistent with the finding that p53–/– mice are highly susceptible to irradiation-induced tumorigenesis (Kemp *et al.*, 1994). This further emphasizes the physiological role of a functional p53 in undifferentiated ES cells in guarding the integrity of the genome in order to prevent propagation of mutations through the germline upon differentiation.

Undifferentiated ES cells with a functional p53 protein proliferate slower than p53–/– ES cells. This indicates that functional p53 is involved in regulating proliferation of these cells *in vitro*. Although differentiation renders the p53 protein inactive, the differentiated cells still proliferate slower than their p53–/– counterparts. In this respect, these cells resemble MEFs, which were shown to have inactive p53 lacking DNA binding activity and which cannot transactivate gene expression, yet these cells proliferate more slowly than p53–/– fibroblasts (Harvey *et al.*, 1993; Hupp *et al.*, 1995). This implies that p53 may regulate proliferation differentially in undifferentiated and differentiated cells *in vitro*, by hitherto not well understood mechanisms. Although p53 has been shown to repress transcription of several genes (Ginsberg *et al.*, 1991; Lechner *et al.*, 1992; Mack *et al.*, 1993; Harris *et al.*, 1996), this may not be the mechanism regulating proliferation of differentiated cells, as transcriptional repression was observed when p53 adopted a wild-type conformation (Ginsberg *et al.*, 1991). This raises the possibility that the inactive p53 might be involved in a complex reaction which may involve several cofactors, thereby regulating proliferation *in vitro* in the absence of transcriptional activation. Nevertheless, p53–/– mice develop normally without showing any signs of hyperproliferation except in tumors, which appear at high incidence (Donehower *et al.*, 1992; Jacks *et al.*, 1994). Although this argues against a general role for p53 as a regulator of proliferation during normal development *in vivo*, it has been shown that a proportion of p53–/– mice develop exencephaly, which has been proposed to be due to increased proliferation of cells of neural tissue (Sah *et al.*, 1995). Furthermore, mice lacking mdm2 die due to presence of p53 (de Oca Luna *et al.*, 1995; Jones *et al.*, 1995). Thus, it is likely that p53 regulates proliferation *in vivo* in a tissue- and stage-specific manner.

ES cells induced to differentiate express p53 in both the wild-type and mutant conformation. Analysis of functional activity revealed that the differentiated cells express non-functional p53, whereas apoptotic cells express functional p53. Thus, it is possible that the differentiated cells express non-functional p53 in the mutant conformation, and that the apoptosing cells maintain functional p53 in the wild-type conformation. This notion is supported by previous work which demonstrated that terminally differentiated cells express p53 in a mutant conformation (Rivas *et al.*, 1992). Furthermore, several studies have indicated that p53 levels remain high in apoptosing cells during the induction of differentiation (Ronen *et al.*, 1996; Soddu *et al.*, 1996), and transcriptionally active p53 has been shown to be both required (Sabbatini *et al.*, 1995; Attardi *et al.*, 1996), and dispensable for apoptosis (Haupt *et al.*, 1995). Moreover, our experiments using ES cell lines transfected with tsp53 and cultured at 39°C showed a significant reduction in the apoptotic rate, indicating that the effect of endogenous wild-type p53, presumably in apoptosing cells, was indeed overruled by the ts mutant. Furthermore, culturing at 32°C did not cause cell cycle arrest (data not shown) or induce apoptosis in undifferentiated ES cells, reiterating that these cells are able to tolerate high levels of functional p53. In addition, there was also no significant increase in apoptosis in differentiating cells cultured at 32°C, suggesting that the exogenous wild-type p53 might be tolerated in mutant conformation-expressing differentiated cells. Although the ratio of transfected to endogenous p53 protein levels, which would influence the experimental outcome, was not determined, the reduction in the apoptotic rate due to the presence of the exogenous p53 in the mutant conformation strongly suggests that modulation of p53 activity regulates the extent of apoptosis

