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Hypoxia attenuates the p53 response to cellular damage

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The tumour suppressor activity of p53 *in vivo* can be subject to pressure from the physiological stress of hypoxia and we report on the development of a cell system to define the p53-dependent stages in the adaptation of cells to hypoxia. $p53^{+/+}$ cells exposed to hypoxia exhibited a transient arrest in G2/M, but escaped from this checkpoint and entered a long-term G_0/G_1 arrest. By contrast, isogenic p53-null cells exposed to hypoxic conditions exhibited a 6–10-fold higher level of apoptosis, suggesting that p53 acts as a survival factor under limiting oxygen concentrations. Surprisingly, hypoxia-dependent growth arrest in $p53^{+/+}$ cells did not result in either $p21^{WAF1}$ or HIF-1 protein stabilization, but rather promoted a significant decrease in Ser^{392}-site phosphorylation at the CK2/FACT site. However, chemically induced anoxia induced Ser^{392}-site phosphorylation as well as stabilization of both p53 and HIF-1 proteins. In contrast to hypoxia, 5-flourouracil (5-FU)-induced p53-dependent cell death correlated with enhanced Ser^{392} phosphorylation of p53 and elevated $p21^{WAF1}$ protein levels. Hypoxia inhibited 5-FU-induced p53-dependent cell death and attenuated p53 phosphorylation at the ATM and CK2/FACT phosphorylation sites. Although anoxia activates the p53 response, hypoxia silences the p53 transactivation pathway and identifies a physiological signalling model to study mechanisms of p53 inactivation under hypoxic conditions.
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Introduction

The p53 pathway is inactivated in most human cancers thus highlighting its central role in tumour suppression. The function of p53 as a tumour suppressor is linked to its activity as a stress-activated transcription factor (Vogelstein *et al*., 2000). The gene products induced by p53 play an important role in growth arrest, cellular repair, and cell death, which maintains tissue integrity and minimizes the propagation of malignant cells (Polyak *et al*., 1997; Zhao *et al*., 2000). Although half of human cancers have been shown to harbour inactivating mutations in p53, half do not, giving hope that the wild-type p53 response may be rescued in some cancers by targeting p53 modifiers. Identifying these epigenetic regulators of p53 has been the subject of basic research aimed at understanding how p53 is activated as a transcription factor.

A concerted set of signalling pathways modify p53 protein activity post-translationally. One signalling branch involves an MDM2-dependent pathway that degrades and inactivates p53 protein (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Thut *et al*., 1997), while the MDM2-antagonizing branch promotes covalent modification of p53 protein by stress-regulated protein kinases that stimulate the sequence-specific transactivation function of p53 (Hupp *et al*., 2000). The binding of transcription coactivator p300 to p53 is stimulated by either Ser^{15} phosphorylation at the ATM site (Lambert *et al*., 1998; Jayaraman and Prives, 1999) or by Ser^{20} phosphorylation at the CHK2 site in the N-terminal transactivation domain of p53 (Shieh *et al*., 2000; Dornan and Hupp, 2001). Such phosphorylation events stabilize the docking of p300 to the transactivation domain of p53 and promote DNA-dependent acetylation of p53 (Dornan *et al*., 2002). p300 inhibitors attenuate the p53 response and block p53 protein stabilization after DNA damage (Yuan *et al*., 1999; Dornan and Hupp, 2001). Complementing phosphorylation of the transactivation domain of p53, C-terminal phosphorylation by CDK2 or CK2/FACT is implicated in modulating sequence-specific DNA binding (Hupp and Lane, 1995; Wang and Prives, 1995; Hansen *et al*., 1996; Sakaguchi *et al*., 1997; Blaydes *et al*., 2001; Keller *et al*., 2001) and activating p53 after UV-irradiation or X-irradiation *in vivo* (Blaydes *et al*., 2000, 2001; Keller *et al*., 2001). These data are consistent with the concerted model that MDM2 inhibition along with activation of p53's DNA binding function and p300-binding activity is required for inducing the p53 response.

The p53-modifying enzymes including PI-3 kinases, CK2/FACT, CDK2, and MDM2 have been the target of experimental therapeutics aimed at determining whether inhibiting these enzymes can modify p53-dependent survival or apoptosis. The p53-inhibitory protein MDM2 is an oncogene that has proven to be an excellent target for developing p53-stimulatory molecules that inhibit the MDM2-dependent degradation of p53 protein (Bottger *et al*., 1997; Lane and Hall, 1997). Such agents also function as effective inducers of

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apoptosis in cells lacking p53 (Kanovsky *et al*., 2001; Wang *et al*., 2002). CDK2 and CK2/FACT kinase pathways play a role in p53 activation after damage and the synthetic CK2/CDK2 kinase inhibitors, roscovitine and DRB, can modify the p53 response (Blaydes and Hupp, 1998; Blaydes *et al*., 2000; Kotala *et al*., 2001). Developing such novel anticancer drugs based on targeting regulators of p53-dependent checkpoint control requires an integrated understanding of the physiological stresses that normally modify p53, as such stresses will complicate the mechanism of how anticancer drugs modify p53 activity. The nature of the DNA damage signalling lesion that activates p53 is more complicated given that agents as diverse as low extracellular pH, hypoxia, or heat shock can activate p53 function without DNA damage (Nitta *et al*., 1997; Giaccia and Kastan, 1998; Williams *et al*., 1999) and the mechanism whereby these physiological damaging agents activate p53 is undefined.

Solid tumours contain heterogeneous populations of cells due in part to a limited blood supply that leads to lowered oxygen concentrations, acidic conditions, and glucose starvation (Williams *et al*., 2002). These stresses within the tumour microenvironment represent physiological signals that can activate survival or apoptotic programmes, the balance of which regulates the rate of either tumour suppression or cancer growth. Although the mechanisms whereby cells adapt or die in the presence of oxygen deprivation are not as well studied as stresses such as DNA damage or heat shock, it is known that two major transcription factors operate during lowered oxygen concentrations: hypoxia-inducible factor-1 (HIF-1) and p53 (Arany *et al*., 1996; Graeber *et al*., 1996; Ashcroft *et al*, 2000; Kung *et al*., 2000; Hammond *et al*., 2002). HIF-1 upregulates the expression of genes in response to hypoxia that are either implicated in generating energy in the absence of oxygen or in inducing the formation of blood vessels to recruit blood flow and thereby oxygen to stressed cells (Wykoff *et al*., 2000; Maxwell, 2002). HIF-1 activity is regulated, in part, by a hypoxia-sensitive proline hydroxylation pathway that controls the binding of the protein to the coactivator p300 (Chan *et al*., 2002; Freedman *et al*., 2002). The gene products induced by HIF-1 allow the adaptation of cancer cells in a microenvironment where tumour-suppressing pathways such as p53 are thought to antagonize such survival pathways (Wenger, 2002). The development of hypoxic centres in solid cancers may provide selection pressures for the survival of clones of cells with mutations in p53.

