Apoptosis, cell cycle progression and gene expression in TP53-depleted HCT116 colon cancer cells in response to short-term 5-fluorouracil treatment

BIRGITTE LID ADAMSEN, KATHERINE L. KRAVIK, OLE PETTER F. CLAUSEN and PAULA M. DE ANGELIS

The Pathology Clinic, Rikshospitalet-Radiumhospitalet Medical Center, Oslo 0027, Norway

Received May 11, 2007; Accepted June 27, 2007

Abstract. Loss of TP53 function may contribute to 5-fluorouracil (5-FU) resistance in colorectal cancer since TP53-deficient cells may be unable to undergo apoptosis in response to 5-FU-induced DNA damage. 5-FU treatment of TP53-deficient cells would provide useful information on the apoptotic response to drug-induced DNA damage in the absence of TP53 and its transcriptional targets. We investigated apoptosis induction and cell cycle alterations in response to short-term treatment with two different 5-FU concentrations following siRNA-mediated knockdown of TP53 in the TP53-proficient HCT116 colon cancer cell line. We focused on high-dose 5-FU treatment to investigate the apoptotic phenotype in 5-FU-treated cultures since this dose resulted in apoptosis induction at 24 h of treatment, whereas clinically-relevant bolus 5-FU treatment of HCT116 cultures did not. Gene expression alterations were also assessed in 5-FU-treated HCT116 cultures using whole genome expression arrays. Compared to 5-FU-treated TP53-proficient HCT116 cultures, 5-FU-treated TP53-depleted HCT116 cultures showed lack of CDKN1A induction, decreased apoptotic levels, decreased FAS and TNFRSF10B transcript levels and cleaved PARP protein levels, G_1/S transition arrests, decreased CCND1 protein levels, and smaller intra-S phase arrests. Alterations in gene expression in 5-FU-treated TP53-depleted HCT116 cultures confirmed previously-reported TP53 target genes and suggested potentially novel TP53 target genes (e.g. *APOBEC3C*, *BIRC3*, *JMJD2B*, *LAMP3*, *MYO1E*, *PRRG1*, *SULF2*, *TACSTD2*, *TncRNA*, *ZFYVE20*) that may play a role in mediating the 5-FU-induced DNA damage response in TP53-proficient cells. Abrogation of TP53 function in 5-FU-treated HCT116 cultures results in reduced apoptosis, TP53- and CDKN1A-independent G_1/S phase arrests that may be protective against apoptosis, smaller intra-S phase arrests, and transcript level decreases of both reported TP53 target genes as well as potentially novel TP53 target genes.

Correspondence to: Dr Paula M. De Angelis, The Pathology Clinic, Rikshospitalet-Radiumhospitalet Medical Center, Oslo 0027, Norway
E-mail: p.d.angelis@medisin.uio.no

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNAs; 5-FU, 5-fluorouracil

Key words: colorectal cancer, HCT116, 5-fluorouracil, TP53, RNA interference, gene expression, cell cycle, apoptosis, pharmacogenomics

Introduction

The fluoropyrimidine 5-FU is widely used in the treatment of a range of cancers including colorectal cancer and breast cancer (1,2), but resistance to the drug remains a major clinical problem. Drug resistance is complex and multi-faceted, involving not only aspects of drug mechanism of action in cells, but also alterations in the way cells respond to DNA damage caused by chemotherapeutic drugs. Stalled replication forks and single- and double-DNA breaks often result from 5-FU exposure (3-5). The TP53 tumor suppressor is a key mediator in the cellular response to DNA damage caused by ionizing radiation, ultraviolet light and chemotherapeutic drugs (6,7), and can be activated by either of the DNA damage checkpoint kinases ATM and ATR (8-10). ATM/ATR phosphorylate and activate TP53 directly or indirectly through the Chk1/Chk2 kinases (9,11). Both Chk1 and Chk2 phosphorylate and inactivate CDC25A phosphatase, leading to its ubiquitin-mediated proteolytic degradation and a subsequent G_1/S arrest, which is a rapid response to DNA damage (9). This rapid response is followed by TP53-mediated G_1/S arrest which becomes fully operational several hours after the detection of DNA damage (9,12), involving transcription of target genes such as CDKN1A (7,11). If drug-induced DNA damage is too extensive to be repaired, TP53 induces apoptosis through up-regulation of pro-apoptotic genes such as *BAX*, *PMAIP1*, *TNFRSF10B*, and *FAS* (7,10,13). A number of studies have shown that disruption of TP53 function contributes to 5-FU resistance, and this has been attributed to an inability to undergo TP53-mediated apoptosis (14,15). Several clinical studies have demonstrated that high levels of TP53 accumulation (a surrogate marker for TP53 mutation) was related to resistance to 5-FU-based chemotherapy (16-18), but other studies found no such relationship (19). Overexpression of proteins affected by 5-FU action, e.g. thymidylate synthetase (20,21), and dihydropyrimidine dehydrogenase (22,23) have

also been shown to be associated with resistance to 5-FU, not surprisingly since inhibition of thymidylate synthetase by FdUMP is the main mechanism of action for 5-FU (3,24).

We investigated apoptosis induction and cell cycle alterations in response to short-term 5-FU treatment following siRNA-mediated knockdown of TP53 in the HCT116 colon cancer cell line, and used comparative gene expression analyses to assess expression levels of altered genes downstream of TP53 in drug-treated TP53-depleted HCT116 cultures. Transient knockdown of TP53 followed by short-term 5-FU treatment should provide useful information about the cellular response to 5-FU-induced DNA damage in the absence of TP53 and its transcriptional targets.

Materials and methods

Cell lines, transfection with siRNAs, drug treatment and viability assays. The human colon cancer cell line HCT116 (wild-type *TP53* gene, American Type Culture Collection #CCL-247) was maintained in RPMI media supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 0.5 mg/ml gentamycin in a humidified incubator at 37˚C with 95% O_2 and 5% CO_2. To achieve transient TP53-depletion in HCT116 cells, cultures were grown in 25-cm^2 cell culture flasks and transfected at 30% confluence with 30 nM siTP53, a pool of siRNAs specific for TP53 (SMARTpool, Dharmacon Inc., Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). After transfection at 37˚C for 1 h, the media were aspirated off and RPMI media supplemented with 2 mM L-glutamine, 0.05 mg/ml gentamycin, and 20% FBS was added. Control cultures were transfected with 30 nM of siControl, a pool of non-specific siRNAs (Dharmacon Inc.) under the same conditions. To assess transfection efficiency, cells were co-transfected with a fluorescein-labeled dsRNA, Block-it-FITC (Invitrogen).