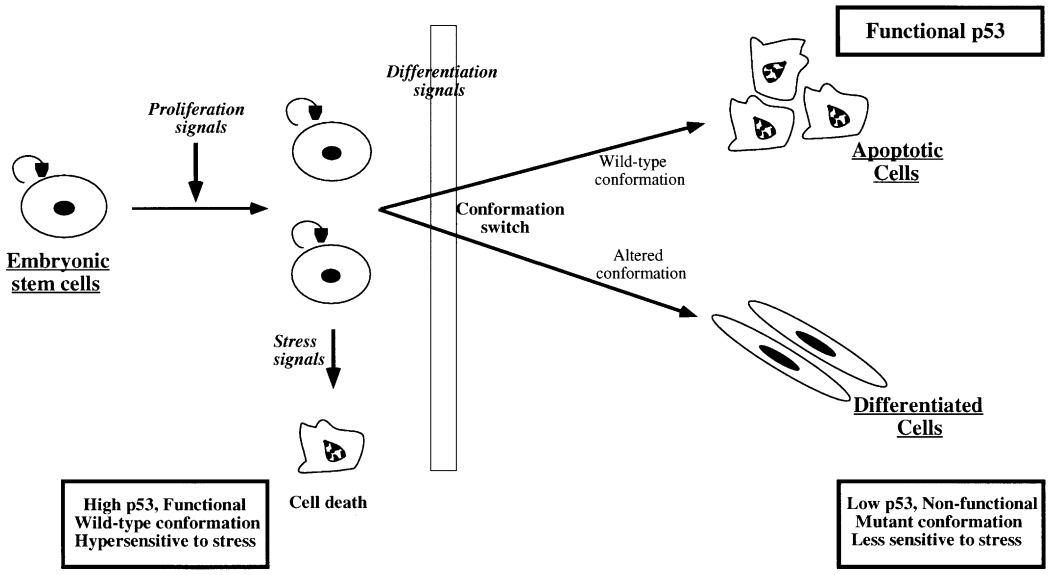

Fig. 10. Proposed model for the role of p53 in ES cell proliferation and differentiation. Undifferentiated ES cells are able to tolerate high levels of functional p53 and continue proliferating. However, they are highly sensitive to genotoxic stress such as UV irradiation, indicating a protective role for p53 in these cells. Upon differentiation induction, the decision is made to either differentiate or undergo apoptosis, and this decision seems to act through p53. Thus, differentiating cells alter their p53 conformational status, which results in modulation of its function, hence promoting cell survival during differentiation. However, cells which maintain the wild-type conformation and function, are programmed to undergo cell death.

accompanying differentiation. Moreover, investigation with a p53–/– ES cell line stably expressing the tsp53 mutant revealed an increase in the rate of apoptosis accompanying differentiation at 32°C as compared with differentiation at 39°C (data not shown). These results suggest that the conformational change of p53, and a consequent functional inactivation, is brought about to prevent differentiating ES cells from apoptosis and to promote cell survival. Thus far, conformation change of p53 has been shown to be associated with changes in its functional activity only in studies using the tsp53 mutant (Michalovitz *et al.*, 1990; Gottlieb *et al.*, 1994; Milner, 1995), although changes in the conformational status of endogenous p53 has been reported in cultured cells (Milner and Watson, 1990). Our study describes an association between the conformational status and functional activity of endogenous p53. Furthermore, we demonstrate directly that apoptosing ES cells maintain functionally active p53. Thus, although mechanisms regulating the conformational change of p53 are at present unclear, conformational change of endogenous p53 might be an important process regulating the homeostatic balance between cell death and survival.

Lutzker and Levine have recently reported that F9 EC cells express high levels of p53 in a wild-type conformation which is functionally inactive, and that differentiation results in transcriptional activation as determined by up-regulation of p21 and mdm2 mRNA levels (Lutzker and Levine, 1996). Differentiated EC cells lacking p53 did not show an increase in p21 and mdm2 mRNA levels, implying that differentiated wild-type EC cells have functional p53. However, p21 and mdm2 have been shown to be regulated independently of p53 during embryonic development (Parker *et al.*, 1995; B.Wasylyk, personal communication). We also observed that p21 protein levels increase upon differentiation in p53–/– ES cell lines (Figure 4). Hence, the observed differences could be attributed to variation in cell type and consequently, different gene expression patterns. EC cells have gone through a stringent selection to form tumors *in vivo*, and it is possible that this selects for mutations of some unknown 'modifier' of p53 function *in vivo*. Hence, the inactivation of p53 in EC cells might be a part of the tumorigenic process, although the molecular mechanisms underlying these differences remain to be elucidated.