In this study, we have set up a cellular experimental system to begin to determine the mechanism of how hypoxia perturbs p53 and how this stress affects p53 activation by a common anticancer drug. We address whether hypoxia is similar to DNA damage via activation of p53 after phosphorylation at key N- and C-terminal sites and whether hypoxia cooperates with or antagonizes the classic 5-fluorouracil (5-FU)-induced p53-dependent apoptotic programme. Our data show that p53 is required for a hypoxia-induced growth arrest pathway, while p53-null cells become sensitive to hypoxia and undergo pronounced p53-independent apoptosis. Further, hypoxia promotes reductions in normally p53-activating phosphorylation sites at Ser^{15} and Ser^{392}, no HIF-1 protein stabilization during growth arrest, and an attenuation of apoptosis in 5-FU-treated cells. In contrast to hypoxia, chemically induced anoxia stabilizes p53 protein, induces its phosphorylation at Ser^{15} and Ser^{392}, promotes induction of gene products like $p21^{WAF1}$ protein, and stabilizes HIF-1 protein. Together, these data indicate that anoxia and hypoxia have opposing effects on the p53 pathway and highlight the complexity of p53 regulation in response to physiological stresses.

Results

A time-dependent change in cell-cycle parameters during the adaptation of cells to hypoxia: p53 functions as a survival factor

To assess the effects of hypoxia on p53-dependent cell-cycle parameters, an isogenic panel of cells was employed, one of which contained wild-type p53 ($HCT116p53^{+/+}$) while the other was null for the p53 locus ($HCT116p53^{-/-}$) (Bunz *et al*., 1999). The change in cell-cycle parameters was monitored under hypoxic conditions over time in $p53^{+/+}$ and $p53^{-/-}$ cells (Figures 1 and 2). $p53^{+/+}$ cells exhibited a transient increase in the number of cells in the G_2/M phase of the cell cycle 24 h after hypoxic treatment (Figure 1c), which is not seen in $p53^{-/-}$ cells (Figure 1d). $p53^{-/-}$ cells exhibited a more pronounced increase in cell death than $p53^{+/+}$ cells 24 or 48 h after hypoxic treatment (Figure 1h *vs* g) (Figure 1h *vs* g). In both cell types the percentage of cells in S phase decreases with time (Figure 1e and f), which can be accounted for by the arrest of cells in G_0/G_1 in the case of $p53^{+/+}$ cells (Figure 1a), and by an increase in cell death in the case of $p53^{-/-}$ cells (Figure 1h). $p53^{-/-}$ cells do not show an increase in the G_0/G_1 phase of the cell cycle and maintain a steady state in the G_2/M phase (Figure 1b, d). Over 80% of the $p53^{+/+}$ cells eventually arrest in G_0/G_1 following 7 days of hypoxic incubation (data not shown).

The FACS analysis profiles of the normoxic and hypoxic cells are displayed in Figure 2. To determine the nature of the hypoxia-induced cell death in $p53^{-/-}$ cells (indicated by the sub-G_0 population of cells in Figure 1h, 2d, 2f) flow cytometry was performed using Annexin V in conjunction with propidium iodide to assess the levels of apoptosis and/or necrosis in the sub-G_0 cell population. In the control population of $p53^{-/-}$ cells (Figure 2g), it can be seen in the lower left (LL) quadrant that most of the cells are neither apoptotic or necrotic with 88% of the cell population remaining healthy and 7–8% of cells being apoptotic. In contrast to this, after 48 h of hypoxic incubation (Figure 2h) the healthy population of cells has diminished to 58% and the level of apoptotic cells increased to 35%. Also, it can be seen in the upper right (UR) quadrant that there is a small population of late apoptotic/necrotic cells (5%).

