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Journal of Cellular Biochemistry 113:3207–3217 (2012)

Journal of Cellular Biochemistry

The Synthetic Purine Reversine Selectively Induces Cell Death of Cancer Cells

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ABSTRACT

The synthetic purine reversine has been shown to possess a dual activity as it promotes the de-differentiation of adult cells, including fibroblasts, into *stem-cell-like* progenitors, but it also induces cell growth arrest and ultimately cell death of cancer cells, suggesting its possible application as an anti-cancer agent. Aim of this study was to investigate the mechanism underneath reversine selectivity in inducing cell death of cancer cells by a comparative analysis of its effects on several tumor cells and normal dermal fibroblasts. We found that reversine is lethal for all cancer cells studied as it induces cell endoreplication, a process that malignant cells cannot effectively oppose due to aberrations in cell cycle checkpoints. On the other hand, normal cells, like dermal fibroblasts, can control reversine activity by blocking the cell cycle, entering a reversible quiescent state. However, they can be induced to become sensitive to the molecule when key cell cycle proteins, e.g., p53, are silenced. J. Cell. Biochem. 113: 3207–3217, 2012.

KEY WORDS: REVERSINE; CELL REPROGRAMMING; SMALL MOLECULES AND STEM CELLS; NEW ANTI-CANCER DRUGS

Stem cell research has received renewed attention with the notion and generation of induced pluripotent stem cells (iPSCs) [Takahashi and Yamanaka, 2006], as it was shown that adult cells can be reprogrammed (or de-differentiated) into *stem cell-like* progenitors. The de-differentiation procedure has been done genetically, by over-expressing specific genes, or by chemical induction, using a combination of synthetic molecules [Anastasia et al., 2010]. The latter approach was pioneered by Rosania and Schultz, in a search for regeneration-inducing compounds [Brockes and Kumar, 2002]. This led to the first screen for synthetic chemical agents that would turn on cell proliferation and induce myotube fission in differentiated C2C12 murine myocyte cultures, which culminated in the discovery of myoseverin [Rosania et al., 2000; Perez et al., 2002]. However, myoseverin's microtubule depolymerizing effect was associated with a marked cell cycle inhibitory effect [Chang et al., 2001], which likely limited the proliferative potential of the fragmented myotubes [Duckmanton et al., 2005]. This led to new screens for compounds that would increase the pluripotency of differentiated cells, which led to the discovery of reversine by Ding and Schultz [Chen et al., 2004]. Among the several compounds used, the synthetic purine "*reversine*" has become the most attractive one, owing to its ability to de-differentiate human fibroblasts [Anastasia et al., 2006], one of the most accessible autologous cell sources for possible applications in regenerative medicine. Reversine-treated cells could be induced to differentiate into adipocytes, skeletal myoblasts, smooth muscle cells, osteoblasts [Chen et al., 2004;

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Manuscript Received: 3 June 2011; Manuscript Accepted: 10 May 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 May 2012

DOI 10.1002/jcb.24197 • © 2012 Wiley Periodicals, Inc.

Anastasia et al., 2006], and even toward neuroectodermal lineage [Lee et al., 2009] and cardiomyocytes [Pikir et al., 2012]. Moreover, it was recently shown that the molecule increase mesenchymal stromal cells plasticity [Conforti et al., 2011] and can improve the efficiency of somatic cell nuclear transfer (SCNT) [Miyoshi et al., 2010]. Interestingly, in our initial study [Anastasia et al., 2006], we observed that reversine, besides inducing de-differentiation, greatly inhibited cell proliferation, leading to the formation of polyploid populations. This was confirmed by a successive mechanistic study, which showed that reversine acts as a dual inhibitor of non-muscle myosin II heavy chain (NMMII) and MEK1, resulting in an altered cell cycle [Chen et al., 2007].

