The R246S hot-spot p53 mutant exerts dominant-negative effects in embryonic stem cells in vitro and in vivo

Ming Kei Lee[1] and Kanaga Sabapathy[1,2,*]

[1]Division of Cellular and Molecular Research, Humphrey Oei Institute of Cancer Research, National Cancer Centre, 11 Hospital Drive, Singapore 169610, Singapore
[2]Department of Biochemistry, National University of Singapore, 8 Medical Drive, Singapore 117597, Singapore
*Author for correspondence (e-mail: cmrksb@nccs.com.sg)

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Summary

***p53* is the most frequently mutated tumour-suppressor gene in human cancers. Mutant p53 is thought to contribute to carcinogenesis by the acquisition of gain-of-function properties or through the exertion of dominant-negative (DN) effects over the remaining wild-type protein. However, the context in which the DN effects are observed is not well understood. We have therefore generated 'knock-in' mouse embryonic stem (ES) cells to investigate the effects of expressing a commonly found hot-spot p53 mutant, R246S – the mouse equivalent of human R249S, which is associated with hepatocellular carcinomas. We demonstrate here that R246S mutant p53 exhibits DN effects with respect to target gene expression, cell survival and cell cycle arrest both in cells that are in the undifferentiated state and upon differentiation. The knock-in cells contain higher levels of p53 that localizes to the nucleus even in the absence of genotoxic stress and yet remains non-functional, reminiscent of mutant p53 found in human tumours. In a model based on carbon-tetrachloride-induced liver injury, these cells were consistently highly tumorigenic in vivo, similar to $p53^{-/-}$ cells and in contrast to both $p53^{+/+}$ and $p53^{+/-}$ ES cells. These data therefore indicate that the DN effects of mutant p53 are evident in the stem-cell context, in which its expression is relatively high compared with terminally differentiated cells.**

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Introduction

Missense mutations in *p53* are the most common genetic alterations seen in human cancers (Olivier et al., 2004; Vousden and Lu, 2002). It is thought that these mutations lead to defects in the tumour-suppressive properties of p53 and further contribute to carcinogenesis through either acquiring novel gain-of-function (GOF) properties or through the exertion of dominant-negative (DN) effects over the remaining wild-type allele, as was proposed more than a decade ago (Oren, 1992). In addition, loss of heterozygosity (LOH) of the remaining wild-type *p53* allele has been noted in both familial and sporadic cancers containing missense mutations (Nishida et al., 1993; Varley et al., 1997; Forslund et al., 2002; Fenoglio-Preiser et al., 2003). The degree to which both LOH and missense *p53* mutations occur in the same tumours varies, and LOH has been suggested to depend on both the mechanism of genotoxicity of the carcinogenic agent and the tissue type (Nishida et al., 1993; Venkatachalam et al., 2001; Forslund et al., 2002; Fenoglio-Preiser et al., 2003). This suggests that total loss of p53 activity, rather than acquisition of GOF or DN properties, might also be more pertinent to tumorigenesis. Nonetheless, evidence points to all three mechanisms being equally important in contributing to the carcinogenic process, although the specific context in which each mechanism operates is unclear.

The phenomenon of DN effects of missense p53 mutants has been intensely studied, mainly through analyzing the properties of the commonly found hot-spot DNA-binding-domain mutations such as R175H, G245S, R248W, R249S, R273H and R282W (Petitjean et al., 2007). Albeit that the DN effects have been extensively described, conflicting data exist to the contrary. Several investigators have used multiple read-outs, including arrest of cellular growth, cellular survival and transactivation ability, the latter using promoter-reporter assays and target gene expression, to evaluate the DN effect of missense mutations. The results have varied, which could be due to the type of cells used as well as to the chosen method of expressing mutant p53, because many initial studies employed transient-transfection protocols (Chan et al., 2004; Davis et al., 1996; Williams et al., 1995). Later studies employed inducible constructs as well as combi-constructs expressing both wild-type and mutant cDNAs in a single vector (Aurelio et al., 2000; Willis et al., 2004). Nonetheless, all the systems thus far have the setback of expressing mutant and wild-type p53 at experimentally high levels, perhaps not precisely reflecting the physiologically relevant situation.

Mice models have also been used to study this phenomenon. For example, the offspring of transgenic mice containing the p53 mutant A135V that were crossed to $p53^{+/-}$ mice developed a higher incidence of tumours compared with $p53^{+/-}$ mice without the transgene, highlighting the DN effects of the exogenous mutant p53 over the endogenous wild-type one (Harvey et al., 1995). In addition, recent studies using 'knock-in' mice that express physiologically relevant levels of the mutant protein rather than

causing its overexpression demonstrated that the mice expressing one allele of the mutant R172H, the human R175H equivalent, had increased rates of metastasis compared with $p53^{+/-}$ mice, although both had a similar tumour spectrum and survival curve, suggesting that the mutant allele has DN effects (Lang et al., 2004). However, this mutant was not observed as having effective DN properties to promote K-ras-initiated lung adenocarcinomas, in contrast to another hot-spot mutant, R270H, the mouse equivalent of the human R273H mutation, which displayed partial DN activity in this context (Jackson et al., 2005). However, LOH was observed in tumours from both knock-in mice, indicative of residual tumour-suppressive function conferred by the remaining wild-type allele of *p53* (Jackson et al., 2005; Lang et al., 2004). Nonetheless, in other cellular contexts, the R270H mutant displayed stronger DN properties: epithelial-specific expression of R270H in the heterozygote state results in an increased incidence of spontaneous and ultraviolet (UV)B-induced skin tumours, affecting latency, multiplicity and progression, although this was not the case with respect to spontaneous tumours in mice expressing the mutant p53 in all tissues (Wijnhoven et al., 2007). Moreover, DN effects were seen in embryonic stem (ES) cells heterozygous for R270H and in primary cells derived from $p53^{+/R270H}$ and $p53^{+/R172H}$ mice (de Vries et al., 2002), suggesting that the DN effects might be cell-type and even signal specific.