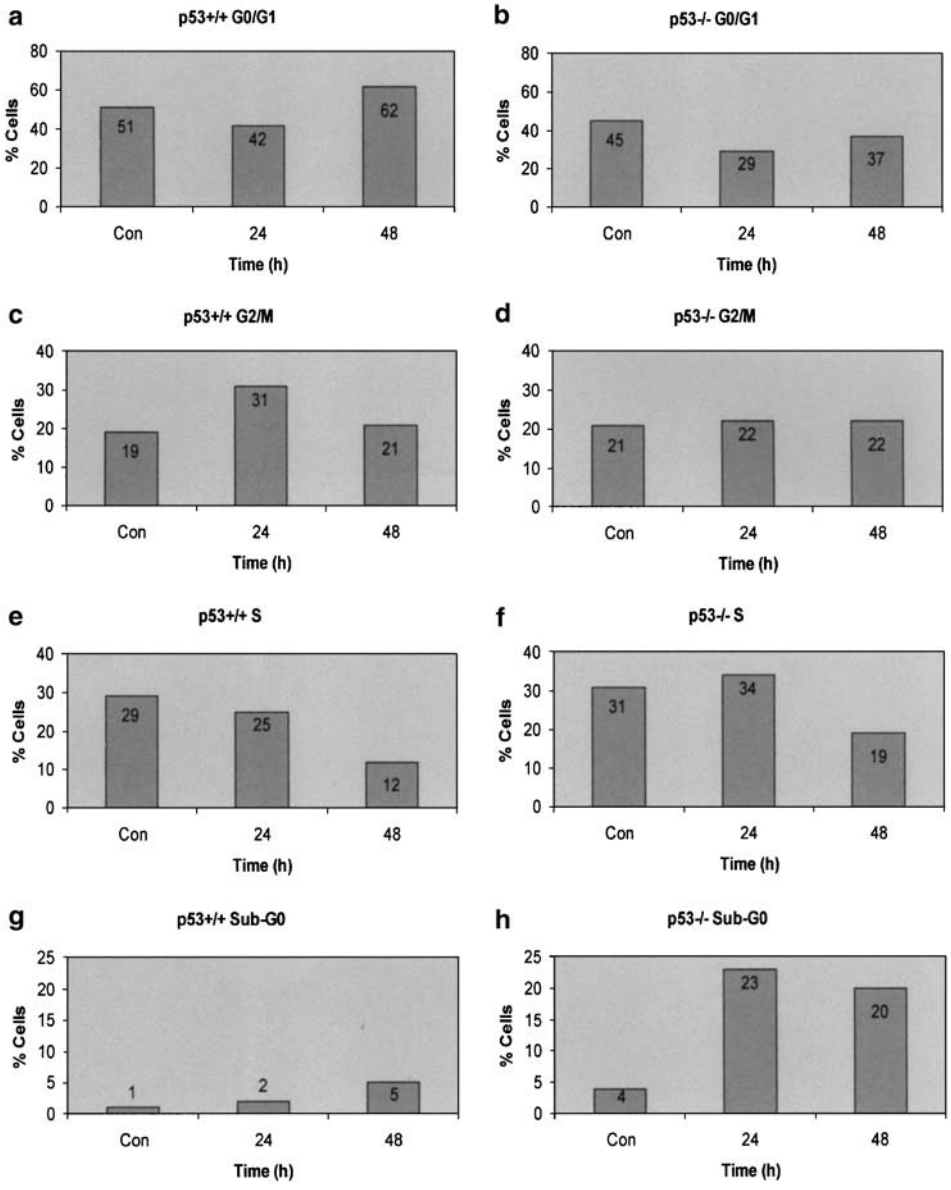

Figure 1 p53-dependent survival pathways are activated by hypoxia. Cells were seeded at 1×10^4 cells/ml, cultured in a hypoxic incubator (1% oxygen) with samples taken at times shown, and treated as stated in Materials and methods. HCT116 are in panels a, c, e, and g, while HCT116$^{-/-}$ (p53-null) cells are in panels b, d, f, and h. The percentage of cells in the indicated stages of the cell cycle (G_0/G_1, G_2/M, S, and sub-G_0) is plotted as a function of time after hypoxic induction from 0 to 48 h

These data indicate that exposure of the p53$^{-/-}$ cells to 1% hypoxia is inducing apoptotic cell death.

Hypoxia downregulates Ser^{392}-site phosphorylation of p53

The data shown in Figures 1 and 2 indicate that hypoxia treatment of cells can activate p53-dependent growth arrest or survival, while the absence of p53 sensitizes cells to apoptotic cell death. This is in apparent contrast to previous reports that show that lowered oxygen activates p53-dependent cell death (Koumenis *et al*., 2001). This discrepancy may be explained by differences in the oxygen concentrations in each study: we have used oxygen concentrations of 1%, while other studies have used chemical inducers of hypoxia (which induce anoxia) or severe hypoxia (0.01–0.1% O_2), which could be considered anoxic. To address this apparent discrepancy, the phosphorylation pattern of p53 was examined under hypoxic or anoxic conditions to determine whether key signal transduction pathways were differentially activated under a hypoxic gradient. Two key regulatory pathways were examined. The first comprises the N-terminal Ser^{15} kinase pathway that is classically induced by the PI3-kinase family including ATR or ATM when cells are exposed to ionizing radiation or UV radiation (Banin *et al*., 1998; Canman *et al*., 1998). The most pronounced effects of this pathway are to stabilize the p53–p300 complex to facilitate transactivation (Lambert *et al*., 1998; Dornan and Hupp, 2001). The second pathway comprises the C-

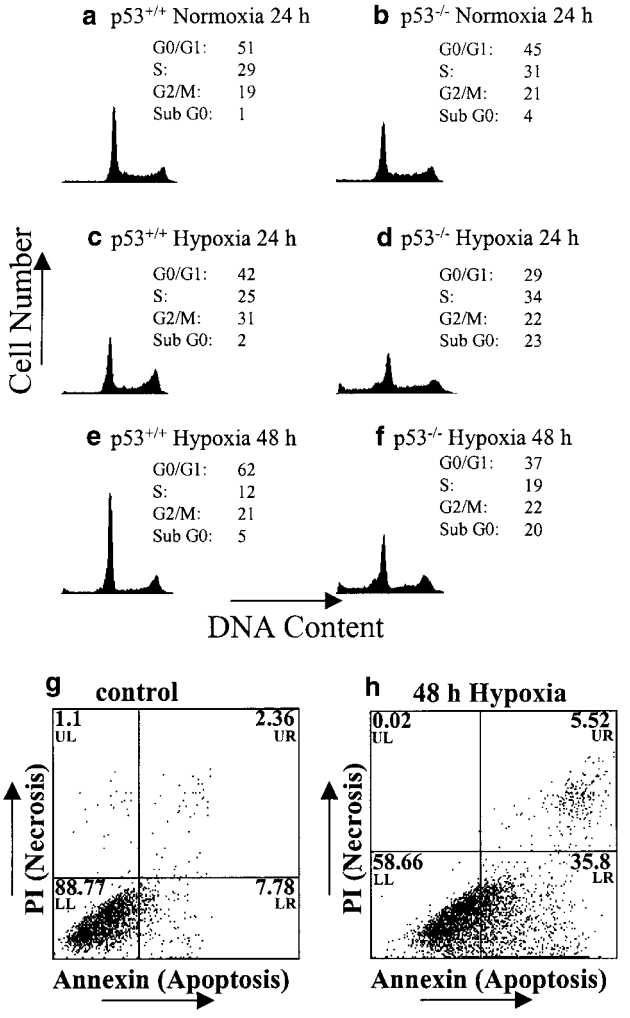

Figure 2 FACS analysis of cell-cycle parameter changes during hypoxia and assessment of hypoxia-induced cell death. Cells were seeded at 1×10^4 cells/ml, cultured in a hypoxic incubator (1% oxygen) with samples taken at times shown, and treated as stated in Materials and methods. HCT116$^{+/+}$ cells are in panels a, c, and e, while HCT116$^{-/-}$ (p53-null) cells are in panels b, d, and f. Numbers showing the percentage of cells in the indicated stages of the cell cycle (G_0/G_1, G_2/M, S, and Sub-G_0) are for 24 or 48 h. p53$^{-/-}$ cells were cultured for: (**h**) 48 h under hypoxic conditions (1% oxygen) or (**g**) control, before assessment of apoptosis and necrosis using the Annexin V assay as described in Materials and methods. UL = upper left, UR = upper right, LL = lower left, and LR = lower right. Numbers in each quadrant represent the percentage of cells detected