It is still unclear whether the de-differentiation activity of reversine is associated with its growth inhibitory effect. Notably, it was shown that reversine growth inhibition is not limited to myoblasts and fibroblasts, but it could also be observed in the prostatic cancer cell lines PC-3, CWR22Rv1, and DU-145 [Hsieh et al., 2007] and, very recently, in thyroid cancer cells [Hua et al., 2012]. Moreover, reversine was shown to inhibit colony formation of human acute myeloid leukemia cells, ultimately prompting cell death [D'Alise et al., 2008]. On the other hand reversine, when employed in the concentration range of 50 nM–5 μM, has been extensively used to induce de-differentiation in normal adult cells, without appreciable toxic effects.

Clearly, the apparent selectivity of reversine for cancer cells needed further elucidation, especially in the perspective of the possible application of the molecule as an anti-cancer drug. Therefore, aim of this study was to investigate the mechanism behind reversine selectivity in inducing cell death of cancer cells to establish the molecular basis for the differential effect of reversine on normal versus malignant cells. On these bases we considered important to assess whether the toxic effect of reversine could be extended to other cancer cells, especially those who are more resistant to current chemotherapies.

In this study, we report that reversine induces massive cell death in several tumor cell lines, including human fibrosarcoma HT1080 [Rasheed et al., 1974], human neuroblastoma SK-N-BE [Amano et al., 1972], human cervical cancer HeLa [Masters, 2002], and, in cancer stem cells (CSCs) from human glioblastoma [Galli et al., 2004]. Our results show that reversine treatment promotes endoreplication in all cancer cells studied, and we elucidated how these induced aberrant-mitoses eventually lead to cell death possibly via mitotic catastrophe. On the other hand, under the same experimental conditions, reversine was not lethal for human fibroblasts, as we also reported in our initial study [Anastasia et al., 2006]. Nonetheless, we demonstrated that fibroblasts could become sensitive to the lethal effects of the compound, when p53, a crucial cell cycle checkpoint regulator, was either chemically inhibited or genetically silenced.

MATERIALS AND METHODS

CELL CULTURES

Primary human dermal fibroblasts were prepared from adult healthy donors, as we previously described [Anastasia et al., 2006]. Human neuroblastoma SK-N-BE, human fibrosarcoma HT1080, and human cervix adenocarcinoma HeLa cell lines were kindly provided by Dr. Petroni from the University of Milan. Human GBM CSC line 0627 was provided by Dr. R. Galli from San Raffaele Hospital Scientific Institute and cultured in serum-free DMEM/F12 medium containing 20 ng/ml of EGF and 10 ng/ml of FGF2 (Sigma), as previously described [Galli et al., 2004]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 4.5 g/L (Sigma) with 10% (v:v) fetal bovine serum (FBS, Sigma), 1 mM sodium piruvate, 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin. All cell cultures were performed at 37°C in a humidified incubator with 5% CO_2 and 95% air.

REVERSINE TREATMENT

Reversine was synthesized in our laboratories according to the published procedure [Chen et al., 2004], on the basis of our consolidated experience in organic synthesis [Anastasia et al., 2001; Negishi and Anastasia, 2003], and its purity (≥98%) was checked by HPLC and LC-MS analysis. All reversine treatments were conducted 24 h after cell seeding in 10% FBS DMEM, the compound being dissolved in DMSO. Controls cells were incubated with DMSO at the same concentration used for dissolving reversine, which was in all cases below 0.05%. With regard to reversine concentration, the used reference concentration, when tumoral cells were compared to human fibroblasts, was 5 μM, the concentration previously shown to induce de-differentiation of adult cells, including human fibroblasts [Chen et al., 2004; Anastasia et al., 2006].

CELL MORPHOLOGY AND GROWTH CURVE

For all experiments, 1×10^5 cells were plated in 100 mm dishes and treated with reversine, dissolved in DMSO, or DMSO alone. Cell morphology was examined daily with a phase-contrast microscope (Axiovert 40cfl, Zeiss). Cells were counted with a Burker's chamber, and the cell viability was determined by trypan blue dye exclusion assay.