One hot-spot *p53* mutation that has a very high tissue-type association is the R249S mutation (Petitjean et al., 2007), which is strongly associated with hepatocellular carcinomas (HCC) in areas of high exposure to the dietary aflatoxin B_1 (AFB_1) (Staib et al., 2003). It is interesting to note that HCCs occurring in areas not-exposed to AFB_1 do not carry the R249S mutation, indicating a high level of specificity that is required for this mutation to occur (Staib et al., 2003). Treatment of mice and rats with AFB_1 have failed to recapitulate the R246S (mouse equivalent of human R249S) mutation in *p53*, even in *Hupki* 'knock-in' mice, which carry the human *p53* locus in their germ line, although animals did succumb to HCCs, suggesting the need for yet-undiscovered mechanisms in the generation of this mutation (Ghebranious and Sell, 1998; Hulla et al., 1993; Tong et al., 2006). Nonetheless, human HCCs with the R249S mutation appear to be more aggressive than those without this mutation, highlighting an important role for this mutation in liver carcinogenesis (Oda et al., 1992; Oda et al., 1994). Analysis of R249S-containing HCCs has revealed both a strong correlation and a lack of correlation with LOH, suggesting that both R249S DN-dependent and -independent mechanisms might be at work in HCC formation (Li et al., 1993; Martins et al., 1999; Oda et al., 1992; Peng et al., 1998).

To ascertain the role of R249S in tumorigenesis and its DN activity in vivo in a physiological context, we generated mouse ES cells carrying a 'knock-in' allele of the human-R249S-equivalent mutation, R246S, by homologous recombination. Data presented here reveal that this mutation acts in a DN manner when assayed for gene transcription, cell death and tumour formation in vivo.

Results

We generated feeder-independent ES cells expressing the R246S mutation at physiological levels by gene targeting. The targeting construct contained a loxP-site-flanked (floxed) neomycin selection cassette and carried the R246S mutation, which was introduced by site-directed mutagenesis (Fig. 1A). G418-resistant ES cell clones were first screened by PCR using two sets of primer pairs priming either at the 5′ or 3′ end, respectively, of the endogenous *p53* locus outside of the targeting construct and on the gene encoding neomycin resistance (data not shown). PCR-positive ES cell clones were further screened by Southern blot hybridization, which gave 17 kb and 8 kb bands for wild-type and mutant alleles, respectively (Fig. 1B, left panel). The homologous-recombination frequency rate was 5.6%. Positive recombinants were further transfected with Cre recombinase to remove the neomycin selection cassette, and the removal was confirmed in G418-sensitive clones by Southern blot hybridization using exon 1 as an external probe (11 kb with and 9 kb without the neomycin cassette) (Fig. 1B, right panel). The presence of the R246S mutation in the genome was confirmed by PCR-RFLP by digestion with *Bsr*BI (data not shown) (please see Materials an Methods for details). Two independently targeted R246S knock-in ES cell clones were analyzed, which gave similar results in all subsequent experiments.

The expression of the mutant allele was first determined by reverse transcriptase (RT)-PCR-RFLP. Similar to other findings (Mendrysa et al., 2003), the presence of the selection cassette strongly suppressed the expression of the targeted allele, which became prominent after removal of the neomycin cassette (Fig. 1C). To confirm the protein expression from the mutant allele, we performed immunoprecipitation analysis using the conformation-specific anti-p53 antibodies, Pab240 (mutant) and Pab246 (wild type) (Sabapathy et al., 1997). Human H1299 cells stably expressing the hot-spot mutant R175H p53 were used as a positive control for the mutant conformation (Fig. 1D, lane 7). Comparison of p53 conformation in $p53^{+/+}$ and $p53^{+/R246S}$ ES cells indicated that p53 adopted a wild-type conformation in both cases (Fig. 1D, compare lanes 2 and 4). To rule out any effects of the wild-type p53 protein over the mutant protein in $p53^{+/R246S}$ ES cells, we used $p53^{-/R246S}$ primary embryonic fibroblasts, which do not express any wild-type protein. We noted that the R246S mutant p53 protein adopted the wild-type conformation as well (Fig. 1D, lane 6), consistent with previous reports investigating the conformation of this R246S mutant p53 protein (Ghebranious et al., 1995), indicating that the mutant allele was indeed expressing the mutant protein in a wild-type conformation. Subsequent analysis of the steady-state levels revealed that the level of p53 protein in $p53^{+/R246S}$ ES cells was higher than in wild-type cells, both before and after doxorubicin treatment (Fig. 1E, compare lane 4 with 1 and 5, and lane 9 with 6 and 10). Furthermore, p53 in $p53^{+/R246S}$ ES cells localized to the nucleus in both normal and stress conditions, in contrast to in $p53^{+/+}$ cells, in which p53 was in the nucleus only after exposure to γ-irradiation (Fig. 1F). The elevated levels and abnormal localization of p53 in $p53^{+/R246S}$ ES cells is reminiscent of mutant p53 expression found in human cancers (Soussi, 2000), suggesting that the mouse R246S mutant p53 resembles the biochemical characteristics of human cancer-cell-derived mutant p53.

To evaluate whether the R246S mutant would exert DN effects over the remaining wild-type protein, we first determined the expression of several p53 target genes, such as *noxa*, *p21* and *mdm2*, before and after genotoxic stress, by quantitative real-time RT-PCR. As expected, the expression of these genes was rapidly induced in a p53-dependent manner in $p53^{+/+}$ and $p53^{+/-}$ ES cells upon doxorubicin treatment and γ-irradiation (Fig. 2A and data not shown). However, the induction of these genes in $p53^{+/R246S}$ ES cells was similar to that observed in $p53^{-/-}$ cells, and was much lower compared with wild-type and $p53^{+/-}$ cells (Fig. 2A), suggesting that the transactivation ability of wild-type p53 was impaired in the presence of the R246S mutant, therefore demonstrating the DN effect over the wild-type protein. It is to be noted that, upon doxorubicin treatment, p53 was abundant in