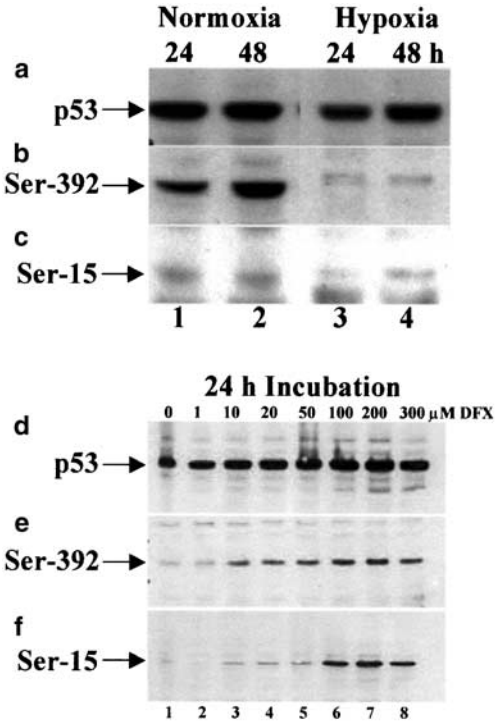

Figure 3 Comparison of hypoxia and anoxia (DFX) on p53 protein levels and on p53 phosphorylation. (**a–c**) Hypoxia attenuates Ser^{392}-site phosphorylation. HCT116$^{+/+}$ cells exposed to hypoxia or normoxia had p53 protein modifications analysed by immunoblotting. p53 protein in normoxic cells are in lanes 1 and 2 and p53 protein in hypoxic cells are in lanes 3 and 4. Cells harvested at indicated time points (lanes 1 and 3 at 24 h and lanes 2 and 4 at 48 h) were immunoblotted and blots probed using (**a**) monoclonal antibody DO-1 (1 : 2000), (**b**) polyclonal antibody antiphosphoserine-392 (1 : 100), or (**c**) polyclonal antibody antiphosphoserine-15 (1 : 1000). (**d–f**) Anoxia induces p53 phosphorylation. Panels d, e, and f show a dose response assay in p53 protein levels and p53 phosphorylation where p53$^{+/+}$ cells were treated with increasing concentrations of DFX (as indicated) and harvested after 24 h. Cells lysates were immunoblotted and blots probed using (**d**) monoclonal antibody DO-1 (1 : 2000), (**e**) polyclonal antibody antiphosphoserine-392 (1 : 100), or (**f**) polyclonal antibody antiphosphoserine-15 (1 : 1000)

terminal Ser^{392} kinase pathway that stimulates the sequence-specific DNA binding function of p53 (Hupp *et al*., 1995; Nichols and Matthews, 2002). This kinase pathway is activated by both ionizing radiation and UV irradiation (Blaydes *et al*., 2000) through the action of CK2-FACT (Keller *et al*., 2001) and/or a DRB-resistant protein kinase (Blaydes and Hupp, 1998).

When cells were grown under normoxic conditions, slight elevations can be seen in the basal Ser^{392}-site phosphorylation at 48 h (Figure 3b, lane 2 *vs* 1) that are not evident at the Ser^{15} site under the same conditions (Figure 3c, lane 2 *vs* 1). This is consistent with an increase in cells undergoing growth arrest as cells reach confluency. A similar induction of Ser^{392}-site phosphorylation can occur in UV-treated cells without increases in p53 protein levels (Blaydes *et al*., 2000). By contrast, a different p53 phosphorylation pattern is observed when cells are incubated in 1% oxygen under conditions where cells growth arrest. In particular, a pronounced downregulation of Ser^{392} phosphorylation occurs 24 h posthypoxia (Figure 3b, lane 3 *vs* 1) as cells enter the growth arrest (Figure 1). This downregulation persists 48 h into hypoxic treatment (Figure 3b, lane 4). A marginal downregulation of Ser^{15} phosphorylation on p53 occurs 24 h posthypoxia (Figure 3c, lane 3 *vs* 1) as cells enter the transient G_2/M arrest (Figure 1c). This downregulation does not persist 48 h into hypoxic treatment (Figure 3c, lane 3 *vs* 4), indicating the Ser^{15} kinase pathway is not being suppressed as effectively the Ser^{392} site kinase pathway. Steady-state levels of total

p53 protein remain unaffected by 48 h of normoxia or 48 h hypoxia (Figure 3a, lanes 1–4).

In contrast to hypoxia, the use of the iron-chelating compound deferoxamine (DFX) to chemically induce anoxia activates the p53 response. A titration of DFX results in a dose-dependent increase in Ser^{392} and Ser^{15} phosphorylation (Figure 3e, f). These data are consistent with previous studies and demonstrates that severe hypoxia/anoxia can activate the classic p53-signalling kinase pathways. As a further control, we investigated whether hypoxia and anoxia differentially affect the levels of the transcription factor HIF-1 (Figure 4). Under conditions where $p53^{+/+}$ cells undergo a growth arrest in hypoxic cells (Figure 1), there is no apparent stabilization of HIF-1 protein (Figure 4a, lanes 2 and 3). Interestingly, HIF-1 protein stabilization is only observed 4 days into hypoxia (Figure 4a, lanes 5 and 6), thus effectively uncoupling hypoxia-dependent growth arrest from HIF-1 protein stabilization. The hypoxia-induced stabilization of HIF-1 protein at 4 and 5 days into hypoxia is p53-independent (Figure 4b). In contrast to hypoxia, chemically induced anoxia results in significant stabilization of HIF-1 protein (Figure 4c, lanes 5–8) at the concentrations where DFX induces p53 phosphorylation (Figure 3). Further, the DFX-induced stabilization of HIF-1 protein is p53-independent (Figure 4d). Together, these data identify hypoxia-responsive signal transduction pathways targeting p53 that are distinct from those induced during anoxia.

p21 is one of the key gene products induced by p53 (el-Deiry *et al*., 1993) and the levels of protein were quantitated in order to determine whether p53's key effector was mediating the growth arrest under conditions where hypoxia promotes decreases in Ser^{392} phosphorylation. p21 protein levels are not elevated under hypoxic conditions when $p53^{+/+}$ cells undergo growth arrest (Figure 5a, lanes 2 and 3 *vs* 1) and a decrease in p21 protein levels begin to occur at 48 h posthypoxia in $p53^{+/+}$ cells (Figure 5a, lane 3 *vs* 1). $p53^{-/-}$ cells show a similar lack of p21 protein induction, albeit at a lower overall level, consistent with the pronounced p53 dependence of p21 (Figure 5b). Together, these data show that p21 protein levels are not induced when cells undergo hypoxia-induced growth arrest and are consistent with data showing that Ser^{392} phosphorylation is substantially reduced in hypoxic cells.