CASPASE ANALYSIS BY WESTERN BLOTTING

After 6, 24, 48 h of reversine (or DMSO) treatment cells were rinsed twice with cold PBS, harvested, lysed in PBS containing aprotinin, leupeptin, and pepstatin, as previously described.[Anastasia et al., 2006] Protein samples corresponding to 40 μg of cell fraction were subjected to SDS/PAGE (12% w/v gel) and subsequently transferred to PVDF transfer membrane (Thermo Scientific) by electroblotting. The membrane was incubated for 1 h in Tris-Buffered Saline/0.1% (w/v) Tween 20 (TBS-T) containing 5% (w/v) dried milk (blocking buffer, BB, Castroni). Blots were incubated overnight with anti-active fragment of caspase 3 monoclonal antibody (1:500) (Cell Signaling), washed with TBS-T, and incubated for 1 h with the appropriate anti-rabbit HRP conjugated IgG (1:3000; Santa Cruz).

CASPASE ANALYSIS BY LUMINESCENCE ASSAY

Cells were plated in white-walled 96-well plates at 2×10^3 cells/well and caspase activation was evaluated at 24, 48, and 72 h after 5 μM reversine treatment by luminescence analysis, according to the manufacturer's instructions (CASPASE-GLO® 3/7 Assay, Promega).

IMMUNOFLUORESCENCE STAINING

Cells were plated in 60 mm culture dishes at 4×10^4 cells/dish for control cells, and 8×10^4 cells/dish for the reversine-treated cells. After 24, 48, and 72 h of reversine treatment, cells where fixed with 4% (w/v) paraformaldehyde at room temperature (RT) for 10 min, permeabilized with 0,1% (w/v) Triton X-100 in PBS for 30 min, and then incubated (1 h) with mouse monoclonal anti-β-tubulin antibody (Sigma-Aldrich, 1:200 dilution) with 0.1% (w/v) Triton X-100, 1% BSA in PBS (blocking solution). After incubation, cells were washed three times in blocking solution and incubated with the appropriate Tetramethyl Rhodamine Isothiocyanate (TRIC)-conjugated secondary antibody (Dako) for 1 h at RT. After washing in the blocking solution, cells were stained with DAPI (Sigma-Aldrich) for 15 min and then analyzed under a fluorescent microscope (Olympus IX51 microscope equipped with a Hamamatsu 1394 camera).

CELL CYCLE ANALYSIS

Cell cycle analysis was performed on at least 30,000 events for each sample with FACSCalibur System (BD Biosciences) and the DNA profile was analyzed by Summit 4.3 (Cytomation Coulter). Cells were treated with 5 μM reversine or 0.05% DMSO for 3, 6, 24, 48, and 72 h, harvested with trypsin-EDTA, collected by centrifugation at 160 rcf for 10 min, and then resuspended with 600 μl of cold PBS. Cells were fixed in 70% ethanol, stained with propidium iodide, and then analyzed by flow cytofluorimetry as described previously [Anastasia et al., 2006].

INHIBITION OF p53 WITH PIFITHRIN-α

Pifithrin-α (Sigma-Aldrich) was dissolved in DMSO, and then added to normal culture medium (10% DMEM-FBS) to a final concentration of 10 μM. DMSO final concentration was 0.05%. Twenty four hours after seeding, human fibroblasts were treated with 10 μM pifithrin-α containing medium for 2 h, and then with a combination of 10 μM pifithrin-α and 5 μM reversine. Controls cells were incubated with 0.05% DMSO alone or with 10 μM pifithrin-α. The culture medium was changed every 48 h for up to 6 days. Expression of p21 was analyzed by real-time PCR (FW-GACTGTGATGCGC-TAATGG, REV-CTCGGTGACAAAGTCGAAGT). Total RNA was isolated with RNeasy Mini kit (QIAGEN). cDNA was synthesized starting from 0.8 μg of RNA, with the iScript cDNA Synthesis Kit (BIO-RAD Laboratories) according to the manufacturer's instructions. Briefly, 10 ng of total RNA was used as template for real-time PCR performed using the iCycler thermal cycler (Bio-Rad Laboratories). PCR mixture included 0.2 μM gene-specific primers for p21 or S14 ribosomal protein, which was used as housekeeper gene, 50 mM KCl, 20 mM Tris/HCl, pH 8.4, 0.8 mM dNTPs, 0.7 U iTaq DNA Polymerase, 3 mM $MgCl_2$, and SYBR Green (iQ SYBR Green Supermix from Bio-Rad Laboratories) in a final volume of 20 μl. Amplification and real-time data acquisition were performed using the following cycle conditions: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C and 30 s at 57°C. DAPI staining for the determination of micronuclei after the combined treatment of cells with 5 μM reversine and 10 μM pifithrin-α was performed according to the manufacturer's recommendations.