One of the major aims of the present work was to investigate the apoptotic response to 5-FU following TP53 knockdown. The 5-FU concentration needed to cause 50% growth inhibition in HCT116 cultures at 24 h of treatment is 90-100 μM (25). We investigated the apoptotic response of HCT116 cultures to two different concentrations of 5-FU (Amersham Biosciences, UK) 24 h after transfection with siTP53 or siControl Smartpools. A (non-clinically relevant) dose of 380 μM was used in order to ensure that apoptosis was induced, as demonstrated in several previous studies (15,42). In addition, a 5-FU bolus dose corresponding to 600 mg/m^2 was also used to simulate clinically-relevant conditions (25). Cell cultures were harvested by trypsinization or scraping at 0, 2, 6, 24, 48 and 72 h after drug addition for further analyses. 5-FU was removed from cultures at 24 h via media shifts. Viability and total cell numbers were assessed using a standard trypan blue viability assay. Data presented are the results of 2 replicate experiments.

Cell death measurements. Trypsinized cells, including floating cells that had detached from the monolayer, were washed once with PBS without Ca and Mg, centrifuged at 1000 rpm for 5 min, and supernatants discarded. Cell pellets were resuspended in Annexin V binding buffer and incubated with Annexin V conjugated to phycoerythrin (Molecular Probes, Eugene, OR) and ToPro-3 (Molecular Probes) for subsequent flow cytometric measurements of apoptosis and necrosis respectively. FITC fluorescence (FL1 parameter) was used to gate transfected cells (transfected with a FITC-labeled non-specific dsRNA) from untransfected cells (FITC-negative) in order to examine these populations individually for apoptosis induction (phycoerythrin fluorescence, FL2 parameter) (Fig. 2A). Apoptotic fractions were measured as the sum of the number of cells in lower right and upper right quadrants on bivariate distributions of Annexin V vs. ToPro-3 positivity. Uptake of ToPro-3 (FL4 fluorescence) indicated necrotic cell death; upper left quadrants on Annexin V vs. ToPro-3 bivariate distributions gave the percentage of necrotic cells. Additionally, trypan blue uptake in drug-treated cultures was also used to assess necrotic cell death.

Cell cycle analyses and determination of mitotic fractions. Transfected cells were sorted from untransfected cells in untreated and drug-treated siTP53- and siControl-transfected HCT116 cultures using a FACSDiVa flow cytometer (BDIS, CA) such that cell cycle analyses could be performed on just the transfected cells in these cultures. ToPro-3 was added to cell samples to label dead cells which were excluded from sorting. Sorted viable cells were fixed in 80% ethanol and stored at -20˚C until analysis. They were then prepared as isolated nuclei stained with propidum iodide using Vindelov's *et al* procedure (26), and analyzed for cellular DNA content using a FACSCalibur flow cytometer (BDIS). WinCycle software (Phoenix Flow Systems, San Diego, CA) was used to determine the percentages of cells in the G_1, S and G_2/M phases of the cell cycle in drug-treated and control samples.

Immunoblotting. Cell cultures were harvested for immunoblotting after 5-FU treatment and boiled for 5 min in standard Laemmli buffer. Protein concentrations were determined, and 10 μg cell samples and 5 μg molecular weight standards were loaded onto 5, 10 or 12% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes using the Criterion Blotter system (Bio-Rad Laboratories). For immunoblotting, the membranes were incubated with the primary mouse or rabbit antibodies overnight at 4˚C. The following primary antibodies were used: mouse monoclonal TP53 (clone 1801) and mouse monoclonal p21 (clone EA10; Calbiochem, San Diego, CA); mouse monoclonal phospho-TP53 (Ser15) (16G8); rabbit polyclonal phospho-Rb (Ser-807/811), and rabbit polyclonal cleaved PARP (Asp214; Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-phospho-histone H3 (Ser-10; clone JBW301; Upstate, Lake Placid, USA); mouse monoclonal FAS (B-10) and rabbit anti-human polyclonal CCND1 (H-295; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated with biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG for 1 h followed by incubation with a streptavidin-biotinylated-alkaline phosphatase complex for 1 h and colorimetric development. Protein levels were quantified using UnScanit gel software version 5.1 for Windows (Silk Scientific Inc., Orem, UT).

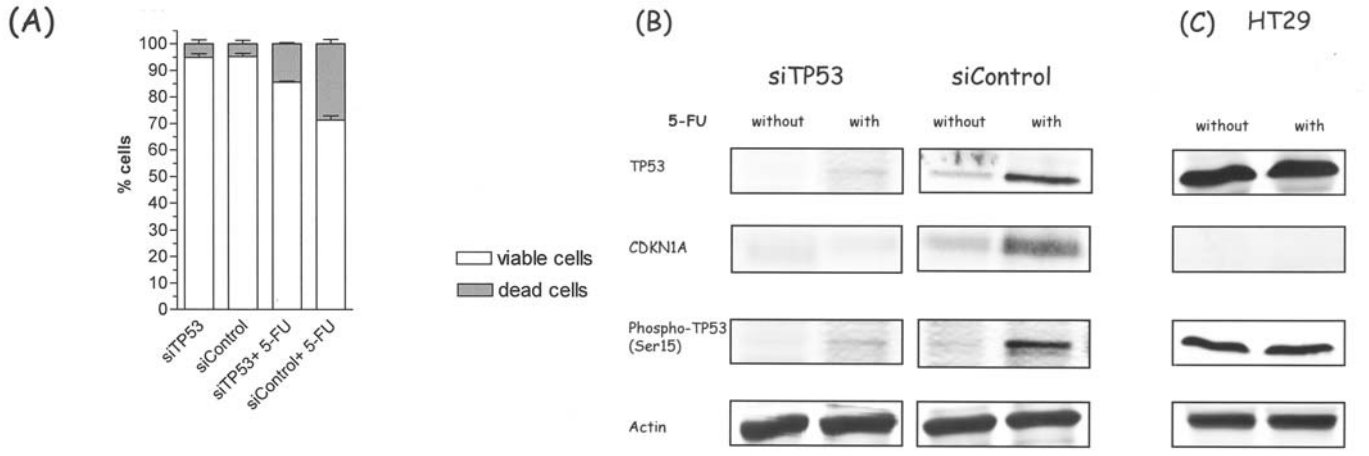

Figure 1. siRNA transfection and TP53 knockdown. (A) Effect of siRNA transfection on cell viability. Percentages of viable cells in siTP53- and siControl-transfected HCT116 cultures following 24-h treatment with 380 μM 5-FU measured using a trypan blue viability assay. (B) Confirmation of TP53 knockdown: immunoblots showing levels of TP53 and CDKN1A proteins at 24 h of treatment with 380 μM 5-FU in HCT116 cultures transfected with siTP53 or siControl. Actin was used as a loading control. (C) 5-FU-treated HT29 cells (have mutated *TP53* gene/non-functional *TP53* protein) are included for comparison.