In conclusion, our studies have revealed that ES cells are unique in that they tolerate high levels of functional p53 in a wild-type conformation without undergoing cell cycle arrest or apoptosis. These cells are hypersensitive to stress stimuli, a response dependent on expression of functional p53, indicating a protective role for p53 which presumably prevents propagation of mutations through the germline (Figure 10). Differentiation results in a reduction of p53 levels, with a concomitant reduction in its functional activity and a shift in its conformational status. In contrast, apoptosis during differentiation is dependent on expression of functional p53. The conformational shift appears to be a mechanism regulating p53 functional activity during differentiation, as interference with the conformational change alters the rate of apoptosis accompanying differentiation, implying a regulatory role for p53 during the early differentiation process (Figure 10). Thus, the conformational switch and functional inactivation of p53 seems to allow the differentiating cell to escape from apoptosis, and highlights the importance of controlling p53 activity during development for cell survival during differentiation. These results provide new insights into the role of p53 as an important regulator in an early differentiation process, controlling the balance between differentiated cells and cells undergoing apoptosis.

Materials and methods

Cells and induction of ES cell differentiation

The following ES cell lines were derived from outgrowth of blastocysts isolated from p53+/– females (129×C57BL/6) mated with p53–/– males

(129×C57BL/6): p2.2 (+/–), p2.4 (+/–), p1.1 (–/–) and p59 (–/–). CCE (+/+) and MBL-1 (+/+) ES cell lines have been described previously (Robertson *et al.*, 1986; Wang *et al.*, 1991). All ES cells were cultured in the presence of LIF as described earlier (Wang *et al.*, 1991). Differentiation was induced either by culturing 1×10^6 undifferentiated ES cells with 3×10^{-7} M *trans* RA for 4 days, or by formation of embryoid bodies in the absence of LIF for 6 days, and replating the embryoid bodies to form monolayers of differentiated cells, as described by Evans and Kaufmann (1981). These differentiated cells were harvested when they appeared morphologically differentiated. Extent of differentiation was determined by staining with anti-ECMA-7 antibody, which recognizes an epitope on undifferentiated ES cells whose expression is lost upon differentiation (Kemler, 1980). Cultures with > 90% of cells that did not stain positive for ECMA-7 were used for the experiments. Primary MEFs were prepared and cultured as described previously (Schreiber *et al.*, 1995). MEFs with or without 40 J/m^2 of UVC treatment were harvested 24 h post-irradiation and used for the experiments. RAC-65, an EC cell line resistant to RA-induced differentiation was cultured in the absence of LIF, as previously described (Pratt *et al.*, 1990).

Plasmids and transfection procedures

To generate stable clones expressing either the pRGCΔfos lacZ or pΔfos lacZ (Frebourg *et al.*, 1992), CCE and p59 cells were co-transfected with the respective plasmids and pPGK-hygromycin (Sibilia and Wagner, 1995) and clones were selected and maintained in 125 μg/ml of hygromycin-B (Calbiochem). For transient assays, 1×10^5 cells were transfected by the $CaPO_4$ coprecipitation method with pPGK (gift from Dr Anton Berns) or pPGKtsp53val135, which was derived from subcloning the *Eco*RI fragment of pLXSNtsp53val135 (Gottlieb *et al.*, 1994) into pPGK, with pPGK-EGFP, derived from subcloning an *Eco*47III–*Sma*I fragment from pCMV-EGFP (Clonetech) into pPGK. Media were changed 18 h later and cells were cultured in the absence or presence of RA to induce differentiation at either 32 or 39°C, as indicated. Cells were harvested 72 h later and analysed for apoptosis.

Assays for apoptosis and cell death

The rate of apoptosis was determined both by sub-2N analysis and by TUNEL reaction. For the former, 1×10^6 cells were fixed in 70% ethanol overnight, centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma) and PI (Sigma). The stained cells were analysed in a FACScan (Becton Dickinson) immediately using the CellQuest program. TUNEL assay was performed as described before (Gottlieb *et al.*, 1994) with minor modifications. Briefly, cells were pelleted and fixed in 2% paraformaldehyde at 4°C for 1 h, washed in PBS and fixed in 70% ethanol at 4°C overnight. Cells were then washed in PBS and the TUNEL reaction was carried out using Cy5-dCTP (Amersham). The cells were analysed in a FACS Vantage (Becton Dickinson) at 670 nm fluorescence for Cy5 and EGFP was detected in the green channel.