SILENCING OF p53 WITH shRNA VIRAL TRANSDUCTION

Silencing of p53 in human dermal fibroblasts was achieved with commercially available shRNA (LTV 451; Cell Biolabs Inc, sequence: GACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACACTGGAGT-CTTTTT).

Viral particles were formed transfecting 3 μg of pLenti-p53 shRNA vector and 9 μg of ViraPower packaging vector mix (K5310-00, Invitrogen) in HEK293FT cells with Lipofectamine™ 2000 reagent (Invitrogen). After 48 h, the culture medium was collected and used to infect human dermal fibroblasts according to the manufacturer's procedure. Infected clones were isolated after selection with Puromycin (1 μg/ml), and the clone exhibiting the highest p53-downregulation by real-time PCR and Western Blot was named "p53-shRNA" and used for further analyses.

ANALYSIS OF HUMAN p53 EXPRESSION BY WESTERN BLOT

Wild-type or p53-shRNA human fibroblasts (control or treated with 5 μM reversine for 72 h), SK-N-BE, HT1080 and HeLa cells were rinsed twice with cold PBS, harvested and lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein samples corresponding to 40 μg of cell fraction were subjected to SDS/PAGE (10% w/v gel) and subsequently transferred to Nitrocellulose membranes (Thermo Scientific) by electro-blotting for 2 h at 100 V. The membranes were incubated for 1 h in TBS- 0.1% (w/v) Tween 20 (TBS-T) containing 5% (w/v) dried milk (blocking buffer; BB). Blots were incubated overnight with anti-human p53 monoclonal antibody (1:1000; OP140; Calbiochem), and incubated for 1 h with the appropriate anti-mouse HRP conjugated IgG antibody (1:2000; Santa Cruz). The membranes were treated with ECL Western blot substrate (Pierce) and signals were detected with the Chemidoc Bio-Rad.

ANALYSIS OF HUMAN p53 ACTIVITY BY TRANSCRIPTION FACTOR ASSAY

Nuclear extracts from human dermal fibroblast, p53-shRNA fibroblasts, SK-N-BE, HT1080, and HeLa cells were obtained following the manufacturer's instructions (Human p53 Transcription Factor Assay Kit, n. 600020, Cayman Chemical Company). To test the activity of human p53, 30 μg of protein from the nuclear extracts were loaded for each replicate and a colorimetric assay was performed according to the manufacturer's protocol (Human p53 Transcription Factor Assay Kit, n. 600020, Cayman Chemical Company). Activity was measured with Victor Light™ multiplate reader (Perkin Ekmer).

CYTOGENETIC ANALYSIS

Cell cultures were treated with Colcemid (10 μg/ml Gibco) prior to harvesting. Following hypotonic treatment with 0.075 M KCl, cells were fixed with methanol/acetic acid (3:1, v/v). Chromosomes were banded using quinacrine (QFQ) and images were captured using a fluorescence microscope equipped with a CCD camera system (Leica). At least 70–100 metaphases were analyzed in each cell line.

DETERMINATION OF CELL VIABILITY BY MTT ASSAY

CSCs were plated onto Matrigel-coated 96-well plates (10,000 cells/well) and treated with DMSO as vehicle or reversine. At every time point,

1 h before collection, the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (5 mg/ml in PBS; Sigma) was added to the medium (final dilution: 500 μg/ml). The pale yellow redox indicator MTT is reduced to a dark blue end product, MTT-formazan, by the mitochondrial dehydrogenases of living cells. Following 1 h-incubation at 37°C, the medium was discarded and cells were lysed by adding 50 μl of DMSO. After 15 min at RT, MTT reduction was measured spectrophotometrically at a wavelength of 550 nm.