Comparative gene expression analyses. RNA was isolated from drug-treated and untreated siTP53- and siControl-transfected HCT116 using TRIzol (Invitrogen). Preparation of fluorescently-labeled cDNAs, hybridization to Affymetrix Gene Chip Human Genome U133 Plus 2.0 expression arrays (include 38,500 human genes), washing, scanning, data collection and analyses were performed according to Affymetrix protocols available on their website (www.affymetrix.com, Affymetrix Inc., Santa Clara, CA). The detection algorithm described whether the transcript was present (P), absent (A) or marginal (M). A change algorithm described a 'change' in transcript level between a baseline array and an experiment array, and was presented as increase (I), decrease (D), marginal increase (MI), marginal decrease (MD) or no change (NC). Gene expression changes were expressed as $\log_2$ ratios. Genes were scored as up-regulated or down-regulated if $\log_2$ ratios were ≥ 0.5 or ≤ -0.5, respectively. A $\log_2$ ratio reflecting the differences between drug-treated siTP53- and drug-treated siControl-transfected HCT116 cultures was calculated by dividing the $\log_2$ ratio of drug-treated siTP53:untreated siTP53 by the $\log_2$ ratio of drug-treated siControl:untreated siControl (essentially a sub-traction of the latter from the former). This newly-derived ratio was used to identify both reported TP53-target genes and potentially novel TP53-target genes.

Real-time RT-PCR. Expression levels for 4 genes, *TP53*, *CDKN1A*, *CCND1* and *EGFR* were determined by real-time RT-PCR for 5-FU-treated and untreated siTP53- and siControl-transfected HCT116 cultures at the 24-h time-point using gene-specific TaqMan probe and primer sets (Applied Biosystems, Foster City, CA) and an ABI PRISM Sequence Detection System model 7500 following manufacturer protocols in order to confirm microarray gene expression results. cDNA (10 ng), which were synthesized from total RNA (2 μg) by use of Superscript III transcriptase (Invitrogen), were employed. The reactions were carried out in triplicate in a 25 μl reaction volume and a 96-well format, and the B2M gene was used as an endogenous control for equal amounts of RNA used.

Statistical analysis. Statistical analyses and plots were generated using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Cell viability, transfection efficiency and TP53 knockdown. Transfection with siRNAs did not adversely affect the viability of untreated control HCT116 cultures (Fig. 1A). Using a non-specific FITC-labeled dsRNA as a transfection indicator, the mean (±SD) percentage of transfected cells in siTP53- and siControl-transfected HCT116 cultures was determined to be 50% (±8.2, n=11). siTP53-tranfected HCT116 cultures treated with 380 μM 5-FU demonstrated very low levels of TP53 accumulation (and TP53 activation) due to the untransfected cells in these cultures which responded to 5-FU, but otherwise showed complete lack of CDKN1A induction at 24 h of drug treatment (Fig. 1B), indicating efficient TP53 knockdown. In contrast, drug-treated siControl-transfected cultures showed high levels of TP53 accumulation (and strong TP53 activation-phosphorylation at Ser15) as well as strong CDKN1A induction at 24 h (Fig. 1B). TP53 and CDKN1A immunoblots for 5-FU-treated HT29 cultures are depicted in Fig. 1C for comparison purposes; this cell line has non-functional TP53 due to gene mutation and expresses high levels of mutant TP53. Note the complete lack of CDKN1A induction in this cell line in response to 5-FU, similar to that seen in the 5-FU-treated TP53-depleted HCT116 cell line. TP53 accumulation and CDKN1A induction in 380 μM-treated siControl-transfected cultures were also detectable at earlier timepoints (data not shown). SiTP53-tranfected HCT116 cultures treated with bolus 5-FU also demonstrated TP53 knockdown at 24 h of drug treatment (data not shown). TP53 knockdown was