For colorimetric quantitation of the amount of LDH released into the culture medium, a CytoTox 96 assay kit (Promega, Madison, WI) was used as described earlier (Schreiber *et al.*, 1995). Briefly, 1×10^4 of both undifferentiated and differentiated cells were plated in triplicate in a 96-well plate and irradiated with 40 J/m^2 of UVC. The survival rate was determined as a function of LDH release 9 h post-irradiation.

Proliferation rates

To determine the proliferation rates of p53+/– and –/– ES cells, 1×10^5 undifferentiated p59, p1.1, p2.2 and p2.4 cells were plated in triplicate in six-well plates and the cell numbers were determined at the indicated time points using a CASY^R cell counter. To determine the proliferation rates of cells treated with RA, 3×10^4 p59, p1.1, p2.2 and p2.4 undifferentiated ES cells were cultured in triplicate in the absence or presence of RA and the cell numbers were determined at indicated time points. Furthermore, to examine the proliferation rates of differentiated cells, 5×10^4 p1.1 and p2.2 cells treated with RA for 3 days (>90% of both cells not expressing ECMA-7 antigen) were re-plated in triplicate and cell numbers determined after a further 3 days.

Immunoblot and immunoprecipitation analysis

For determination of the steady-state p53 and p21 levels, cell extracts were prepared, and 20 μg of extracts were analysed by Western blotting using rabbit polyclonal anti-p53 antibody CM5 (Novocastro Labs Ltd), or rabbit polyclonal anti-p21 antibody (M19; Santa Cruz) as described previously (Gottlieb *et al.*, 1994). Immunoprecipitations were carried out according to Gannon *et al.* (1990) using 100 μg of whole cell extracts, with PAb246 and PAb240 antibodies (Oncogene Science), and were subsequently immunoblotted and probed with CM5 antibody. Quantitations were performed using the ImageQuant program.

Electrophoretic mobility shift assays

Nuclear extracts were prepared and gel retardation assays (EMSAs) were performed as described by Funk *et al.* (1992). Briefly, 10 μg of nuclear extract were incubated in 5% glycerol, 20 mM HEPES–KOH pH 7.9, 50 mM KCl, 1 mM DTT, 1 mg BSA and 1 μg poly(dI–dC) with 1 ng of radioactively labelled probe for 30 min at room temperature, followed by separation of DNA–protein complexes on 4% Tris–glycine gels. In experiments to further confirm the specificity of the binding reaction, anti-p53 antibody (100 ng of PAb 421; Oncogene Science) was added to the reaction mixture. The following double-stranded oligonucleotides were used as probes: wild-type: 5′-AGCTTA-GACATGCCTAGACATGCCTA-3′ ; random Oligo : 5′-TATGTCTAA-GGGACCTGCGGTTGGCATTGATCTTG-3′.

Northern blot and RT–PCR analysis

RNA analyses were carried out using standard Northern and RT–PCR techniques (Schreiber *et al.*, 1995). Probes for p21 and mdm2 were generated by RT–PCR using the following primers: p21, 5′-GGA-GCAAAGTGTGCCGTTGTC-3′ and 5′-GAGGAAGTACTGGGCCTC-TTG-3′; mdm2, 5′-GGCTGTAAGTCAGCAAGACTC-3′ and 5′-CCAGGTAGCTCATCTGTGTTC-3′. RT–PCR analysis were performed using the following primers: cyclin G, 5′-GGTACAGGCGAA-GCATCTTG-3′ and 5′-GCAAGGTGTCGTGAACGAGTG-3′; bax-1a, 5′-GGCGAATTGGAGATGAACTGG-3′ and 5′-GCTAGCAAAGTA-GAAGAGGGC-3′; gadd45, 5′-GAGCAGAAGACCGAAAGGATG-3′ and 5′-GTGAAATGGATCTGCAGAGCC-3′; cyclin D1, 5′-GCTGCG-AAGTGGAGACCATC-3′ and 5′-CTTCTGCTCCTCACAGACCTC-3′; and β-tubulin, 5′-CAACGTCAAGACGGCCGTGTG-3′ and 5′-GACA-GAGGCAAACTGAGCACC-3′.