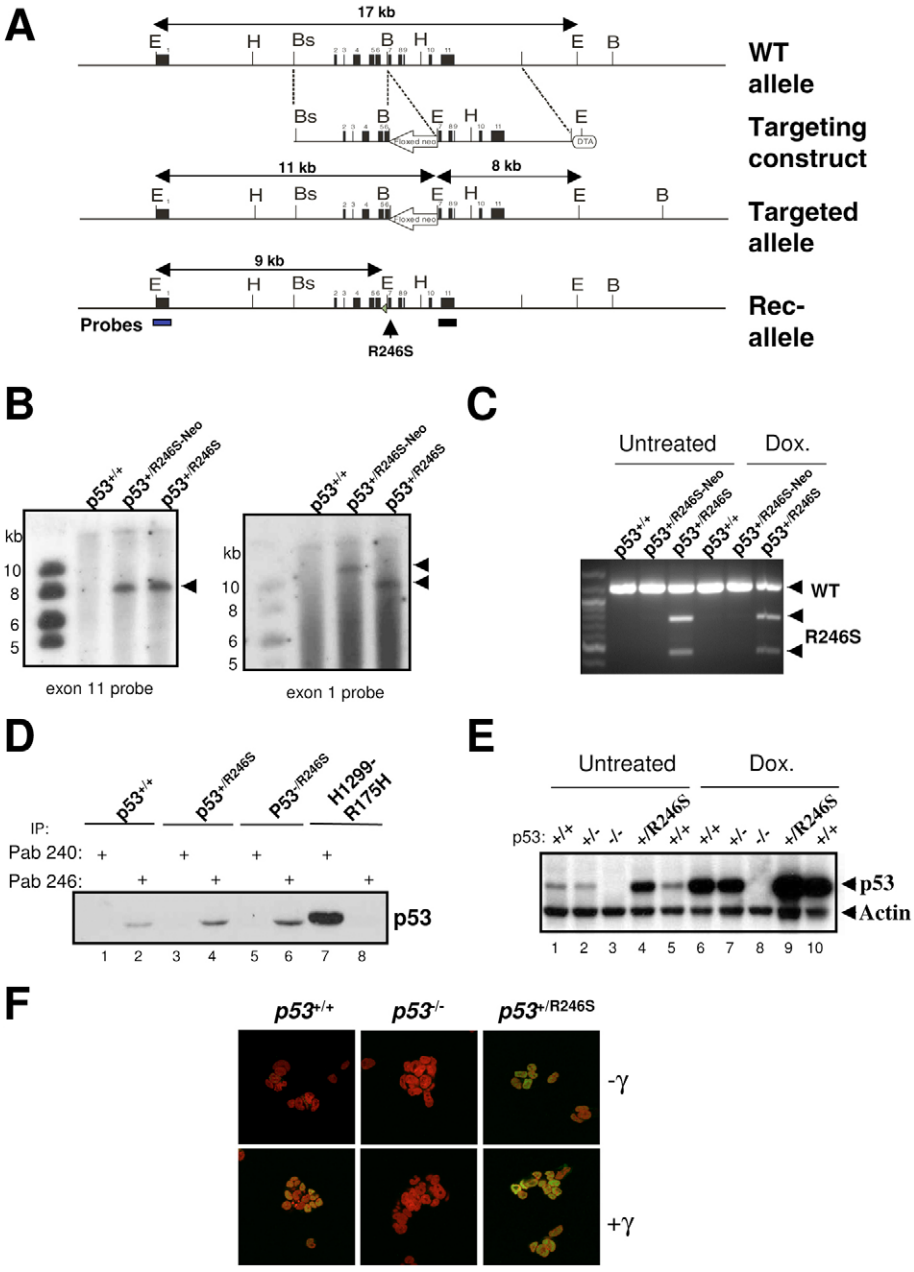

Fig. 1. Generation and characterization of R246S knock-in ES cells. (A) Schematic of the targeting strategy. Black boxes represent *p53* exons (exon numbers are indicated above). The positions of the probes used for Southern blotting are indicated below the diagram. Floxed neomycin and DTA expression cassettes are also indicated. Restriction sites are indicated as follows: E, H, Bs and B represents *Eco*RI, *Hin*dIII, *Bss*HII and *Bam*HI, respectively. Expected fragment sizes for Southern screening are indicted by the arrows and the position of the R246S mutation is indicated with the arrowhead. WT, wild type; Rec, after Cre-mediated recombination. The small green triangle represents the single loxP site remaining after Cre-mediated recombination. (B) Results of Southern blot hybridization, using exon 11 as the internal probe (left panel) and exon 1 as the external probe (right panel), after *Eco*RI digestion are shown for one representative targeted clone ($p53^{+/R246S\text{-}Neo}$) and one clone with the targeted- and neomycin-cassette removed ($p53^{+/R246S}$). Arrowheads point to the position of the expected bands for the mutant allele. (C) Expression of the mutant *p53* allele in ES cells was analyzed by RT-PCR-RFLP. ES cells of various genotypes were treated with 0.5 μg/ml doxorubicin (Dox.) for 3 hours and *p53* transcripts were PCR amplified prior to digestion with *Bsr*BI. Restriction fragments generated from the respective alleles are indicated with arrowheads. (D) Conformation of R246S mutant p53 protein was determined by immunoprecipitation using conformation-specific antibodies Pab240, which recognizes the mutant, and Pab246, which recognizes the wild-type conformation, followed by immunoblotting. H1299 cells expressing the R175H human p53 mutant were used as a positive control for mutant conformation. (E) Protein level of p53 and actin was determined by western blotting. (F) Localization of p53 was analyzed by fluorescence confocal microscopy. ES cells were mock (–γ) or 20-Gy (+γ)-irradiated and harvested 3 hours later. Representative images are shown in which the green fluorescence represents p53 protein and red fluorescence represents genomic DNA.

$p53^{+/R246S}$ cells (Fig. 1E), yet it was unable to result in the activation of target gene expression. These results were further confirmed by northern blot analysis of *mdm2* expression (Fig. 2B). Next, analysis of short-term cellular survival rates upon exposure to genotoxic stress revealed that $p53^{+/R246S}$ ES cells were almost as resistant as $p53^{-/-}$ cells to doxorubicin (Fig. 2C) and UV (Fig. 2D) treatment over a range of doses, as indicated. However, consistent with previous reports indicating a lack of a role for p53 in the long-term survival of ES cells (Chao et al., 2000), cell counting (supplementary material Fig. S1A) and colony-formation assays (supplementary material Fig. S1B) revealed that p53 status did not affect the long-term survival of ES cells after irradiation. Together, these results demonstrate the DN effect of the R246S mutant protein over its wild-type counterpart in $p53^{+/R246S}$ ES cells, with respect to target gene activation and short-term cellular survival.