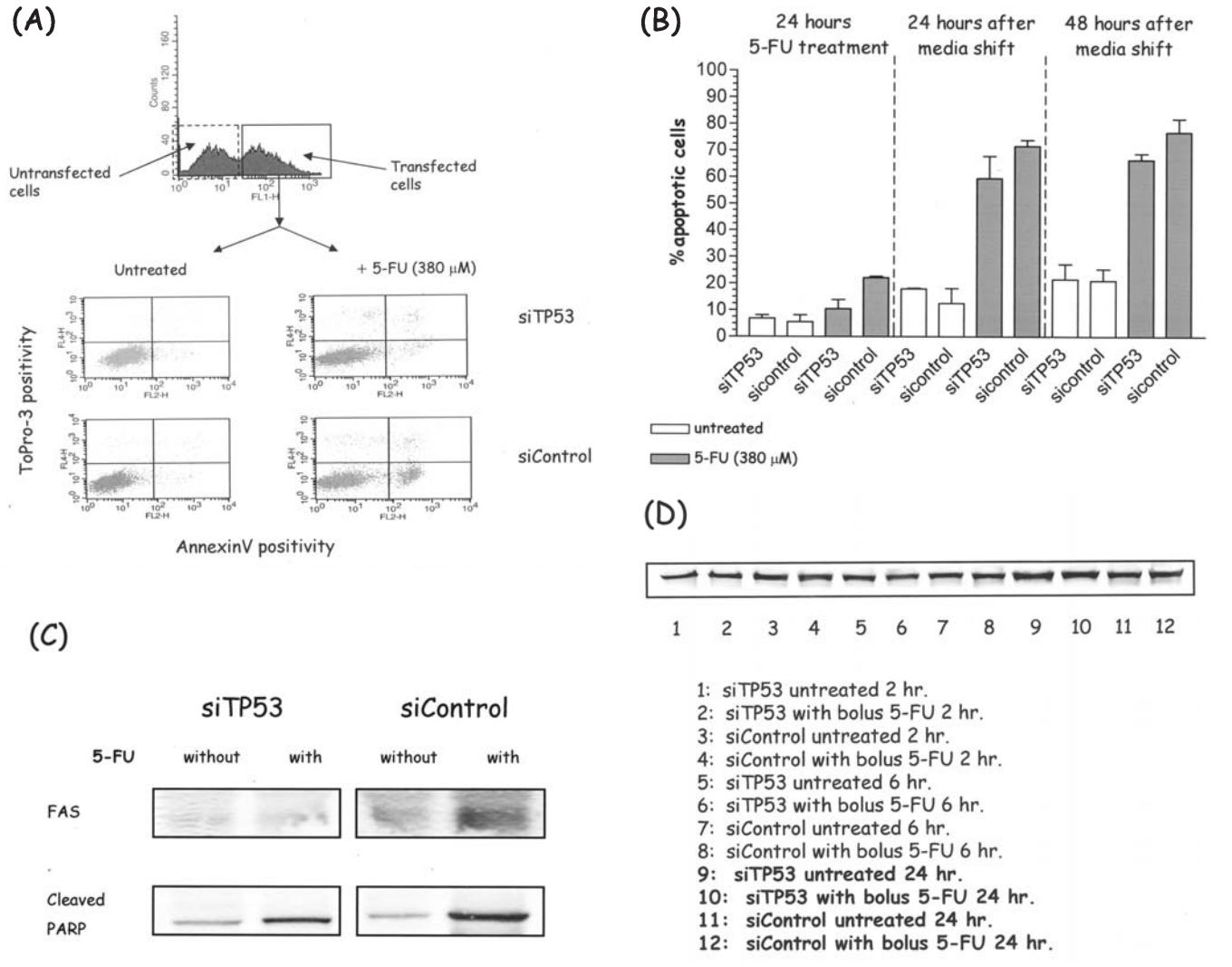

Figure 2. Apoptosis induction in siTP53- and siControl-transfected HCT116 cultures treated with 5-FU. (A) An FL1 histogram depicting transfected cells that are positively labeled with FITC-conjugated non-specific dsRNA and untransfected cells (FITC-negative). These populations were gated separately in order to quantify apoptosis and necrosis in transfected and untransfected cell populations. Bivariate distributions of Annexin V-positivity (apoptosis, FL2 parameter) vs. ToPro-3 positivity (necrosis, FL4 parameter) for 5-FU-treated and untreated transfected cells were generated for each population. Percentages of apoptotic cells were measured as the sum of AnnexinV-positive cells in the upper right and lower right quadrants of the correlated bivariate distributions. Cells in the upper left quadrants were necrotic cells. (B) These analyses were used to generate percentages of transfected apoptotic cells in HCT116 cultures at 24 h of 5-FU treatment and at 24 and 48 h of recovery following drug removal via media shift. Data (means ± SD) are from 2 replicate experiments. (C) Immunoblots showing levels of FAS and cleaved PARP proteins at 24 h in drug-treated and untreated HCT116 cultures. (D) Cleaved PARP levels in bolus 5-FU-treated transfected HCT116 cultures showing lack of apoptosis induction at 2, 6 and 24-h timepoints compared to corresponding untreated controls.

also observed in siTP53-transfected HCT116 cultures when γ-irradiation (12 Gy) was used as a DNA damaging agent (data not shown).

Since all immunoblotting data in the present work will presumably reflect the 1:1 mixture of transfected and untransfected cells, several attempts were made to flow cytometrically sort transfected from untransfected cells in untreated and 5-FU-treated cultures in order to be able to (optimally) assess expression levels of TP53 (and TP53-inducible proteins) separately for these sorted fractions, but the mechanical stress caused by cell sorting resulted in TP53 ubiquitination in some of the sorted fractions in 2 replicate experiments, so this approach was not pursued.

Cell death. Apoptotic levels were 2-fold higher in 5-FU-treated siControl-transfected cultures compared to drug-treated siTP53-transfected HCT116 cultures at 24 h (Fig. 2A and B). Apoptosis was not detected at earlier timepoints in siControl-transfected HCT116 cultures treated with 380 μM 5-FU. Decreased FAS and cleaved PARP levels were detected in 5-FU-treated siTP53-transfected HCT116 cultures at 24 h relative to 5-FU-treated siControl-transfected HCT116 cultures (Fig. 2C). There was little necrotic cell death at 24 h in 5-FU-treated siTP53-transfected- and siControl-transfected HCT116 cultures (3.2%±0.2 and 2.4%±1.1, respectively). Bolus 5-FU treatment did not result in apoptosis induction in siControl-transfected HCT116 cultures as indicated by similar (unchanged) levels of both cleaved

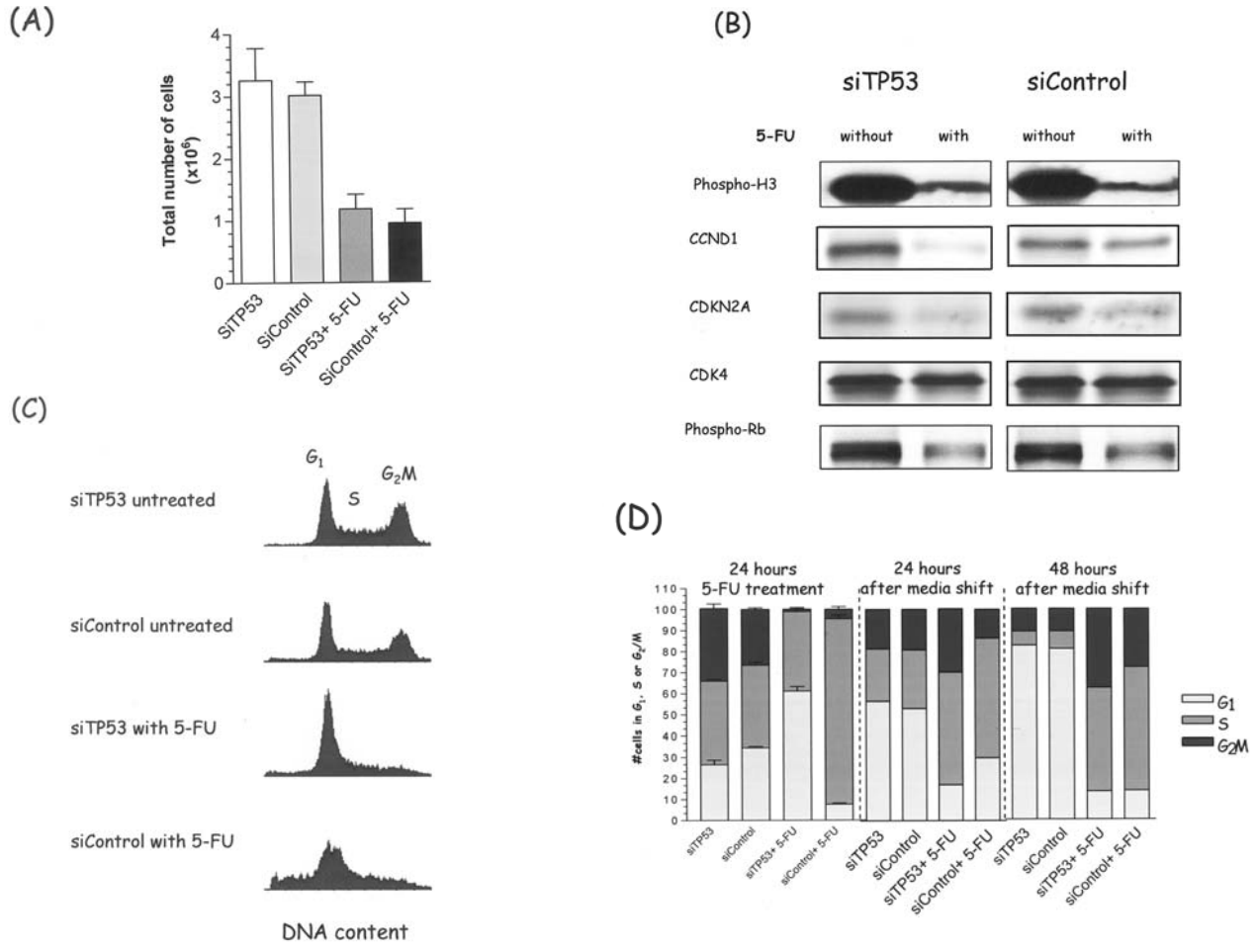

Figure 3. Growth inhibition and cell cycle progression in siTP53- and siControl-transfected HCT116 cultures treated with 5-FU. (A) total number of cells in siTP53- and siControl-transfected HCT116 cultures at 24 h of treatment with 380 μM 5-FU. Data (means ± SD) are from 2 replicate experiments. (B) immunoblots showing levels of phospho-H3, CCND1, CDKN2A, CDK4, and phospho-Rb proteins in drug-treated and untreated transfected HCT116 cultures at 24 h of treatment with 380 μM 5-FU. (C) Representative cell cycle distributions of sorted transfected cells from untreated and 5-FU-treated siControl- and siTP53-transfected HCT116 cultures at 24 h of drug treatment. (D) Bar plots summarizing the percentages of cells in G_1, S, and G_2/M phases in HCT116 cultures at 24 h of 5-FU treatment, and at 24 and 48 h of recovery following drug removal via media shift. Data (means ± SD) are from 2 replicate experiments.