RESULTS

CELL GROWTH ANALYSIS

Reversine effects on cell morphology and proliferation were tested on HT1080, SK-N-BE, and HeLa tumor cell lines, while primary cultures of human dermal fibroblasts were used as normal control cells. Controls were treated with DMSO at the same concentration used to dissolve reversine, which in all cases was below 0.05%, and gave the same results as untreated controls. Human fibroblasts were treated for 4 days with reversine at different concentrations (50 nM–50 μM) (Fig. 1 and Suppl. Fig. 1). Reversine treatment, at the lowest used concentration (50 nM), started to reduce cell proliferation but did not cause significant cell death up to 5 μM, which is the concentration used to induce de-differentiation [Chen et al., 2004; Anastasia et al., 2006] although cell growth was completely arrested and cells entered a quiescent state, as expected from our previous studies [Anastasia et al., 2006; Fania et al., 2009]. The response of tumor cells (SK-N-BE, HT1080, and HeLa cells) to 5 μM reversine treatment was remarkably different from that of normal fibroblasts: Although cell proliferation was arrested, as in the case of fibroblasts, abundant formation of vacuoles, (Fig. 1A) and extensive cell death was observed (Fig. 1B).

FLOW CYTOMETRY

Cell cycle analysis was performed daily in all cells treated with 5 μM reversine for one to 4 days. Flow cytometry allowed to distinguish diploid from tetraploid populations, and in particular to discriminate diploid G_2/M and tetraploid G_1 cells. Cell cycle analysis of reversine-treated fibroblasts revealed the formation of a polyploid population already after 24 h of treatment, reaching a diploid/tetraploid ratio of 60:40, which did not change during the 4-day treatment (Suppl. Fig. 2 and Fig. 2). Cytometry analyses of