We next investigated whether mutant p53 would also exhibit DN effects in differentiated cells. Molecular analysis of differentiation-related genes confirmed that knock-in cells were as pluripotent as wild-type cells, as determined by the expression of *nanog*, *rex 1*, *oct3/4*, *gata4* and *pax6* (Fig. 3A, left panel), and morphological analysis showed that cultures had a typical round colony-like morphology (Fig. 3A, right panel). The rate of differentiation, as determined by the changes in *nanog* and *oct3/4* levels, was also found to be similar between wild-type, $p53^{+/R246S}$ and $p53^{-/-}$ cells (Fig. 3B). Similar results were obtained upon DMSO-induced differentiation (data not shown), suggesting that the expression of R246S mutant p53 does not affect the pluripotency and differentiation potential of the ES cells; these results are congruent with the lack of differentiation defects in $p53^{-/-}$ mice and in cells from other p53 hot-spot mutant knock-in mice (Jacks et al., 1994;

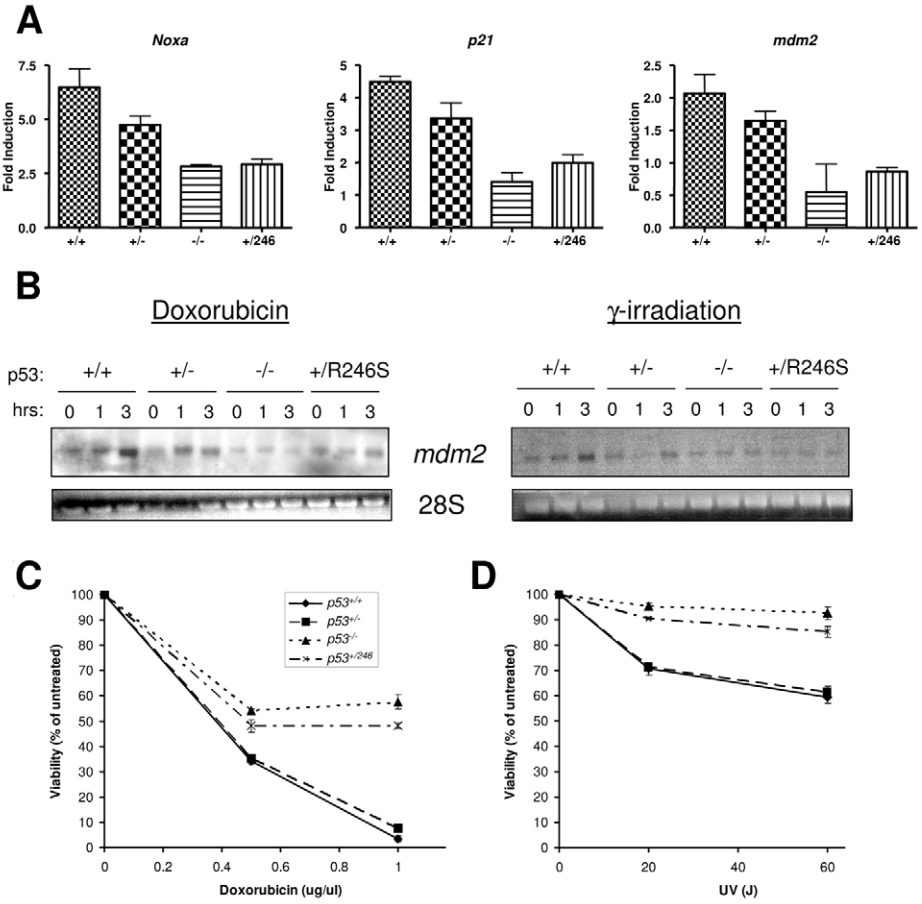

Fig. 2. R246S mutant protein exhibits DN effects in ES cells. (A) The expression of p53 target genes *noxa*, *p21* and *mdm2* was analyzed by quantitative real-time PCR 6 hours after doxorubicin (0.5 μg/ml) treatment. The expression level of each gene was normalized with the expression of *gapdh* and fold induction was calculated. Data represents mean ± s.e.m. of four independent experiments, each performed in duplicate. (B) Expression of *mdm2* mRNA was analyzed by northern blot hybridization after doxorubicin (0.5 μg/ml) or γ-irradiation (5 Gy). Intensity of 28S ribosomal RNA on agarose gel is shown for the loading control. (C,D) Cell-death analysis upon genotoxic stresses. Undifferentiated ES cells were treated with various doses of doxorubicin (C) or UV (D) for 12 hours and cell death was determined by flow cytometry after staining with annexin-V/propidium iodide. Data represents the percentage survival normalized to untreated samples (mean ± s.e.m.) from four independent experiments, each performed in duplicate.

Lang et al., 2004; Olive et al., 2004). Immunofluorescence analysis revealed that the R246S mutant protein localized to the nucleus of differentiated ES cells even in the absence of any genotoxic stress (Fig. 3C), similar to that noticed in undifferentiated ES cells, indicating that R246S mutant p53 might also exert DN effects over wild-type p53 in the differentiated state. We therefore examined the ability of $p53^{+/R246S}$ cells to undergo p53-dependent cell cycle arrest upon γ-irradiation, as has been shown for differentiated wild-type ES cells (Chao et al., 2000). As expected, the proportion of S-phase cells dramatically reduced after γ-irradiation in wild-type cultures but not in $p53^{-/-}$ cultures (% reduction of S-phase cells in $p53^{+/+}$ vs $p53^{-/-}$ cultures: 68.59±1.13% vs 23.34±5.11%) (Fig. 3D). Importantly, $p53^{+/R246S}$ cultures did not show a significant reduction in the proportion of S-phase cells, similar to that observed with $p53^{-/-}$ cultures ($p53^{+/R246S}$ vs $p53^{-/-}$ vs $p53^{+/-}$ cells: 29.11±5.47% vs 23.34±5.11% vs 58.74±0.88%), suggesting that cell cycle regulation of $p53^{+/R246S}$ cells was impaired (Fig. 3D). To further confirm these results, we analyzed the proportion of cells positive for BrdU staining ($BrdU^+$), which is a measure of the amount of cells undergoing DNA replication during the S-phase of the cell cycle, before and after doxorubicin treatment. Whereas there was no significant difference among the various untreated cell types, doxorubicin treatment resulted in a significant decrease in the number of $BrdU^+$ $p53^{+/+}$ and $p53^{+/-}$ cells ($BrdU^+$ $p53^{+/+}$ vs $p53^{+/-}$ cells after doxorubicin treatment: 9.34±1.48% vs 10.44±0.13%) (Fig. 3E). By contrast, the decrease was subtle and less pronounced in both $p53^{+/R246S}$ and $p53^{-/-}$ cells ($BrdU^+$ $p53^{+/R246S}$ vs $p53^{-/-}$ cells after doxorubicin treatment: 18.225±2.96% vs 17.7±0.46%) (Fig. 3E), confirming the DN effect of the mutant allele over the wild-type allele. Together, the data suggest that the R246S mutant protein acts in a DN manner in both undifferentiated and differentiated ES cells.