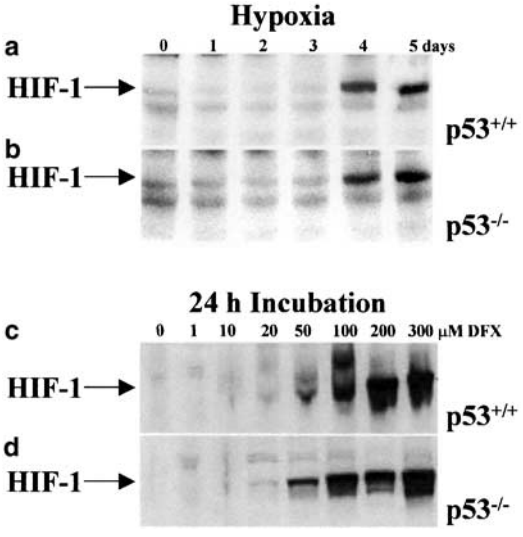

Figure 4 Hypoxia (1%) does not stabilize HIF-1 protein. HCT116 and HCT116-$P53^{-/-}$ cells were exposed to hypoxia (1%) for up to 5 days (**a, b**) or DFX for 24 h (**c, d**) had HIF-1 protein levels examined by immunoblotting using anti-HIF-1 monoclonal antibodies

Hypoxia attenuates p53 phosphorylation and p53-dependent apoptosis induced by 5-FU

Based on data presented so far, oxygen tension may have significant influences on p53 activation and tumour response *in vivo*. Hypoxia would attenuate p53 and remove the selection pressure for p53 mutation, while anoxia will activate p53 and place a selective burden on the p53 pathway. Solid tumours will have gradients of differing oxygen concentration because of altered blood flow and thus anticancer drug response will be obviously altered by the p53 response in an oxygen-depleted microenvironment. We therefore studied the significance of the hypoxia-dependent perturbation of p53 by examining whether the anticancer drug 5-flourouracil (5-FU) is dominant over hypoxia or is attenuated by hypoxia.

When $p53^{+/+}$ cells were exposed to 5-FU, significant increases in p53 protein levels and p21 protein levels occurred 24 h posttreatment (Figure 6a, b, lanes 2–6 *vs* 1). This elevation in p53 protein and p21 protein levels also correlates with increases in Ser^{392} and Ser^{15} phosphorylation of p53 (data not shown, see below). This increase in p53 protein in response to 5-FU correlates with induction of p53-dependent cell death as measured using FACS by the number of cells in sub-G_0 (Figure 7d, 37% of $p53^{+/+}$ cells in sub-G_0 *vs* Figure 8d, 14% of $p53^{-/-}$ cells in sub-G_0). In a time course over 2 days, it took 24 h before a time-dependent accumulation in dead cells was observed in $p53^{+/+}$ cells (Figure 7d, 24, 36, and 48 h). These data are consistent

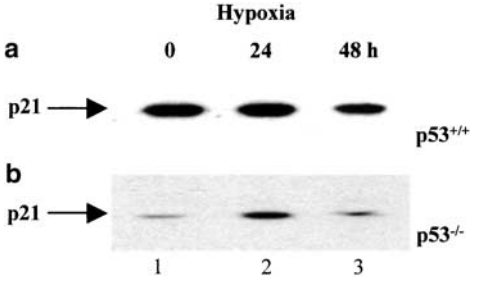

Figure 5 Hypoxia does not induce p21. The steady-state levels of p21 protein were quantified as indicated in Materials and methods from normoxic cells (lane 1) or hypoxic cells (lanes 2 and 3) in $HCT116^{+/+}$ cells (**a**) or HCT116-$p53^{-/-}$ cells (**b**) (lane 1 *vs* 2 and 3). The samples are matched to the cell-cycle parameters in Figure 1 and the p53 protein levels in Figure 4. The arrows indicate the position of p21 protein

with reports that 5-FU induces apoptosis, that is p53-dependent (Bunz *et al*., 1999).

Hypoxia was able to attenuate the p53-dependent cell death in $p53^{+/+}$ cells at 24–48 h after hypoxic incubation of 5-FU-treated cells (Figure 7d). The level of cell death at 24–36 h posthypoxic treatment in 5-FU-treated cells was half that observed in 5-FU-treated cells only. By 48 h posthypoxia in 5-FU-treated $p53^{+/+}$ cells, the level of cell death (Figure 7d, 23%) was similar to the amount of p53-independent cell death (in $p53^{-/-}$ cells) induced by 5-FU in the presence of hypoxia (Figure 8d, 15 and 21%, respectively). The presence of p53 can therefore reduce cell death and promote survival in the presence of hypoxia (Figure 1), while p53-dependent apoptotic cell death induced by a damaging agent is blocked by hypoxia (Figures 7 and 8).

In order to determine whether the mechanism of attenuation of 5-FU-dependent cell death is upstream of p53, changes in the levels of p53 phosphorylation at the Ser^{15} and Ser^{392} activation sites were examined in 5-FU-treated cells without or with hypoxic incubation. Within 6 h of 5-FU addition, p53 protein was stabilized and this persisted up to 48 h post-5-FU treatment (Figure 9a, lanes 2–5 *vs* 1). After 6 h of hypoxic incubation in 5-FU-treated cells, the induction of p53 protein was significantly reduced and this persisted up to 48 h (Figure 9a, lanes 7–10 *vs* 6). Ser^{15} phosphorylation was also significantly attenuated in hypoxic cells from 6 h post-5-FU treatment (Figure 9b, lanes 7–10 *vs* 2–5) and similar attenuation in Ser^{392} site phosphorylation was observed from 6 to 48 h of hypoxic treatment (Figure 9c, lanes 7–10 *vs* 2–5). To further examine the down-regulation of 5-FU-induced p53 protein by hypoxia, we also assessed the levels of two other p53-inducible genes, namely $p21^{WAF1}$ and MDM2. As can be seen in Figure 9d, e, these two gene products are stabilized marginally by 5-FU treatment of cells (lanes 2–4) but hypoxia attenuates $p21^{WAF1}$ and MDM2 induction by 5-FU (lanes 7–10). The attenuation of $p21^{WAF1}$ and MDM2 protein induction by hypoxia is most pronounced 48 h after 5-FU addition (lane 10 *vs* 5). These data indicate that hypoxic stress can antagonize the