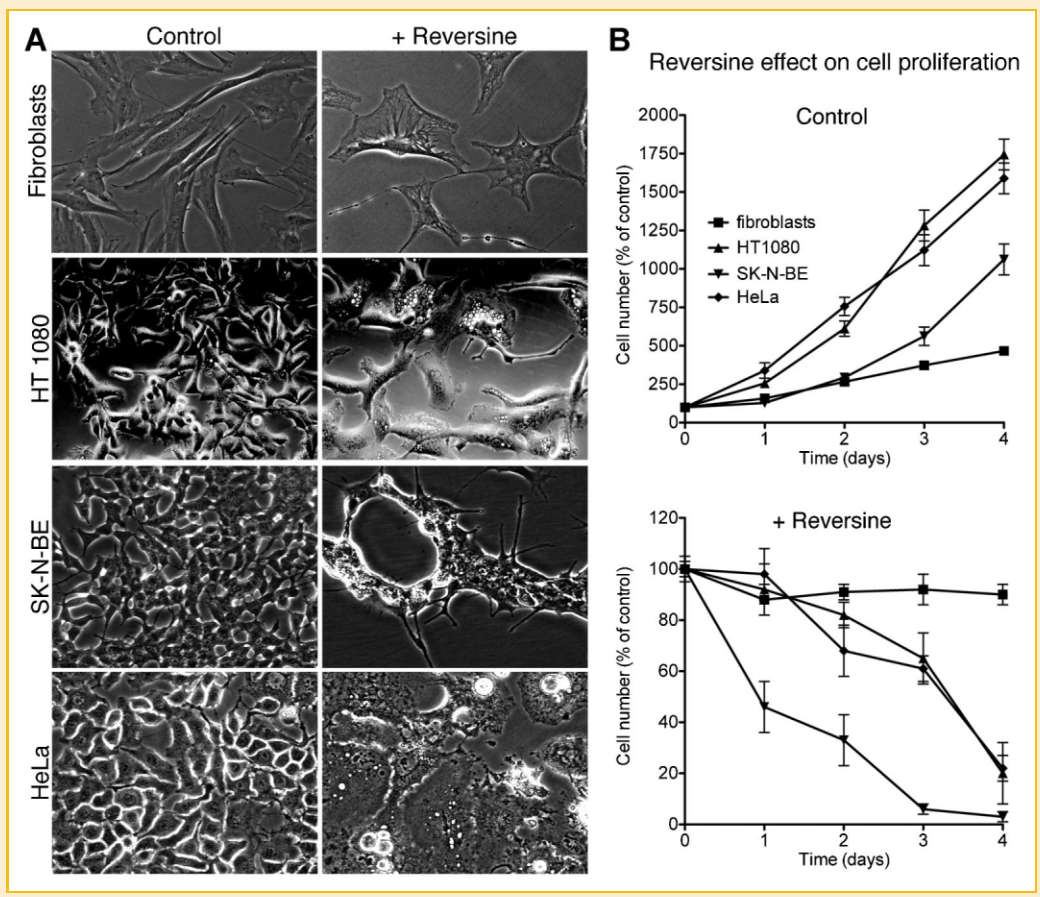

Fig. 1. Reversine effects on cell morphology and cell proliferation. A: Phase-contrast microphotographs of fibroblasts and cancer cells (HT1080, SK-N-BE, and HeLa) after 4-day treatment with DMSO 0.05% (Control) and 5μM reversine (+Reversine); (B) cell count was plotted versus days of culture. Control cells (treated with DMSO) and 5 μM reversine-treated cell proliferation curves are shown. All experiments were performed in triplicates. A typical result is shown. Error bars show mean and SD.