The general tumorigenic potential of $p53^{+/R246S}$ cells was next investigated. ES cells have been shown to form teratomas, homing to the liver in mice with liver injury caused by carbon tetrachloride (CCl_4) treatment (Yamamoto et al., 2003). We therefore used this model to test whether $p53^{+/R246S}$ ES cells would display higher tumorigenicity in vivo compared with wild-type p53-containing cells. All ES cell lines were transfected stably with a green-fluorescent protein (GFP) expression vector to monitor their homing capacity. As shown in Fig. 4A, injection of $p53^{+/+}$ and $p53^{+/-}$ ES cells into *scid* mice resulted in the formation of micronodules in the liver, which were usually not visible to the naked eye, whereas $p53^{-/-}$ and $p53^{+/R246S}$ ES cells formed numerous tumour nodules, with an average diameter greater than 5 mm, in all of the four mice tested in each group. We therefore further investigated, by florescence microscopy, whether the tumours were derived from the injected ES cells and found that the teratoma cells were indeed GFP-positive, confirming their ES-cell origin (Fig. 4B). Detailed histological analysis revealed that the injected ES-cell-derived tumours contained heterogeneous cells types, including bone and cartilage cells, columnar epithelial cells, fibroblast-like cells and muscle cells (Fig. 4C). Because it has also been reported that the injected ES cells will also differentiate into hepatocytes, we analyzed the expression of hepatocyte markers, such as α1-antitrypsin, albumin, tryptophan-2,3-dioxygenase, transthyretin and α-fetal protein, and found their expression to be similar among the