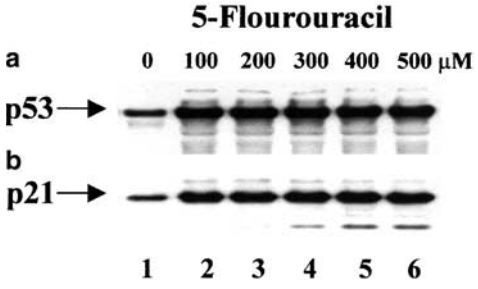

Figure 6 5-FU induces p53 protein and p21 protein. $HCT116^{+/+}$ cells were grown in drug-free conditions for 24 h prior to addition of 5-FU at the stated concentrations for 24 h. Cells were then harvested, immunoblotted, and blots probed using (**a**) monoclonal antibody DO-1 (1 : 2000) for p53 or (**b**) monoclonal antibody AB-1 for p21

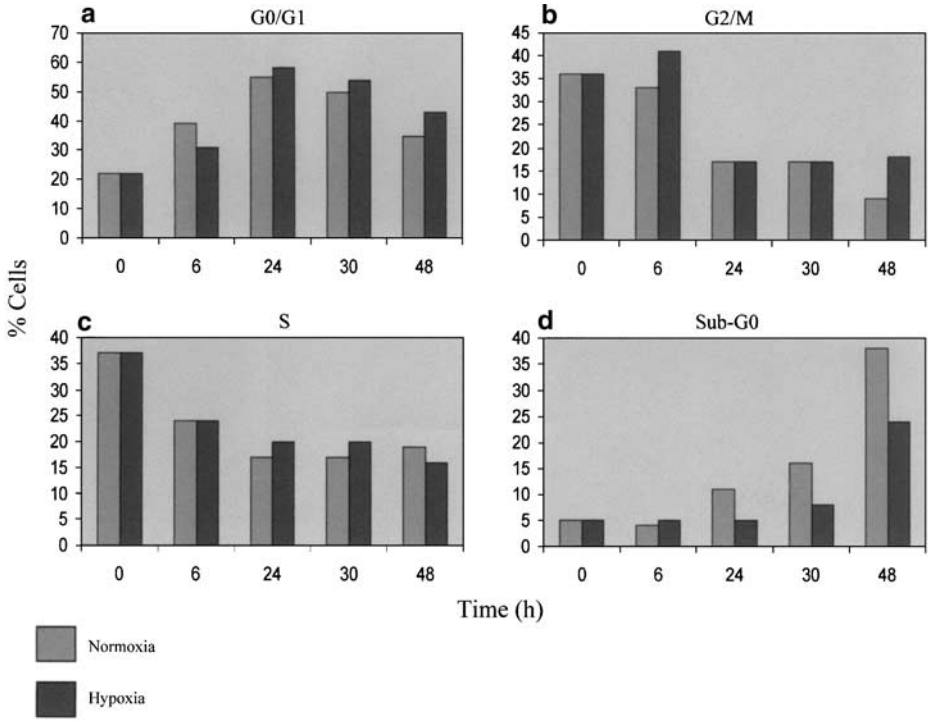

Figure 7 Hypoxia attenuates 5-FU induced p53-dependent apoptosis. $HCT116^{+/+}$ cells were seeded at 1×10^4 cells/ml and grown in McCoys 5A medium in drug-free normoxic conditions for 24 h prior to addition of 5-FU at 100 μM and transfer to a hypoxic incubator (1% oxygen). Samples were then taken at times shown, and treated as stated in Materials and methods

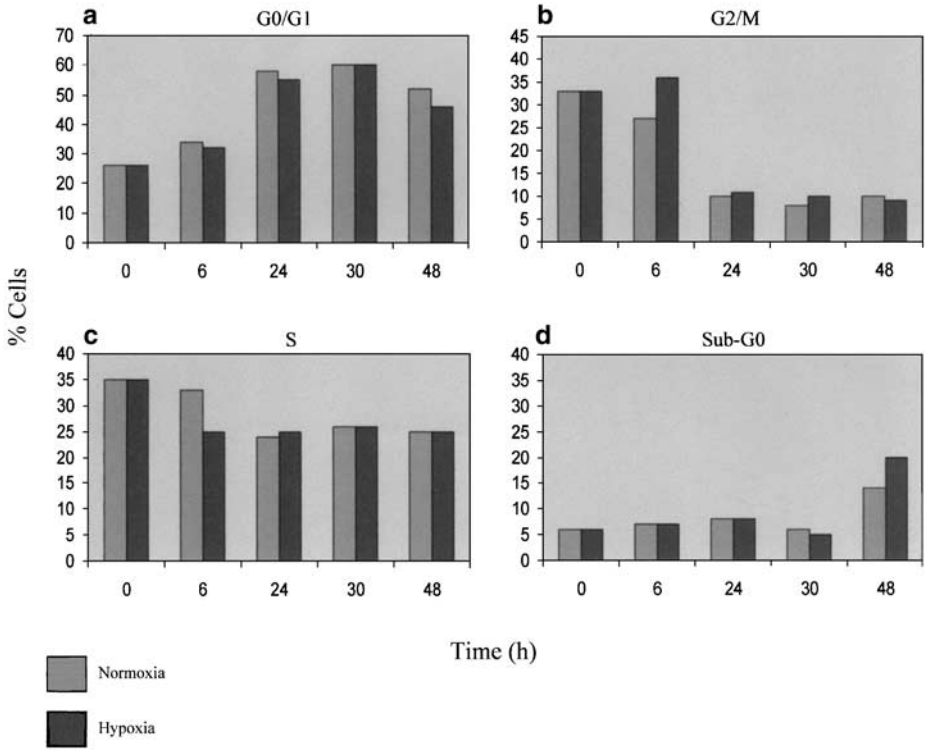

Figure 8 FACS analysis of cell-cycle parameter changes in $p53^{-/-}$ cells treated with 5-FU under normoxic and hypoxic conditions. $HCT116^{-/-}$ (p53-null) cells were seeded at 1×10^4 cells/ml and grown in McCoys 5A medium in drug-free normoxic conditions for 24 h prior to addition of 5-FU at 100 μM and transfer to a hypoxic incubator (1% oxygen). Samples were then taken at times shown, and treated as stated in Materials and methods

5-FU-dependent kinase signalling and apoptotic pathway and suggest that the reduced cell death in 5-FU-damaged hypoxic cells is due, in part, to the reduction in p53 phosphorylation at key activating kinase sites.