Determination of β-galactosidase activity

Cells were subjected to X-Gal staining as described by Sibilia and Wagner (1995). For detection of β-galactosidase activity in early apoptotic cells, cells were washed twice in PBS, resuspended in 0.1 ml of PBS containing 4% serum and loaded with 0.1 ml of 2 mM CMFDG (Molecular Probes) at 37°C, as per the manufacturer's instructions. The cells were incubated at 37°C for 2 min, after which they were resuspended in normal culture medium and incubated at 37°C for 30 min. The cells were then stained with 5 μg/ml of PI and 5 μg/ml Hoechst-33342, and analysed immediately in a FACS Vantage (Becton Dickinson) using the CellQuest program.

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References

Attardi,L.D., Lowe,S.W., Brugarolas,J. and Jacks,T. (1996) Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene mediated apoptosis. *EMBO J.*, **15**, 3693–3701.

Banerjee,D., Lenz,H.J., Schnieders,B.D., Manno,J, Ju,J.F., Spears,C.P., Hochhauser,D., Danenberg,K., Danenberg,P. and Bertino,J.R. (1995) Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. *Cell Growth Differ.*, **6**, 1405–1413.

Belloc,F., Dumain,P., Boisseau,M.R., Jalloustre,C., Reiffers,J., Bernard,P. and Lacombe,F. (1994) A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry*, **17**, 59–65.

Brenner,L., Munoz-Antonia,T., Vellucci,V.F., Zhou,Z. and Reiss,M. (1993) Wild-type p53 tumor suppressor gene restores differentiation of human squamous carcinoma cells but not the response to transforming growth factor β. *Cell Growth Differ.*, **4**, 993–1004.

Chen,X., Ko,L.J., Jayaraman,L. and Prives,C. (1996) p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev.*, **10**, 2438–2451.

de Oca Luna,R.M., Wagner,D.S. and Lozano,G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203–206.

Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery,C.A.,Jr, Butel,J.S. and Bradley,A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215–221.

Dulic,V., Kaufmann,W.K., Wilson,S.J., Tlsty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53 dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023.

Ehinger,M., Nilsson,E., Persson,A.M., Olsen,I. and Gullberg,U. (1995) Involvement of the tumor suppressor gene p53 in tumor necrosis factor-induced differentiation of the leukemic cell line K562. *Cell Growth Differ.*, **6**, 9–17.

El-Deiry,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,J.A. and Vogelstein,B. (1992) Definition of a consensus binding site for p53. *Nature Genet.*, **1**, 45–49.

Evans,M.J. and Kaufman,M. (1981) Establishment in culture of pluripotent cells from mouse embryos. *Nature*, **292**, 154–156.

Evans,M.J., Lovell-Badge,R.H., Stern,P.L. and Stinnakre,M.G. (1979) Cell lineage of the mouse embryo and embryonal carcinoma cells: Forssman antigen distribution and patterns of protein synthesis. In Le Douarin,N. (ed.), *Cell Lineages, Stem Cells and Cell Determination*. INSERM Symposium No. 10. Elsevier/North-Holland Biomedical Press. Amsterdam, The Netherlands, pp. 115–129.

Feinstein,E., Gale,R.P., Reed,J. and Canaani,E. (1992) Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene*, **7**, 1853–1857.

Frebourg,T., Barbier,N., Kassel,J., Ng,Y.S., Romero,P. and Friend,S.H. (1992) A functional screen for germ line p53 mutations based on transcriptional activation. *Cancer Res.*, **52**, 6976–6978.

Friedlander,P., Haupt,Y., Prives,C. and Oren,M. (1996) A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. *Mol. Cell. Biol.*, **16**, 4961–4971.

Funk,W.D., Pak,D.T., Karas,R.H., Wright,W.E. and Shay,J.W. (1992) A transcriptionally active DNA-binding site for human p53 protein complexes. *Mol. Cell. Biol.*, **12**, 2866–2871.

Gannon,J.V., Greaves,R. and Lane,D. (1990) Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595–1602.

Generoso,W.M., Rutledge,J.C. and Aronson,J. (1990) Developmental anomalies: Mutational consequence of mouse zygote exposure. In Allen,J.A., Bridges,B.A., Lyon,M.F., Moses,M.J. and Russell,L.B. (eds), *Biology of Mammalian Germ Cell Mutagenesis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 34, pp. 311–320.