PARP (Fig. 2D) and FAS (data not shown) in both drug-treated and corresponding untreated siControl-transfected cultures at all experimental timepoints.

Since high-dose rather than bolus 5-FU treatment resulted in apoptosis induction in HCT116 cultures, we focused on investigations of the apoptotic (and cell cycle) responses of siRNA-transfected HCT116 cultures to high-dose 5-FU treatment. Accordingly, all data presented from this point on are those resulting from use of 380 μM 5-FU.

Cell growth and cell cycle progression during treatment. SiTP53- and siControl-transfected HCT116 cultures treated with 380 μM 5-FU demonstrated similar levels of growth inhibition and similar total cell numbers at 24 h relative to their respective untreated controls (Fig. 3A). Phosphorylated histone H3 levels were substantially decreased in drug-treated transfected HCT116 cultures compared to their respective untreated controls at 24 h of drug treatment, irrespective of TP53 knockdown (Fig. 3B), indicating cessation of mitosis. CCND1 levels were decreased substantially in 5-FU-treated siTP53-transfected HCT116 cultures, whereas CCND1 levels were unchanged in drug-treated siControl-transfected HCT116 cultures at 24 h compared to their respective untreated controls (Fig. 3B). CDKN2A levels were decreased in drug-treated siTP53-transfected and drug-treated siControl-transfected HCT116 cultures relative to their untreated controls (Fig. 3B). CDK4 levels were unchanged in drug-treated siTP53-transfected and siControl-transfected HCT116 cultures relative to their respective untreated controls (Fig. 3B). Phospho-Rb (Ser807/811) levels were decreased in both drug-treated siTP53- and siControl-transfected HCT116 cultures relative to their untreated controls (Fig. 3B). Cell cycle analyses of 5-FU-treated cultures at the 24-h timepoint indicated the presence of arrests in different phases of the cell cycle in these cultures that could explain the observed growth inhibitions. A large population of S phase-arrested cells (over 80%) was seen in 5-FU-treated siControl-transfected HCT116 cultures, whereas drug-treated siTP53-transfected cultures had larger G_1 fractions and S phase fractions that were half the size of those in drug-treated siControl-transfected cultures (Fig. 3C and D). G_2M fractions were smallest in drug-treated siTP53-transfected

Table I. Expression levels of reported TP53 target genes in 5-FU-treated TP53-depleted HCT116 cultures.

(A) Gene symbol	(B) siTP53 vs. siControl Log_2 ratio	(C) siControl + 5-FU vs. siControl untreated Log_2 ratio	(D) siTP53 + 5-FU vs. siTP53 untreated Log_2 ratio	(E) Expression difference between columns D and C (D/C)
TP53	-0.8	0.1	-0.7	-0.8
MDM2	-0.7	3.5	1.7	-1.8
DRAM	0.0	1.9	0.5	-1.4
FDXR	0.1	2.3	1.0	-1.3
SESN1	-0.2	2.2	1.0	-1.2
P53CSV	0.1	2.3	1.1	-1.2
TP53I3	-0.5	4.3	3.1	-1.2
SERPINB5	0.2	3.4	2.3	-1.1
TAP1	0.5	1.5	0.4	-1.1
DDB2 ///				
LHX3	-0.4	1.6	0.6	-1.0
IKIP	-0.4	2.6	1.6	-1.0
TNFRSF10B	0.2	2.1	1.1	-1.0
TP53INP1	-0.4	2.0	1.0	-1.0
CCNG1	0.3	0.9	0.0	-0.9
CYFIP2	-0.3	1.6	0.7	-0.9
FAS	-0.2	3.0	2.1	-0.9
WIG1	0.0	1.9	1.0	-0.9
PPM1D	-0.2	2.2	1.4	-0.8
BTG2	-0.1	2.2	1.5	-0.7
EGFR	0.3	-1.2	-1.9	-0.7
GPX1	0.0	1.2	0.6	-0.6
PLK2	-0.4	1.9	1.3	-0.6
SFN	0.2	2.3	1.7	-0.6
GADD45A	-0.2	1.1	0.6	-0.5
CDKN1A	-0.5	2.5	2.0	-0.5
S100A2	-0.2	1.8	1.3	-0.5
SCD	0.1	-1.4	-0.9	0.5
CCNB1	-0.6	-2.4	-1.9	0.5
VEGF	-0.3	-2.1	-1.6	0.5
ANLN	-0.1	-1.5	-0.9	0.6
BRCA1	-0.1	-1.1	-0.5	0.6
CRYZ	0.0	-1.4	-0.7	0.7
EEF1A1	-0.4	1.4	2.2	0.8
ECT2	-0.2	-1.4	-0.4	1.0

cultures. The cell cycle distributions for untransfected cells in drug-treated siTP53- and siControl-transfected HCT116 cultures were similar to those seen for transfected cells in drug-treated siControl-transfected HCT116 cultures (data not shown).

Gene expression in drug-treated TP53-depleted HCT116 cultures. Comparative gene expression analyses were used to identify genes down-stream of TP53 that were affected by TP53 knockdown and subsequent 5-FU treatment in HCT116 cultures. Complete expression data for genes with detectable transcripts are available upon request. We first identified transcript level alterations of genes reported to be TP53 target genes, taking into account an average transfection efficiency of 50% and then focusing on genes with the highest log_2 ratio differences. Genes that showed 2-fold transcript level differences (log_2 ratio differences of 1) in 5-FU-treated siTP53- vs. 5-FU-treated siControl-transfected cultures were considered to be (putative) TP53-target genes. The actual log_2 ratio differences for a number of reported

Table II. Expression levels of genes in 5-FU-treated TP53-depleted HCT116 cultures that may be putative TP53 target genes.