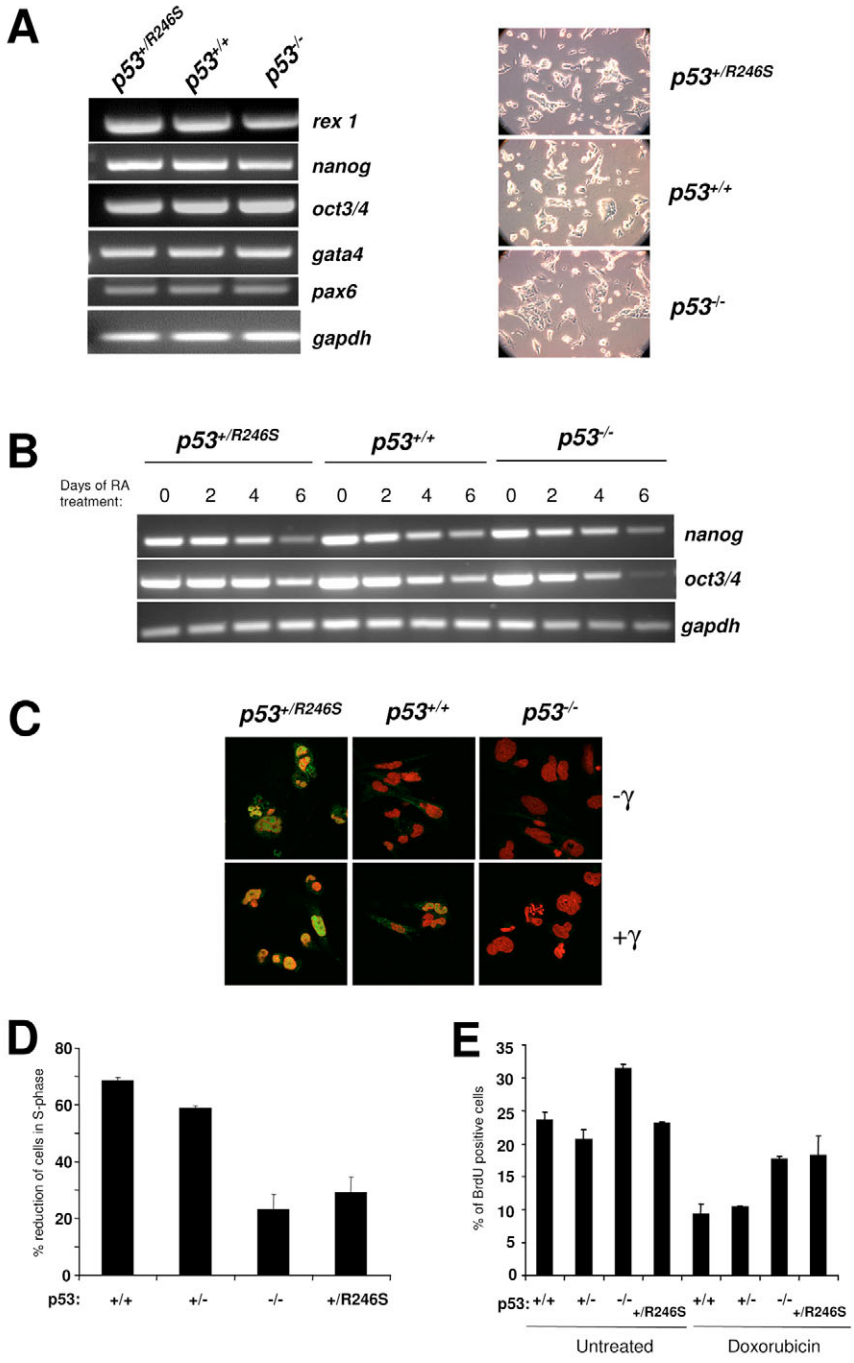

Fig. 3. R246S mutant protein does not affect differentiation of ES cells but also exerts DN effects in the differentiated state. (A) Pluripotency of ES cells is not affected by expression of R246S mutant p53. The expression of various stem-cell markers was analyzed by semi-quantitative RT-PCR (left panel). The morphology of the undifferentiated ES-cell colonies was routinely monitored by phase-contrast microscopy and representative images of ES cells of various *p53* genotypes are shown (right panel). (B) The expression of stem-cell markers was analyzed during retinoic-acid-induced (0.1 μM) differentiation of ES cells. (C) Localization of p53 in ES cells differentiated with 0.1 μM retinoic acid for 6 days, after treatment with (+γ) or without (–γ) 20 Gy irradiation, was analyzed by fluorescence confocal microscopy. The green fluorescence represents p53 protein; red fluorescence represents genomic DNA. (D,E) Differentiated ES cells were irradiated with 20 Gy and harvested 24 hours later. (D) Cell-cycle analysis was performed by flow cytometry and ModFit cell cycle analysis software. (E) Cellular proliferation was determined by BrdU staining of cells (treated without or with doxorubicin) followed by flow-cytometric analysis. Data represents the percentage reduction of S-phase cells (D) or the percentage of $BrdU^{+}$ cells (E) (mean ± s.e.m.) from three independent experiments, each performed in duplicate.

different genotypes (data not shown), suggesting that the rate of differentiation was not altered in vivo. The data therefore indicate that, although the mutation in *p53* does not affect differentiation rates, the tumorigenic potential of $p53^{+/R246S}$ ES cells is similar to that of $p53^{-/-}$ ES cells and in contrast to wild-type and $p53^{+/-}$ ES cells, indicating that the R246S mutant p53 is capable of exerting its DN effect over the wild-type p53 both in vivo and in vitro.

To assess whether the presence of other oncogenic stimuli will affect the growth properties of ES cells of the various *p53* genotypes, we generated K-ras^{v12}-expressing ES cells (supplementary material Fig. S2A). Interestingly, similar to previously published results (Brooks et al., 2001), expression of K-ras^{v12} did not promote the growth of unstressed wild-type ES cells (supplementary material Fig. S2B). Similarly, there was no effect of H-ras^{v12} expression on cellular growth (supplementary material Fig. S2B) or on colony formation in vitro (data not shown) in ES cells of the various *p53* genotypes, indicating that the presence of a further oncogeneic signal does not affect the growth properties of fast-growing ES cells with intrinsic tumorigenic potential.

Discussion

Taken together, the data presented here demonstrate that the mouse equivalent of the commonly found human R249S hot-spot p53 mutant is capable of DN effects over the wild-type protein both in

undifferentiated and differentiated ES cells, as assessed by multiple parameters – including transactivation of target genes, cellular survival, cell-cycle arrest after genotoxic stress and tumorigenic potential – therefore confirming the potency of the mutant protein in supporting tumorigenicity. This mutant, although incapable of transactivation potential both in the context of cultured cells and in transgenic mice (Ghebranious et al., 1995), maintains its capacity to inhibit the wild-type protein, again highlighting the relevance of DN effects of hot-spot mutants in tumorigenesis.

It is noteworthy that the effects of mutant p53 are probably not due to its ability to inhibit the other p53 family members, p63 or p73. ES cells do not express high levels of p63 (compared with fibroblasts) (supplementary material Fig. S3A), and inhibition of p73 expression by siRNA-mediated silencing did not affect cell death even in wild-type ES cells (supplementary material Fig. S3B), suggesting that the effect of this hot-spot mutant p53 is indeed through its ability to function in a DN manner over the remaining wild-type allele.

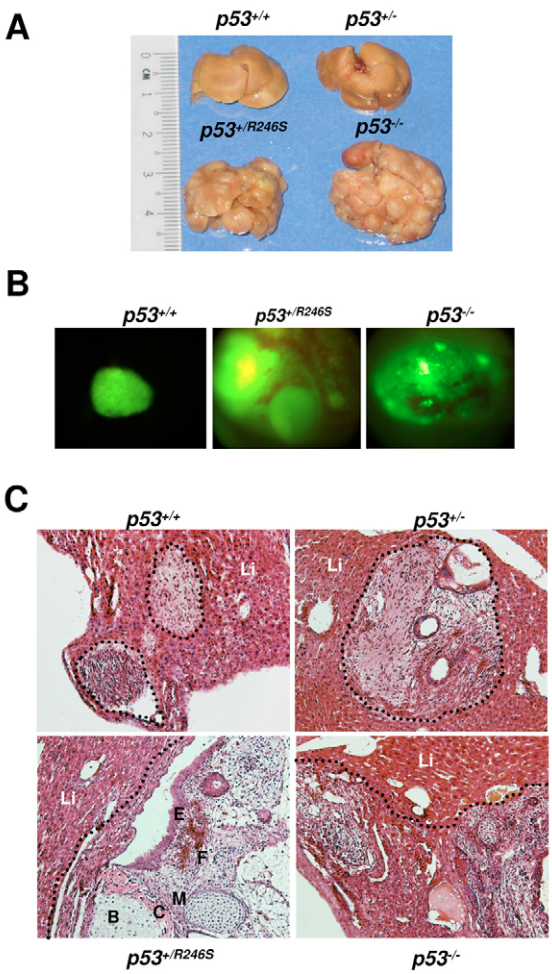

Fig. 4. $p53^{+/R246S}$ ES cells are as highly tumorigenic as the $p53^{-/-}$ cells. (A) Representative images of livers from *scid* mice injected with ES cells of various *p53* genotypes. Four mice per group were used, all of which gave consistent results. (B) Fluorescence microscopy of ES-cell-injected livers before fixation. Green fluorescence indicates the expression of GFP in the tumour nodules in livers. (C) Histological analysis of livers injected with ES cells of various *p53* genotypes. The tumour portions derived from the differentiated ES cells are highlighted by dotted lines. Normal liver is indicated by 'Li', whereas 'B', 'C', 'E', 'F' and 'M' indicate the bone, cartilage, epithelium, fibroblast-like cells and smooth muscles, respectively.