Ginsberg,D., Mechtor,F., Yaniv,M. and Oren,M. (1991) Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl Acad. Sci. USA*, **88**, 9979–9983.

Gottlieb,E., Haffner,R., von Ruden,T., Wagner,E.F. and Oren,M. (1994) Down-regulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. *EMBO J.*, **13**, 1368–1374.

Gottlieb,E., Haffner,R., King,A., Asher,G., Gruss,P., Lonai,P. and Oren,M. (1997) Transgenic mouse model for studying the transcriptional activity of the p53 protein: age and tissue dependent changes in radiation-induced activation during embryogenesis. *EMBO J.*, **16**, 1381–1390.

Gottlieb,M.T. and Oren,M. (1996) p53 in growth control and neoplasia. *Biochim. Biophys. Acta*, **1287**, 77–102.

Harris,L.C., Remack,J.S., Houghton,P.J. and Brent,T.P. (1996) Wild-type p53 suppresses transcription of the O^6-methylguanine-DNA methyltransferase gene. *Cancer Res.*, **56**, 2029–2032.

Harvey,M., Sands,A.T., Weiss,R.S., Hegi,M.E., Wiseman,R.W., Pantazis,P., Giovanella,B.C., Tainsky,M.A., Bradley,A. and Donehower,L.A. (1993) In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, **8**, 2457–2467.

Haupt,Y., Rowan,S., Shaulian,E., Vousden,K. and Oren,M. (1995) Induction of apoptosis in HeLa cells by transactivation-deficient p53. *Genes Dev.*, **9**, 2170–2183.

Haupt,Y., Maya,R., Kazaz,A. and Oren,M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296–299.

Hunter,T. (1993) Braking the cell-cycle. *Cell*, **75**, 839–841.

Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) Regulation of the specific DNA binding function of p53. *Cell*, **71**, 875–886.

Hupp,T.R., Sparks,A. and Lane,D. (1995) Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell*, **83**, 237–245.

Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T. and Weinberg,R.A. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.*, **4**, 1–7.

Jones,S.N., Roe,A.E., Donehower,L.A. and Bradley,A. (1995) Rescue of embryonic lethality on Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206–208.

Kastan,M.B., Onyekwere, O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991a) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.

Kastan,M.B., Radin,A.I., Kuerbitz,S.J., Onyekwere,O., Wolkow,C.A., Civin,C.I., Stone,T., Woo,K.D., Ravindranath,Y. and Craig,R.W. (1991b) Levels of p53 increase with maturation in human hematopoietic cells. *Cancer Res.*, **51**, 4279–4286.

Kemler,R. (1980) Analysis of mouse embryonic cell differentiation. In Saner,H.W. (ed.), *Progress in Developmental Biology*. Gustav Fischer Verlag, Stuttgart, Germany, band 26, pp. 175–181.

Kemp,C.J., Wheldon,T. and Balmain,A. (1994) p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nature Genet.*, **8**, 66–69.

Knippschild,U., Oren,M. and Deppert,W. (1996) Abrogation of wild-type p53 mediated growth-inhibition by nuclear exclusion. *Oncogene*, **12**, 1755–1765.

Knudson,A.G. (1992) Stem cell regulation, tissue ontogeny, and oncogenic events. *Semin. Cancer Biol.*, **3**, 99–106.

Ko,L.J. and Prives,C. (1996) p53: puzzle and paradigm. *Genes Dev.*, **10**, 1054–1072.

Kubbutat,M.H.G., Jones,S.N. and Vousden,K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, **387**, 299–303.

Lechner,M.S., Mack,D.H., Finicle,A.B., Crook,T., Vousden,K.H. and Lamins,L.A. (1992) Human papillomavirus E6 protein bind p53 *in vivo* and abrogate p53-mediated repression of transcription. *EMBO J.*, **11**, 3045–3052.

Louis,J.M., McFarland,V.W., May,P. and Mora,P.T. (1988) The phosphoprotein p53 is down-regulated post transcriptionally during embryogenesis in vertebrates. *Biochim. Biophys. Acta*, **950**, 395–402.

Lu,X. and Lane,D. (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell*, **75**, 765–778.