(A) Gene symbol	(B) siTP53 vs. siControl Log_2 ratio	(C) siControl + 5-FU vs. siControl untreated Log_2 ratio	(D) siTP53 + 5-FU vs. siTP53 untreated Log_2 ratio	(E) Expression difference between columns D and C (D/C)
LOC400581	0.5	3.2	1.8	-1.4
JMJD2B	-0.2	-1.1	-2.5	-1.4
MYO1E	0.5	2.3	0.9	-1.4
APOBEC3C	0.2	2.1	0.8	-1.3
TACSTD2	0.8	2.8	1.5	-1.3
TncRNA	-0.2	-1.0	-2.2	-1.2
LOC128977	-0.2	-0.3	-1.5	-1.2
BIRC3	0.0	2.6	1.5	-1.1
SULF2	-0.1	2.1	1.0	-1.1
PRRG1	0.0	-1.2	-2.2	-1.0
KIAA1026	0.0	-2.1	-3.1	-1.0
LAMP3	0.5	1.7	0.7	-1.0
PPM1A	0.2	2.6	1.6	-1.0
RAI1	-0.6	-2.6	-3.6	-1.0
ZFYVE20	-0.4	1.7	0.7	-1.0
MICAL-L1	0.2	-0.4	-1.4	-1.0
ATAD2	-0.3	-1.5	-0.5	1.0
DLG1	-0.4	-1.6	-0.6	1.0
ITSN1	0.1	-1.7	-0.7	1.0
PHLDB2	-0.3	-1.5	-0.5	1.0
RHOBTB3	0.1	-2.1	-1.1	1.0
SFRS11	-0.5	-1.2	-0.2	1.0
TACC3	0.2	-1.7	-0.7	1.0
TIA1	-0.2	-3.1	-2.1	1.0
XBP1	-0.5	-1.5	-0.5	1.0
ATXN1	-0.2	-1.7	-0.6	1.1
CDCA7	0.0	-2.0	-0.8	1.2
CDS1	-0.1	-1.7	-0.5	1.2
KIF11	-0.2	-2.2	-1.0	1.2
NFXL1	0.0	-2.0	-0.7	1.3
C9orf91	-0.3	-2.0	-0.6	1.4
CCNT2	-0.4	-2.8	-1.4	1.4
OCLN	-0.1	0.9	2.4	1.5

TP53 target genes ranged from -1.8 to 0.8 (Table I, column E), e.g. *MDM2, DRAM (FLJ11259), FDXR, SESN1, P53CSV, TP53I3, SERPINB5, TAP1, DDB2, IKIP, TP53INP1, TNFRSF10B, CCNG1, CDKN1A, CYFIP2, EGFR, FAS, PPM1D, WIG1*. Table II lists potentially novel TP53 target genes with focus on those genes that had log_2 ratio differences either ≤-1 or ≥1 (Table II, column E). Column B in Tables I and II lists the log_2 ratios for genes in untreated siTP53 vs. siControl cultures and is an indication of whether siTP53 transfection affected transcript levels of TP53 target genes in the absence of DNA damage caused by 5-FU. Expression levels for 4 genes, *TP53, CDKN1A, CCND1* and *EGFR*, determined by real-time RT-PCR for 5-FU-treated vs. untreated siTP53- and siControl-transfected HCT116 cultures at the 24-h timepoint, showed strong correlation with Affymetrix microarray expression level measurements for the same genes (Pearson r=0.85, p=0.008) (drug-treated vs. untreated siRNA-transfected cultures) (Table III).

Apoptosis levels and cell cycle progression following drug removal. 5-FU-treated cell cultures were monitored for apoptosis induction and cell cycle progression at 24 and 48 h following removal of 5-FU (corresponding to 72 and 96 h

Table III. Comparison of expression levels for four genes analyzed using Affymetrix whole genome microarrays and real-time RT-PCR.

Gene symbol	siControl + 5-FU vs. siControl untreated Log_2 ratio		siTP53 + 5-FU vs. siTP53 untreated Log_2 ratio	
	Affymetrix microarrays	RT-PCR	Affymetrix microarrays	RT-PCR
TP53	0.1	-1.8	-0.7	-1.4
CDKN1A	2.5	2.9	2.0	2.1
CCND1	0.1	-1.1	-0.2	-0.8
EGFR	-1.2	-1.2	-1.9	-0.9

The results are presented as log_2 ratios of signal intensities.

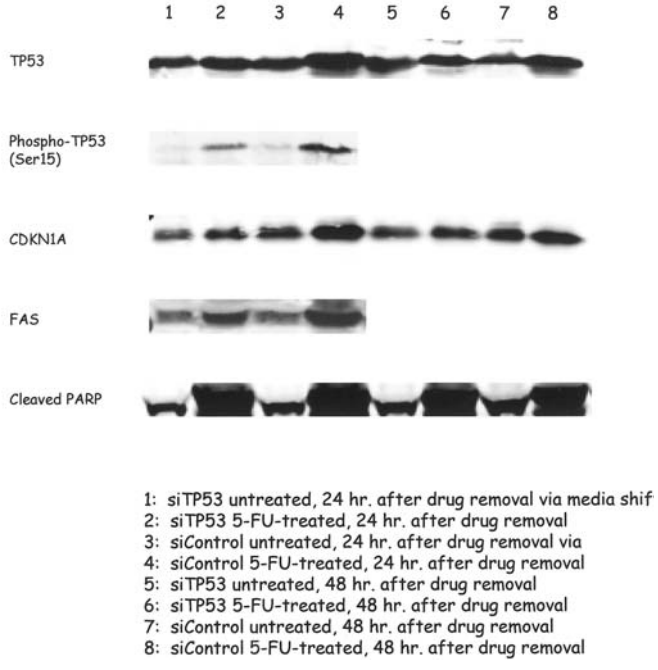

Figure 4. Recovery of HCT116 cultures following 5-FU removal. Levels of TP53 (unphosphorylated and phosphorylated), CDKN1A, FAS and cleaved PARP at 24 and 48 h following drug removal via medium shift. TP53 knock-down was no longer in effect at these timepoints as indicated by detection of TP53 accumulation/activation, and CDKN1A and FAS induction in drug-treated siTP53-transfected cultures. Note the high levels of cleaved PARP (apoptosis) in all drug-treated cultures at these timepoints.

after transfection with siRNAs, respectively). TP53 function was gradually restored during this time period, as indicated by accumulation of TP53 [and detection of activated TP53 (Ser15)] in drug-treated siTP53 cultures (Fig. 4). This was again confirmed by the induction of both CDKN1A and FAS in these cultures already at 24 h following drug removal (Fig. 4). Apoptosis (cleaved PARP levels) increased markedly in both drug-treated siTP53- and siControl-transfected HCT116 cultures following 5-FU removal (Figs. 2B and 4). Untreated siTP53- and siControl-transfected monolayer cultures demonstrated increasing levels of (spontaneous) apoptosis throughout the recovery period compared to untreated control cultures at 24 h as they became confluent.