Although the DN activity of mutant p53 has been acknowledged as a contributory mechanism for tumorigenesis, experimental studies both in cultured cells and in mice have resulted in conflicting results. Many reasons have been put forth to explain the lack of DN effects when it was not observed, ranging from tissue-type specificity to the expression levels of the mutant protein in the cell. It is interesting to note that a recent study revealed that at least three molecules of mutant p53 are required to inhibit the function of a wild-type protein, highlighting the requirement for threshold levels for the DN effects of the mutant protein to be observed (Chan et al., 2004). Consistently, the DN effect was not observed in primary tissues from other mutant p53 knock-in mice, in which there was no accumulation of the mutant protein, in contrast to the tumour cells from these mice (Lang et al., 2004; Olive et al., 2004). In this respect, ES cells are different and are known to express extremely high levels of p53 compared with differentiated cell types such as fibroblasts (Sabapathy et al., 1997). Expression of endogenous mutant p53 are higher in these cells (this report), which coincided with the DN effects, noted also in the case of the R270H mutation (de Vries et al., 2002). Therefore, it is plausible that higher steady-state protein level in both normal and stressed conditions, as seen in the context of ES cells, might be essential for the mutant p53 protein to manifest its DN behaviour.

Results from previously generated mutant p53 knock-in mice revealed that there might be a cell-type bias for mutant p53-mediated DN function. Whereas the DN effect was not observed in mouse embryonic fibroblasts (MEFs), it was observed partially in thymocytes and very strongly in the developing brain of these mice (Olive et al., 2004). It is interesting to note that the DN effect is seen in cell types that are not terminally differentiated, such as in thymocytes, which contain primarily double-positive immature T cells, and neural stem cells in developing brain (Brazel et al., 2003; Hayday and Pennington, 2007). p53 levels are elevated upon γ-irradiation in thymocytes, which are known to undergo apoptosis in a p53-dependent manner (Clarke et al., 1993). Similarly, *p53* is highly expressed in the neural-stem-cell niche of adult brain, and absence of *p53* promotes proliferation, survival and self-renewal of the neural stem cells (Meletis et al., 2006). Therefore, it is not unconceivable that stem cells and less-differentiated cells rely on the activity of p53 more than do terminally differentiated somatic cells, and, hence, the DN effects are more evident in them. Further indirect support for this idea comes from R270H conditional knock-in mice, which only showed partial DN effects in vivo when most of the mutant protein was expressed in the matured epithelial cells by adenoviral induction (Jackson et al., 2005). Similarly, partial DN effects upon UV-irradiation-induced skin carcinogenesis was observed in $p53^{R270H}$/*K14-Cre* double-transgenic mice, in which the mutant protein was expressed in keratinocytes (Wijnhoven et al., 2007). It will therefore be interesting to determine whether expression of the mutant protein in stem-cell niches will lead to stronger DN effects.

In conclusion, the data presented here demonstrate that the commonly found hot-spot p53 mutant, R246S, displays strong DN properties in mouse ES cells, resembling the situation in human tumours. Because cancer is often considered a disease of stem cells, and because mutant p53 is very stable in cancer cells, the ES cell model presented here fulfils both criteria and provides a best-fit to understand mutant p53 properties. It will therefore be interesting to next evaluate whether either or both properties, i.e.

pluripotency and high levels of protein, are required for the DN effects of mutant p53 to be manifested, using the knock-in mice for analysis.

Materials and Methods

Generation of targeting construct

The genomic clones of murine *p53* (G2, exon 2-6 and G10, exon 7-11) were kindly provided by A. de Vries (National Institute of Public Health and the Environment, The Netherlands) (de Vries et al., 2002), and the floxed neomycin-resistant gene cassette (pKSloxpNT) and diphtheria toxin A (DTA) expression cassette were gifts from M. Sibilia (University of Vienna, Austria). The DTA cassette was PCR amplified with a pair of primers containing the *Xho*I site and was sub-cloned into the *Xho*I site at the 3′ end of the genomic clone G10 to generate the intermediate clone, G10-DTA. The floxed neomycin-resistance gene was released by *Eco*RI-*Kpn*I digestion and G10-DTA was linearized by *Xba*I. Blunt ends of all fragments were generated using Klenow and the floxed neomycin-resistant gene cassette was sub-cloned into the *Xba*I site by blunt-end ligation to generate the second intermediate clone, *neo*-G10-DTA. The R246S mutation (CGA to TCT) was generated by site-directed mutagenesis on the *neo*-G10-DTA clone by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation creates a novel *Bsr*BI cut site, which was used for screening constructs and ES cells. A 4 kb *Bss*HII fragment of G2 was sub-cloned into cloning vector pSL1180 (Amersham Biosciences, Buckinghamshire, UK) to obtain an additional *Hpa*I and *Not*I site at the 5′ end of the fragment (pSLG2). The 4-kb G2 fragment from pSLG2 was released by *Not*I digestion and *neo*-G10(R246S)-DTA was linearized with *Not*I. These fragments were ligated together to generate the targeting construct (Fig. 1A). All the exons and the splice junctions in the targeting construct have been verified by sequencing to ensure that no additional unwanted mutations were introduced during cloning.

Cell culture, gene targeting and in vitro differentiation

CCE ($p53^{+/+}$), p2.4 ($p53^{+/-}$) and p1.1 ($p53^{-/-}$) ES cells were cultured and differentiated with 0.1 μM retinoic acid for 6 days as described previously (Lee et al., 2005). 30 μg of *Hpa*I-linearized construct was electroporated into exponentially growing ES cells and selected with 250 μg/ml G418 for 10-14 days. Homologous recombinants were identified by PCR and Southern blot screening, and were transiently transfected with pCAG-Cre to remove the neomycin selection cassette. G418-sensitive ES cells clones were screened to confirm the removal of selection cassette. The presence of R246S mutant allele was determined by PCR/RFLP and the expression of mutant allele was confirmed by RT-PCR-RFLP.

The $p53^{R246S/-}$ MEFs were obtained from 13.5 dpc embryos by mating $p53^{+/R246S}$ and $p53^{+/-}$ mice. R175H-expressing H1299 cells were generated in our laboratory as described previously (Vikhanskaya et al., 2007). The $p63^{-/-}p73^{-/-}$ mouse embryonic fibroblasts were a kindly provided by E. Flores (MD Anderson, TX).