Lu,X., Park,S.H., Thompson,T.C. and Lane,D.P. (1992) ras-induced hyperplasia occurs with mutation of p53, but activated ras and myc together can induce carcinoma without p53 mutation. *Cell*, **70**, 153–161.

Ludwig,R.L., Bates,S. and Vousden,K.H. (1996) Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. *Mol. Cell. Biol.*, **16**, 4952–4960.

Lutzker,S.G. and Levine,A.J. (1996) A functionally inactive p53 protein in teratocarcinoma cells is activated by either DNA damage or cellular differentiation. *Nature Med.*, **2**, 804–810.

MacCallum,D.E., Hupp,T.R., Midgley,C.A., Stuart,D., Campbell,S.J., Harper,A., Walsh,F.S., Wright,E.G., Balmain,A., Lane,D.P. and Hall,P.A. (1996) The p53 response to ionising radiation in adult and developing murine tissues. *Oncogene*, **13**, 2575–2587.

Mack,D.H., Vartikar,J., Pipas,J.M. and Laimins,L.A. (1993) Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature*, **363**, 281–283.

MacQueen,H.A. (1979) Lethality of radioisotopes in early mouse embryos. *J. Embryol. Exp. Morphol.*, **52**, 203–208.

Maltzman,W. and Czyzyk,L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol. Cell. Biol.*, **4**, 1689–1694.

Martinez. J., Georgoff,I., Martinez,J. and Levine,A.J. (1991) Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev.*, **5**, 151–159.

Michalovitz,D., Halevy, O. and Oren,M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, **62**, 671–680.

Milner,J. (1984) Different forms of p53 detected by monoclonal antibodies in non-dividing and dividing lymphocytes. *Nature*, **310**, 143–145.

Milner,J. (1995) Flexibility: the key to p53 function? *Trends Biochem. Sci.*, **20**, 49–51.

Milner,J. and Medcalf,E.A. (1991) Cotranslation of activated mutant p53 with wild-type drives the wild-type p53 protein into the mutant conformation. *Cell*, **65**, 765–774.