Twenty-four and 48 hours after drug removal, siTP53-transfected and siControl-transfected cultures had similarly-sized S phase fractions and larger G_2/M fractions (Fig. 3D), indicating that G_1/S)-arrested cells in drug-treated siTP53 cultures had moved into S phase where they arrested, but also that some S phase cells had moved into G_2/M phase. We did not track recovery further in these cultures.

Discussion

TP53 plays a crucial role in determining the cellular sensitivity to a number of chemotherapeutic agents, such as 5-FU and CPT-11 (15,27,28). Inability to undergo TP53-mediated apoptosis due to loss of TP53 function may contribute to 5-FU resistance (14,15). In the present work, HCT116 cultures transiently depleted for TP53 and treated with 380 μM 5-FU for 24 h demonstrated reduced apoptosis induction, consistent with reduced cleaved PARP and FAS protein levels and reduced transcript levels of the apoptosis inducer TNFRSF10B compared to untreated control cultures. Our data are in agreement with previous studies that reported an association between abrogation of TP53 function and decreased levels of apoptosis in response to drug treatment (14,15,28-33). The observed G_1/S border arrests in TP53-depleted cultures at 24 h of drug treatment were TP53-independent, consistent with results of previous studies of 5-FU-treated TP53-deficient cells (15,30). TP53 function was gradually restored during 72 to 96 h after transfection with siTP53, concomitant with release of G_1/S border arrests and movement of cells into S phase (and G_2/M phase) where they arrested and underwent apoptosis, consistent with detection of very high levels of apoptosis in these cultures during recovery. The G_1/S border arrests in 5-FU-treated TP53-depleted cultures appeared to be protective against cell death, because as long as cells were arrested at the G_1/S transition, apoptotic levels were reduced, whereas upon restoration of TP53 function, these arrests were released and cells moved into S (and G_2/M) phases where they arrested and underwent apoptosis. The high apoptotic levels measured for both cultures during recovery indicate inability to repair 5-FU-induced DNA damage, not surprising since the 5-FU concentration used was considerably high. Use of this

concentration was justified in the present work given that a bolus 5-FU dose did not result in apoptosis induction and that we were specifically interested in investigating apoptosis induction in TP53-depleted HCT116 cells treated with the chemotherapeutic drug 5-FU. The HCT116 cell line tolerates fairly high doses of 5-FU in order for apoptosis to be induced, in agreement with previous reports (15,25,34).

Generally, progression through G_1 can be blocked either by preventing Rb phosphorylation or by silencing CCNE-CDK2 activity. The inhibition of CDK4 by CDKN2A prevents the formation of CCND1-CDK4 complexes and subsequent phosphorylation of Rb, leading to G_1 arrest (35-37). Rb phosphorylation is necessary for the dissociation of Rb from the E2F transcription factor and the subsequent expression of E2F-regulated genes such as *CCNA, CCNE, DHFR*, and *TYMK* (38) that drive cell cycle progression forward. The observed G_1/S arrests in TP53-deficient HCT116 cultures could not be explained by decreases in phosphorylated Rb or CDK4 levels, since phosphorylated Rb levels were similarly decreased in both drug-treated TP53-deficient and -proficient HCT116 cultures, and CDK4 levels in both drug-treated cultures were similar. However, the marked reduction in CCND1 levels in drug-treated TP53-depleted HCT116 cultures compared to drug-treated TP53-proficient cultures could be an explanation for the observed G_1/S arrests. This is consistent with the results of a recent study that showed that CCND1 suppression by RNAi resulted in G_1 arrest (39). Our data also support the findings of Agami and Bernards (40) who demonstrated that DNA damage causes an immediate and TP53-independent G_1 arrest mediated by (rapid) proteolysis of CCND1. These authors also showed that if CCND1 degradation/G_1 arrest did not occur, cells were more susceptible to DNA damage, indicating that CCND1 degradation was an essential component of the cellular response to genotoxic stress. How fast this (TP53-independent) initiation of G_1 arrest mediated by CCND1 proteolysis occurs, and what causes CCND1 degradation in TP53-deficient cells are currently under investigation in our laboratory.

Gene expression alterations in 5-FU-treated TP53-depleted (relative to TP53-proficient) HCT116 cultures confirmed previously-reported TP53 target genes and suggested potentially novel TP53 target genes. Taking into account an average transfection efficiency of 50%, we found that transcript levels of genes such as *CCNG1, CYFIP2, DDB2*, *DRAM, EGFR, FAS, FDXR, IKIP, MDM2, TP53CSV*, *PPM1D, SERPINB5, SESN1, TAP1, TNFRSF10B*, *TP53I3*, *TP53INP1* and *WIG1* in drug-treated siTP53-transfected HCT116 cultures were generally 2-fold or more reduced (Table I) compared to those in drug-treated siControl-transfected cultures. Identification of these TP53-target genes is in agreement with previous reports identifying (some of) these genes as transcriptional targets for TP53 (2,32,34,41-50). One reported TP53 target gene, *EEF1A1*, had transcript levels in drug-treated TP53-depleted cultures that were higher compared to those in drug-treated TP53-proficient cultures. Using the same range of $\log_2$ ratio differences and focusing on the highest difference values, we identified numerous potentially novel direct or indirect TP53 target genes (Table II). Many of these genes are involved in regulation of apoptosis (*BIRC3, TIA1*), cell division (*KIF11, CCNT2*), cell adhesion/cytoskeletal functions (*MYO1E*, *DLG1, OCLN*), ion binding/transport (*PRRG1, ZFYVE20*, *MICAL-L1*), signal transduction (*LOC400581, ITSN1*, *TACC3, LAMP3, SULF2, CDS1, RHOBTB3*), transcription (*JMJD2B, RAI1, NFE2L2, CDCA7, XBP1, NFXL1*) and mRNA processing (*APOBEC3C, 5FRS11*). Future studies are planned to investigate whether these genes are in fact TP53-target genes, and what role some of them play in mediating the response to DNA damage, in apoptosis, and in 5-FU resistance.

In conclusion, we have shown that transient loss of normal TP53 function in HCT116 cultures treated with 5-FU resulted in reduced apoptosis, TP53- and CDKN1A-independent G_1/S phase arrests that appear to be protective against apoptosis, smaller S phase arrests, and transcript level decreases of many reported TP53-target genes. We have also identified a number of potentially novel TP53 target genes.

Acknowledgments

We thank Siv Tunheim at the Core Facility for Gene Expression, Rikshospitalet-Radiumhospitalet Medical Center, for performing the microarray hybridizations. This work was generously supported by Stiftelsen UNI and Norwegian Cancer Society grant B02092.