Discussion

Studies of p53 activation by cellular stresses have historically focused on the DNA damage response and have been based on genetic studies centred on p53 activation mechanisms. Mice that are null for the p53 locus develop spontaneous cancers at an elevated rate and are compromised with respect to induction of ionizing radiation-dependent apoptosis in thymocytes, splenocytes, and intestinal cells (Hupp, 2000). Independent studies demonstrated that radiation-induced checkpoint defects in cells from patients with genetic defects in ATM or CHK2 predispose patients to reduced repair of damaged DNA and elevated risk of cancer development (Jayaraman and Prives, 1999). Further, the reduced induction of p53 by UV radiation in cells from XP-A, but not XP-C mutant cells (Yamaizumi and Sugano, 1994), indicates a role for coupled transcription repair as a major pathway that signals to p53 (Ljungman *et al*., 1999). Quantitative studies have shown that there is both an oxidative damage component and an RNA polymerase signalling component for the synergistic production of p53 with a high specific activity as a transcription factor (Blaydes *et al*., 2000). Together these studies highlight key genetic factors that modulate the p53 response to DNA damage.

The DNA damage or 'guardian of the genome' model for p53 activation was an important milestone in marking p53 as a stress-regulated transcription factor (Lane, 1992). Studies into physiologically relevant stresses that may activate p53 function *in vivo* such as hypoxia or acidification have been carried out recently. Acidification is reported to stabilize p53 protein and induce a p53-dependent growth arrest (Williams *et al*., 1999). Hypoxia has been shown using *in vivo* models to be involved in p53-dependent tumour suppression and in the induction of p53-dependent apoptosis. Our data appear to contrast with some reports describing how hypoxia impinges on the p53 pathway. The first significant difference is that hypoxia was reported previously to induce p53-dependent apoptosis (Graeber *et al*., 1996; Giaccia and Kastan, 1998). By contrast, we show paradoxically that loss of p53 sensitizes cells to hypoxia-dependent cell death and that hypoxia attenuates the known activation of p53 by 5-FU. Further, hypoxia was reported to have no effect on ionizing-radiation-induced gene expression by p53 (Koumenis *et al*., 2001), while we show that hypoxia attenuates the p53 response in response to 5-FU. p53 protein is also reportedly stabilized by hypoxic treatment (Ashcroft *et al*., 2000), while our studies show no increases in p53,

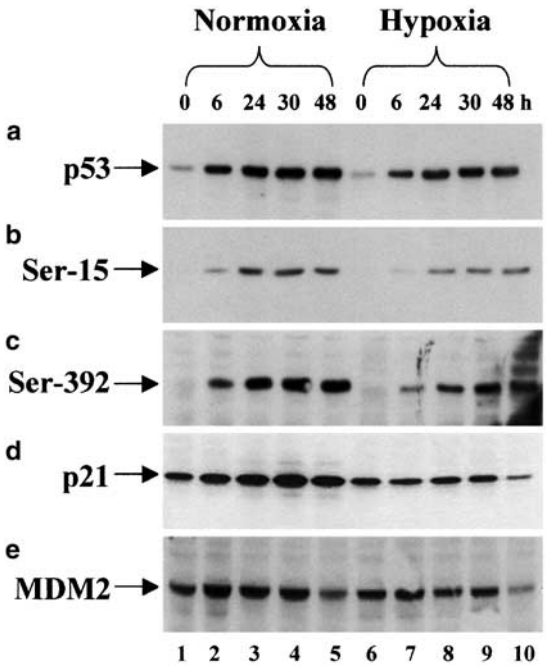

Figure 9 Hypoxia attenuates 5-FU-induced phosphorylation of p53 and induction of $p21^{WAF1}$ and MDM2. $HCT116^{+/+}$ cells were seeded at 1×10^4 cells/ml and grown in McCoys 5A medium in drug-free normoxic conditions for 24 h prior to addition of 5-FU at 100 μM and transfer to a hypoxic incubator (1% oxygen). Samples were then taken at times shown, and treated as stated in Materials and methods. Cells were then harvested, immunoblotted, and blots probed using (**a**) monoclonal antibody DO-1 (1 : 2000), (**b**) polyclonal antibody antiphosphoserine-15 (1 : 1000), (**c**) polyclonal antibody antiphosphoserine-392 (1 : 100), (**d**) anti-p21 monoclonal antibody AB-1, or (**e**) anti-MDM2 monoclonal antibody 4B2

but in fact decreases in basal phosphorylation most pronounced at the CK2/FACT site at Ser^{392}. A notable difference in the methods may account for these opposing results. Much work using hypoxia is carried out under conditions where oxygen concentrations are lowered to 0.1% or 0.01% or chemical mimetics of hypoxia (Hammond *et al*., 2002), rather than the 1% O_2 used in the studies described here. These lower concentrations of oxygen in other reports may represent anoxic rather than hypoxic conditions. In fact, when we lower our oxygen concentrations to 0.5% rather than 1%, we can begin to see induction of p53 protein (data not shown). Further, the induction of severe hypoxia or anoxia by the iron chelator DFX induces significant p53 phosphorylation and HIF-1 protein induction (Figures 3 and 4), thus resolving our studies with previous reports: hypoxia induces a p53-dependent growth arrest without HIF-1 stabilization and via an attenuation of p53, while anoxia induces significant HIF-1 protein stabilization and p53 activation. In a sense, then, the use of 1% hypoxia has allowed for the uncoupling of hypoxic-induced growth arrest from HIF-1 and p53 protein activation/induction.