Milner,J. and Watson,J.V. (1990) Addition of fresh medium induces cell cycle and conformation changes in p53, a tumor suppressor protein. *Oncogene*, **5**, 1683–1690.
Morgenbesser,S.D., Williams,B.O., Jacks,T. and DePinho,R.A. (1994) p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature*, **371**, 72–74.
Nevins,J.R. (1992) E2F; a link between Rb tumor suppressor protein and viral oncoproteins. *Science*, **258**, 424–429.
Oren,M., Maltzman,W. and Levine,A.J. (1981) Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Mol. Cell. Biol.*, **1**, 101–110.
Oren,M., Reid,N.C. and Levine,A.J. (1982) Regulation of the cellular p53 tumor antigen in teratocarcinoma cells and their differentiated progeny. *Mol. Cell. Biol.*, **2**, 443–449.
Parker,S.B., Eichele,G., Zhang,P., Rawls,A., Sands,A.T., Bradley,A., Olsen,E.N., Harper,J.W. and Elledge,S.J. (1995) p53-independent expression of p21 Cip in muscle and other terminally differentiating cells. *Science*, **267**, 1024–1027.
Perry,M.E. and Levine,A.J. (1993) Tumor suppressor p53 and the cell cycle. *Curr. Opin. Genes Dev.*, **3**, 50–54.
Pierce,G.B. and Speers,W.C. (1988) Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation. *Cancer Res.*, **48**, 1996–2004.
Pratt,M.A.C., Kralova,J. and McBurney,M.W. (1990) A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Mol. Cell. Biol.*, **10**, 6445–6453.
Purdie,C.A., Harrison,D.J., Peter,A., Dobbie,L., White,S., Howie,S.E.M., Salter,D.M., Bird,C.C., Wylie,A.H., Hooper,M.L. and Clarke,A.R. (1994) Tumor incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene*, **9**, 603–609.
Rivas,C.I., Wisniewski,D., Strife,A., Perez,A., Lambek,C., Bruno,S., Darzynkiewicz,Z. and Clarkson,B. (1992) Constitutive expression of p53 protein in enriched normal human marrow blast cell populations. *Blood*, **79**, 1982–1986.
Robertson,E., Bradley,A., Kuehn,M. and Evans,M. (1986) Germ line transmission of genes introduced into cultured pluripotent cells by retroviral vector. *Nature*, **323**, 445–448.
Rogel,A., Popliker,M., Webb,C.G. and Oren,M. (1985) p53 cellular tumor antigen: Analysis of mRNA levels in normal adult tissues, embryos, and tumors. *Mol. Cell. Biol.*, **5**, 2851–2855.
Ronen,D., Schwartz,D., Teitz,Y., Goldfinger,N. and Rotter,V. (1996) Induction of HL-60 cells to undergo apoptosis is determined by high levels of wild-type p53 protein whereas differentiation of the cells is mediated by lower p53 levels. *Cell Growth Differ.*, **7**, 21–30.
Rutledge,J.C., Shourbaji,A.G., Hughes,L.A., Polifka,J.E., Cruz,Y.P., Bishop,J.B. and Generoso,W.M. (1994) Limb and lower-body duplications induced by retinoic acid in mice. *Proc. Natl Acad. Sci. USA*, **91**, 5436–5440.
Sabbatini,P., Lin,J., Levine,A.J. and White,E. (1995) Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes Dev.*, **9**, 2184–2192.
Sah,V.P., Attardi,L.D., Mulligan,G.J., Williams,B.O., Bronson,R.T. and Jacks,T. (1995) A subset of p53-deficient embryos exhibit exencephaly. *Nature Genet.*, **10**, 175–180.
Savatier,P., Huang,S., Szekely,L., Wilman,K.G. and Samarut,J. (1994) Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. *Oncogene*, **9**, 809–818.
Savatier,P., Lapillonne,H., van Grunsven,L.A., Rudkin,B.B. and Samarut,J. (1995) Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene*, **12**, 309–322.
Schreiber,M., Baumann,B., Cotten,M., Angel,P. and Wagner,E.F. (1995) Fos is an essential component of the mammalian UV response. *EMBO J.*, **14**, 5338–5349.
Shaulsky,G., Goldfinger,N., Peled,A. and Rotter,V. (1991) Involvement of wild-type p53 in pre-B-cell differentiation *in vitro*. *Proc. Natl Acad. Sci. USA*, **88**, 8982–8986.
Sibilia,M. and Wagner E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*, **269**, 234–237.
Soddu,S., Blandino,G., Citro,G., Scardigli,R., Piaggio,G., Ferber,A., Calabretta,B. and Sacchi,A. (1994) Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood*, **83**, 2230–2237.
Soddu,S., Blandino,G., Scardigli,R., Coen,S., Marchetti,A., Rizzi,M.G., Bossi,G., Cimino,L., Crescenzi,M. and Sacchi,A. (1996) Interference with p53 protein inhibits hematopoietic and muscle differentiation. *J. Cell Biol.*, **134**, 193–204.
Symonds,H., Krall,L., Remington,L., Saenz-Robles,M., Lowe,S., Jacks,T. and Dyke,T.V. (1994) p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. *Cell*, **78**, 703–711.
Ueda,H., Ullrich,S.J., Gangemi,D., Kappel,C.A., Ngo,L., Feitelson,M.A. and Jay,G. (1995) Functional inactivation but not structural mutation of p53 causes liver cancer. *Nature Genet.*, **9**, 41–47.
Vogelstein,B. and Kinzler,K. (1992) p53 function and dysfunction. *Cell*, **70**, 523–526.
Wang,Q.M., Jones,J.B. and Studzinski,G.P. (1996) Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D_3 in HL60 cells. *Cancer Res.*, **56**, 264–267.
Wang,Z.Q., Grigoriadis,A.E., Mohle-Steinlein,U. and Wagner,E.F. (1991) A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. *EMBO J.*, **10**, 2437–2450.
Weinberg,W.C., Azzoli,C.A., Chapman,K., Levine,A.J. and Yuspa,S.H. (1995) p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein. *Oncogene*, **10**, 2271–2279.
Wilman,K.G. (1993) The retinoblastoma gene: role in cell cycle control and cell differentiation. *FASEB J.*, **7**, 841–845.
Wolkowicz,R. and Rotter,V. (1997) The DNA binding regulatory domain of p53: See the C. In Soussi,T. (ed.), *Pathologie-Biologie.* in press.

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