References

1. Longley DB, Allen WL, McDermott U, *et al*: The roles of thymidylate synthase and p53 in regulating Fas-mediated apoptosis in response to antimetabolites. Clin Cancer Res 10: 3562-3571, 2004.
2. Zhang L, Yu J, Park BH, *et al*: Role of BAX in the apop-totic response to anticancer agents. Science 290: 989-992, 2000.
3. Peters GJ, van Triest B, Backus HH, *et al*: Molecular downstream events and induction of thymidylate synthase in mutant and wild-type p53 colon cancer cell lines after treatment with 5-fluorouracil and the thymidylate synthase inhibitor raltitrexed. Eur J Cancer 36: 916-924, 2000.
4. Van der Wilt CL, Smid K, Aherne GW, *et al*: Biochemical mechanisms of interferon modulation of 5-fluorouracil activity in colon cancer cells. Eur J Cancer 33: 471-478, 1997.
5. Sampath D, Rao VA and Plunkett W: Mechanisms of apoptosis induction by nucleoside analogs. Oncogene 22: 9063-9074, 2003.
6. Prives C and Hall PA: The p53 pathway. J Pathol 187: 112-126, 1999.
7. Vogelstein B, Lane D and Levine AJ: Surfing the p53 network. Nature 408: 307-310, 2000.
8. Motoyama N and Naka K: DNA damage tumor suppressor genes and genomic instability. Curr Opin Genet Dev 14: 11-16, 2004.
9. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, *et al*: Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73: 39-85, 2004.
10. Zhivotovsky B and Kroemer G: Apoptosis and genomic instability. Nat Rev Mol Cell Biol 5: 752-762, 2004.
11. Ljungman M: Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. Neoplasia 2: 208-225, 2000.
12. Shiloh Y: ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3: 155-168, 2003.
13. Harms K, Nozell S and Chen X: The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci 61: 822-842, 2004.
14. Longley DB, Boyer J, Allen WL, *et al*: The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res 62: 2644-2649, 2002.
15. Bunz F, Hwang PM, Torrance C, *et al*: Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 104: 263-269, 1999.

16. Elsaleh H, Powell B, McCaul K, *et al*: P53 alteration and microsatellite instability have predictive value for survival benefit from chemotherapy in stage III colorectal carcinoma. Clin Cancer Res 7: 1343-1349, 2001.
17. Liang JT, Huang KC, Cheng YM, *et al*: P53 overexpression predicts poor chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV colorectal cancers after palliative bowel resection. Int J Cancer 97: 451-457, 2002.
18. Ahnen DJ, Feigl P, Quan G, *et al*: Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. Cancer Res 58: 1149-1158, 1998.
19. Paradiso A, Simone G, Petroni S, *et al*: Thymidilate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. Br J Cancer 82: 560-567, 2000.
20. Johnston PG, Drake JC, Trepel J, *et al*: Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. Cancer Res 52: 4306-4312, 1992.
21. Peters GJ, Backus HH, Freemantle S, *et al*: Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta 1587: 194-205, 2002.
22. Takebe N, Zhao SC, Ural AU, *et al*: Retroviral transduction of human dihydropyrimidine dehydrogenase cDNA confers resistance to 5-fluorouracil in murine hematopoietic progenitor cells and human $CD34^+$-enriched peripheral blood progenitor cells. Cancer Gene Ther 8: 966-973, 2001.
23. Salonga D, Danenberg KD, Johnson M, *et al*: Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 6: 1322-1327, 2000.
24. Kohne CH and Peters GJ: UFT: mechanism of drug action. Oncology 14: 13-18, 2000.
25. De Angelis PM, Fjell B, Kravik KL, *et al*: Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. Int J Oncol 24: 1279-1288, 2004.
26. Vindelov LL, Christensen IJ and Nissen NI: A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3: 323-327, 1983.
27. Magrini R, Bhonde MR, Hanski ML, *et al*: Cellular effects of CPT-11 on colon carcinoma cells: dependence on p53 and hMLH1 status. Int J Cancer 101: 23-31, 2002.
28. Arango D, Wilson AJ, Shi Q, *et al*: Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. Br J Cancer 91: 1931-1946, 2004.
29. Hayward RL, Macpherson JS, Cummings J, *et al*: Enhanced oxaliplatin-induced apoptosis following antisense Bcl-xl down-regulation is p53 and Bax dependent: genetic evidence for specificity of the antisense effect. Mol Cancer Ther 3: 169-178, 2004.
30. Boyer J, McLean EG, Aroori S, *et al*: Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin and irinotecan. Clin Cancer Res 10: 2158-2167, 2004.
31. Perego P, Giarola M, Righetti SC, *et al*: Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Res 56: 556-562, 1996.
32. Fan S, El-Deiry WS, Bae I, *et al*: p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 54: 5824-5830, 1994.
33. Gallagher WM, Cairney M, Schott B, *et al*: Identification of p53 genetic suppressor elements which confer resistance to cisplatin. Oncogene 14: 185-193, 1997.
34. Kho PS, Wang Z, Zhuang L, *et al:* p53-regulated transcriptional program associated with genotoxic stress-induced apoptosis. J Biol Chem 279: 21183-21192, 2004.
35. Lukas J, Parry D, Aagaard L, *et al*: Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 375: 503-506, 1995.
36. Guan KL, Jenkins CW, Li Y, *et al*: Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev 8: 2939-2952, 1994.
37. Koh J, Enders GH, Dynlacht BD, *et al*: Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature 375: 506-510, 1995.
38. Johnson DG and Walker CL: Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 39: 295-312, 1999.
39. Alarcon-Vargas D, Zhang Z, Agarwal B, *et al*: Targeting cyclin D1, a downstream effector of INI1/hSNF5, in rhabdoid tumors. Oncogene 25: 722-734, 2006.
40. Agami R and Bernards R: Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 102: 55-66, 2000.
41. Endo Y, Fujita T, Tamura K, *et al*: Structure and chromosomal assignment of the human cyclin G gene. Genomics 38: 92-95, 1996.
42. Tan T and Chu G: p53 Binds and activates the xeroderma pigmentosum DDB2 gene in humans but not mice. Mol Cell Biol 22: 3247-3254, 2002.
43. Crighton D, Wilkinson S, O'Prey J, *et al*: DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 126: 121-134, 2006.
44. Ludes-Meyers JH, Subler MA, Shivakumar CV, *et al*: Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol 16: 6009-6019, 1996.
45. Hofer-Warbinek R, Schmid JA, Mayer H, *et al*: A highly conserved proapoptotic gene, IKIP, located next to the APAF1 gene locus, is regulated by p53. Cell Death Differ 11: 1317-1325, 2004.
46. Kern SE, Fearon ER, Tersmette KW, *et al*: Clinical and pathological associations with allelic loss in colorectal carcinoma (corrected). JAMA 261: 3099-3103, 1989.
47. Budanov AV, Sablina AA, Feinstein E, *et al*: Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. Science 304: 596-600, 2004.
48. Okamura S, Arakawa H, Tanaka T, *et al*: p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis. Mol Cell 8: 85-94, 2001.
49. Varmeh-Ziaie S, Okan I, Wang Y, *et al*: Wig-1, a new p53-induced gene encoding a zinc finger protein. Oncogene 15: 2699-2704, 1997.
50. Yu J and Zhang L: The transcriptional targets of p53 in apoptosis control. Biochem Biophys Res Commun 331: 851-858, 2005.