Undifferentiated or differentiated ES cells were treated with 0.1 μg/ml doxorubicin for 24 hours prior to BrdU addition (to a final concentration of 10 μM), incubated for a further 1 hour and fixed in 70% ethanol overnight.

For gene silencing experiments, 1×10^5 undifferentiated ES cells were transfected with scrambled or *p73* siRNA for 36 hours by RNAiFect, following the manufacturer's protocol (Qiagen, Germany), and treated with 1 μg/ml doxorubicin for 12 hours prior to determination of cell viability by flow cytometry.

Growth-curve analysis and colony-formation assay

2×10^4 undifferentiated ES cells were plated onto six-well plates and γ-irradiated at various doses. Irradiated cells were cultured for a further 8 days and the total number of surviving cells was counted. For Ras-expressing cells, 1×10^5 cells were plated onto six-well plates and counted daily. Independent experiments were performed, in duplicate, at least thrice and data represent mean ± s.e.m.

Long-term survival of ES cells was assayed by colony-formation assays. Essentially, 1×10^3 undifferentiated ES cells were irradiated as mentioned above and cultured for 8-10 days. To determine the effects of H-ras expression, undifferentiated ES cells were transfected with linearized pBabe-Ras^{V12} plasmid as described previously (Lee et al., 2005) and selected with 1 μg/ml puromycin for 8-10 days. Surviving cells were pooled and maintained in 0.5 μg/ml puromycin at all times. For colony-formation assay, 100 cells were plated and allowed to grow for 10 days. Surviving colonies were stained with crystal violet solution (MERCK, Whitehouse Station, NJ) as described previously (Vikhanskaya et al., 2007).

Southern blot hybridization, PCR-RFLP and RT-PCR-RFLP

10 μg of ES cell genomic DNA was *Eco*RI-digested, separated by 0.7% agarose gel electrophoresis and transferred to positively charged nylon membrane (Amersham Biosciences). PCR-amplified exon 1 and 11 probes were used for Southern blot hybridization as described (Luo et al., 2001; Olive et al., 2004).

For RT-PCR-RFLP, total RNA from ES cells was used for first-strand cDNA synthesis. *p53* cDNA and *p53* exon 7 from genomic DNA were PCR amplified as described previously (Lee et al., 2005), and were digested with *Bsr*BI for RFLP analysis.

Quantitative and semi-quantitative RT-PCR and northern blot hybridization

Quantitative real-time PCR was performed using gene-specific primers and Quantitect real-time PCR reagent (Qiagen) in Cobett real-time PCR machine (Cobett Research, Sydney, Australia) as described previously (Lee et al., 2005). mRNA expression of target genes were normalized with *gapdh* expression and fold induction was calculated with reference to untreated samples. For semi-quantitative RT-PCR, 1 μl of cDNA was used to amplify the gene of interest as described previously (Lee et al., 2005).

The expression of mouse *p63* and *p73* genes was analyzed by semi-quantitative RT-PCR using *p63*-specific primers (forward, 5′-CACAGAATAGCGTGACG-GCGCC-3′ and reverse, 5′-CTCTGCCTTCCCGTGATAGGATC-3′) and *p73*-pecific primers (forward, 5′-GAGCACCTGTGGAGTTCTCTAGA-3′ and reverse, 5′-GTGACAGGGTCATCCACGTACTGG-3′). *p73* siRNA has been described (Vikhanskaya et al., 2007).

Expression of Ras was determined by semi-quantitative RT-PCR (Ras forward primer, 5′-AGAAGGCATCCTCCACTCC-3′ and reverse primer, 5′-CCATCA-ACCAACACCCAAG-3′).

20 μg of total RNA was used for northern blot analysis. Probe was labelled and purified as described above.

Immunoblotting, immunoprecipitation and immunocytochemistry

Whole-cell lysates from ES cells were used for western blotting performed as described previously (Lee et al., 2005), with either anti-p53 antibody (CM5; Novocastra, Newcastle, England) or anti-actin antibody (Sigma, St Louis, MO).

500 μg of whole-cell protein lysate was used for immunoprecipitation with the following conformation-specific anti-p53 antibodies: Pab240 (Calbiochem, San Diego, CA), which recognizes the denatured and mutant conformation, and Pab246 (Calbiochem), which recognizes the native wild-type conformation, followed by anti-mouse IgG agarose beads. Immunoprecipitates were then separated by 10% SDS-PAGE and western blot analysis was performed with a mixture of anti-p53 antibody 1C12 (Cell Signaling, Danvers, MA) and DO-1 (Santa Cruz, CA).

For immunocytochemistry, ES cells were fixed in 4% paraformaldehyde 3 hours after irradiation, stained with anti-p53 antibody Pab240 (Calbiochem) followed by Alexa-Fluor-488-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR) and propidium iodide. Fluorescence confocal microscopy was performed using the LSM 510 laser scanning confocal microscope (Zeiss, Jena, Germany).

Flow cytometry

Apoptosis was measured by annexin-V binding assay and flow cytometry according to the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ). For cell cycle analysis, cells were fixed in 70% ethanol at 4°C overnight, washed with PBS once and incubated with 0.2 mg/ml RNase and 50 μg/ml PI in PBS for 20 minutes, prior to flow cytometric analysis with ModFit cell cycle analysis software. The percentage of $BrdU^+$ cells was determined by flow cytometry after staining with FITC conjugated anti-BrdU antibody (BD Biosciences), as per the manufacturer's instruction.

Induction of teratoma and histological analysis

ES cells of various *p53* genotypes were first transfected with GFP-encoding plasmid pRNAT-U6.1/Hygro (GenScript Corp., Piscataway, NJ), and hygomycin-resistant ES cells were used to induce teratoma as described previously using 6-week- old *scid* mice treated with CCl_4 (Yamamoto et al., 2003). All mice were sacrificed 3 weeks later and livers were fixed in 4% neutral-buffered formaldehyde, followed by dehydration and paraffin embedding. Histological analysis was carried out on 5-μm sections stained with haematoxylin and eosin (Sigma). All animal experiments were carried with the approval of the Institutional Animal Care and User Committee

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