Despite our data showing that hypoxia (1%) activates p53-independent apoptosis (Figure 1) or severe hypoxia activates p53-dependent apoptosis (Koumenis *et al*., 2001), the agreement between three different studies is that p53 is not activated as a positive transcription factor by hypoxia (Figure 3) (Ashcroft *et al*., 1999; Koumenis *et al*., 2001). A sin3A-dependent transrepression mechanism may account for the activation of p53-dependent pathways by lowered oxygen concentrations (Koumenis *et al*., 2001). One of the most striking changes we observed in the early stages of hypoxia is alterations in Ser^{392} phosphorylation, which is known to affect sequence-specific DNA binding (Hupp *et al*., 1995), transcription in contact-inhibited fibroblasts (Hao *et al*., 1996), and p53 transrepression (Hall *et al*., 1996). Together, these data suggest that Ser^{392} phosphorylation changes may be modulating p53 function as a transrepressor of gene expression under hypoxic conditions (Koumenis *et al*., 2001). However, although such activation of p53 as a transrepressor may account for the early cell cycle perturbations, $p53^{+/+}$ cells escape the G_2/M block and enter a longer-term G_0/G_1 arrest for at least 7 days (data not shown). Under these longer incubation times where cells remain growth arrested, there is an HIF-1 protein induction (Figure 4) and re-phosphorylation of p53 at Ser^{392} (data not shown). Thus, although alanine mutation of the Ser^{392} phosphorylation site of p53 reduces its activity as a transcription factor (Keller *et al*., 2001), this mutation can also paradoxically reduce its activity as a transrepressor of transcription (Hall *et al*., 1996). Such a requirement of p53 as a transactivator and a transrepressor has been revealed by global changes in p53-dependent gene expression using differential display techniques, SAGE, or microarray analysis (Polyak *et al*., 1997; Vogelstein *et al*., 2000; Zhao *et al*., 2000).

The complex physiology of the hypoxia response *in vivo* will obviously then depend upon the severity of oxygen starvation as well as the fluctuations that occur in blood pressure of patients and oxygen concentration gradients in areas of limiting blood supply. The striking differences in p53 protein phosphorylation under conditions of hypoxia or anoxia suggest a significant heterogeneity in the p53 response even within a homogenous tumour population due to oxygen gradients. One of the central questions in cancer progression is the nature of the environmental driving force for the production of clones with mutation in oncogenes and tumour suppressors. Hypoxia is a logical selection pressure for mutating p53 in solid cancer cells (Graeber *et al*., 1996), but our data indicating that hypoxia attenuates p53 are not apparently compatible with this hypothesis. However, an elegant study looking at the p53 mutation frequency in solid cancers *vs* ascites tumours has shown that p53 mutations occur not in the solid cancer but in the ascites outgrowth (Magnusson *et al*., 1998). The ascites microenvironment is considered anaerobic and it may be that such anoxic conditions, rather than hypoxic signals, favour the evolution of cells with p53 mutations. Thus, it may be possible that p53 is silenced *in vivo* in the hypoxic, oxygen-deprived cancer cell and that its tumour suppressor activity is recruited in the anoxic, oxygen-free microenvironment.

Materials and methods

Cell culture

HCT116 human colorectal carcinoma cell lines ($p53^{+/+}$ and $p53^{-/-}$) were a kind gift of Dr Bert Vogelstein (Waldman *et al*., 1995; Bunz *et al*., 1999) (Johns Hopkins University, USA) and maintained in a monolayer culture in McCoys 5A modified medium (GibcoBRL, 26600-023) supplemented with 10% foetal bovine serum (GibcoBRL, 10099-141) and 1% penicillin (10 000 i.u./ml)–streptomycin (10 000 μg/ml) (GibcoBRL, 15140114) in a Heraeus Hera Cell humidified incubator at 37°C in 5% CO_2.

Hypoxia assay

Cells were seeded at appropriate density in Nunc T80 culture flasks and incubated at 37°C with 5% CO_2 and 1% O_2 in a Napco 7000 incubator. DFX was resuspended in ddH_2O and added at the indicated concentrations. Cultures were then removed at the times stated and immediately had medium removed, washed using ice-cold PBS, trypsinized using trypsin-EDTA (GibcoBRL, 25300-054) for 1 min at 37°C, and all components combined in a 50 ml plastic tube held on ice. Cells were then centrifuged at 1000 r.p.m. for 4 min in an MSE Mistral 1000 centrifuge followed by two washes using 10 ml ice-cold PBS, before resuspension in 1.0 ml ice-cold PBS. Each sample was then split with 0.4 ml reserved for FACS analysis and 0.6 ml used for Western analysis. FACS analysis samples were fixed by the addition of 4.0 ml 70% ice-cold ethanol with vigorous mixing and held at 4°C for up to 1 week.

Western analysis

Western analysis samples were centrifuged at 13 000 r.p.m. for 1 min at room temperature and pellets resuspended in 100 μl urea lysis buffer (8 M urea, 1% Triton, 100 mM DTT, 50 mM HEPES, pH 7.6), held on ice for 20 min, and then centrifuged at 14 000 r.p.m. at 4°C for 20 min using an Eppendorf S417R centrifuge. The use of urea as a core component of the lysis buffer prevents the dephosphorylation of p53 during lysis. Supernatant was then retained for protein analysis using the Bradford method. Equal amounts of protein (20–50 μg) were added to an equal volume of Laemmli sample buffer (5% SDS, 25% glycerol, 125 mM Tris, few grains bromophenol blue, 1 M DTT, pH 8.0) and then subjected to electrophoresis using an Invitrogen Novex mini cell system with NuPage precast 4–12% gradient gels (NP0323). Following transfer to nitrocellulose, samples were then subjected to protein immunoblotting using the following p53 antibodies: Ser20-phospho-sensitive monoclonal antibody DO-1 (Craig *et al*., 1999), DO-12 (Mab) to detect total p53 (Craig *et al*., 1999), FP3 (Mab) Ser^{392}, FP3 (Poly) (Blaydes and Hupp, 1998; Blaydes *et al*., 2000), and FPS-15 (Poly) ATM site (Canman *et al*., 1998). Immunoreactive proteins were viewed using enhanced chemiluminescence. p21 protein levels were detected using the antibody AB-1 (Scott *et al*., 2000).

FACS analysis

Each sample fixed in 70% ethanol was centrifuged at 1000 r.p.m. for 5 min at room temperature and pellet resuspended in 1.0 ml PBS. Cells were counted using a Neubauer haemocytometer and adjusted to 7.5×10^5 cells ml in PBS. RNAse A (100 μl of 1 mg/ml) (Sigma) and propidium iodide (100 μl of 400 μg/ml) (Sigma) were added to 1.0 ml of cells in an FACS tube (Falcon) and incubated at 37°C for 30 min. Samples (10 000 cells) were then immediately analysed using Cellquest software on a Beckton Dickinson FACScan flow cytometer. For apoptosis detection, cells were cultured under hypoxic conditions (1% oxygen) for up to 48 h before harvesting, followed by assessment of apoptosis using Oncogene Annexin V FITC Apoptosis Detection Kit (Cat # PF032-1EA) according to the manufacturer's instructions.

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