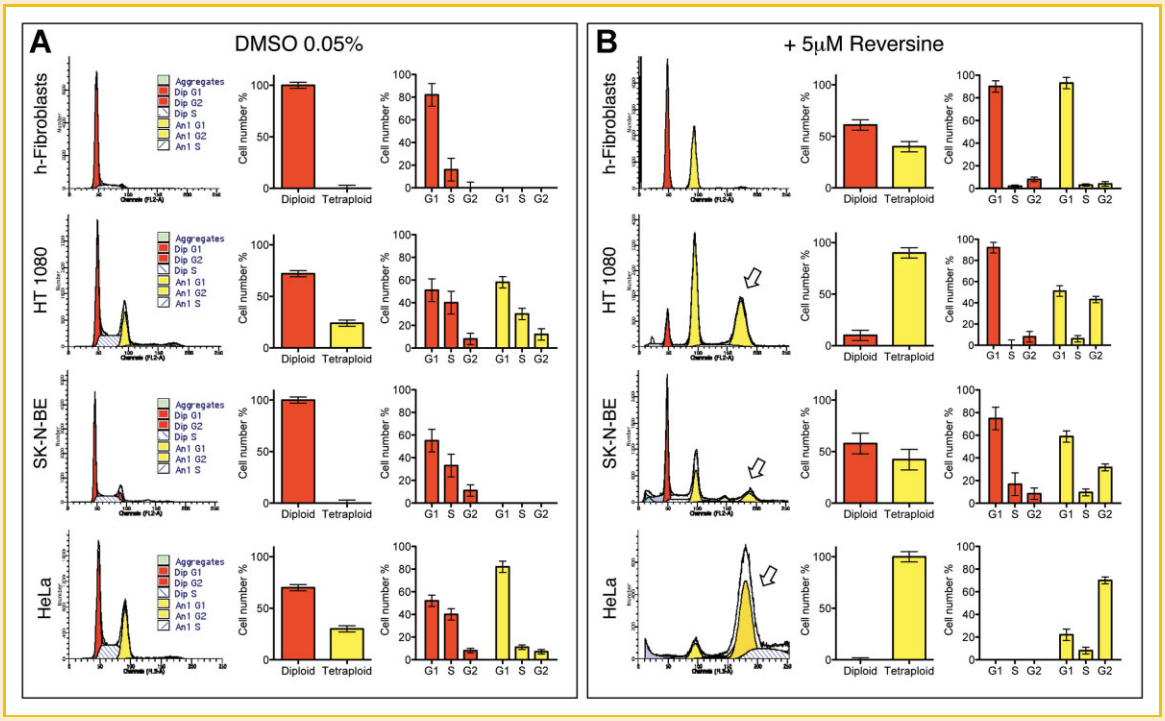

Fig. 2. Effects of reversine treatment on cell cycle. Flow cytometry analysis performed after 72 h of 0.05% DMSO (A) or 5 μM reversine treatment (B). After reversine treatment, fibroblasts form a mixed diploid/tetraploid population were blocked in their respective G1 phases, while all cancer cells undergo endo-replication and form polyploid populations which are cycling, as shown by the formation of G2 tetraploid peaks (panel B, white arrows). Three experiments were performed. Error bars show mean and SD. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

reversine-treated fibroblasts showed the formation of two distinct diploid and tetraploid populations, both arrested in their respective G_1 phases. In the case of cancer cells, reversine treatment caused the formation of a predominant tetraploid population, which was above 90% in the case of HT1080 and HeLa cells (Fig. 2B). However, 5 μM reversine did not cause cell cycle arrest in cancer cells, as it did in fibroblasts, but induced endoreplication, as the tetraploid populations in all treated cancer cells appeared to be also in the tetraploid G2 phase (Fig. 2B, white arrows).

CELL DEATH ANALYSIS

To further investigate the type of cell death induced by reversine, we started by assessing the extent of apoptosis by measuring chromatin condensation by means of DAPI staining in all reversine treated and control cells (Suppl. Fig. 3). Although 5 μM reversine caused massive cell death in all cancer cells, only reversine-treated SK-N-BE cells revealed chromatin condensation (Fig. 3A), typical of apoptotic cells. In all other cases no significant difference in chromatin condensation could be observed between reversine-treated and untreated cells, beside the presence of polynucleated cells in all reversine-treated cancer cells (Suppl. Fig. 3). Apoptosis activation was also tested with Caspase-Glo® 3/7 Assay, which is a luminescent assay that measures caspase-3 and -7 activities (Fig. 3B). Reversine-treated SK-N-BE neuroblastoma cells at 24 h showed a marked caspase activation typical of apoptosis, which was confirmed by cytofluorimetric analysis (Fig. 3C) and western Blot (Fig. 3D) with active-caspase 3 antibody. No significant caspase activation could be detected for HT1080 and HeLa cells (Fig. 3B), even at 48 and 72 h of reversine-treatment (data not shown).

Upon reversine suspension, fibroblasts appeared to return to their normal phenotype and proliferation rate within 3 weeks (Fig. 3E), as we previously observed [Anastasia et al., 2006]. On the contrary, cancer cells that survived a 72 h reversine-treatment did not recover but gradually died over time, even when treatment was suspended after 72 h, and cells were switched to normal growth medium without reversine (Fig. 3E). To further investigate the type of cell death induced by reversine in HT1080 and HeLa cells, staining with anti-β – tubulin antibody was performed. Reversine-treated cells showed an altered distribution of cell microtubules, revealing the presence of multiple centrosomes in mitotic cells (Fig. 3F). The presence of micronulei, which is typical of aberrant mitoses due to incorrect spindle formation and endoreplication [Edgar and Orr-Weaver, 2001], could be observed in both reversine-treated HT1080 and HeLa cells (Fig. 3F, DAPI nuclei staining), as further confirmed by co-staining HT1080 cells with DAPI and anti-human nuclei antibody (Fig. 3G).

CHROMOSOME ANALYSIS

Chromosome analysis of untreated HT1080 cells showed a modal chromosome number of 46 in 80% of cells, and exhibited chromosomal aneuploidy ranging from 37 to 92 chromosomes in 20% of metaphases (10% hypodiploid and 10% hypertriploid) (Fig. 3H). After 24 h of reversine treatment, cells showed increased chromosome aneuploidy, ranging from 38 to 92 chromosomes in

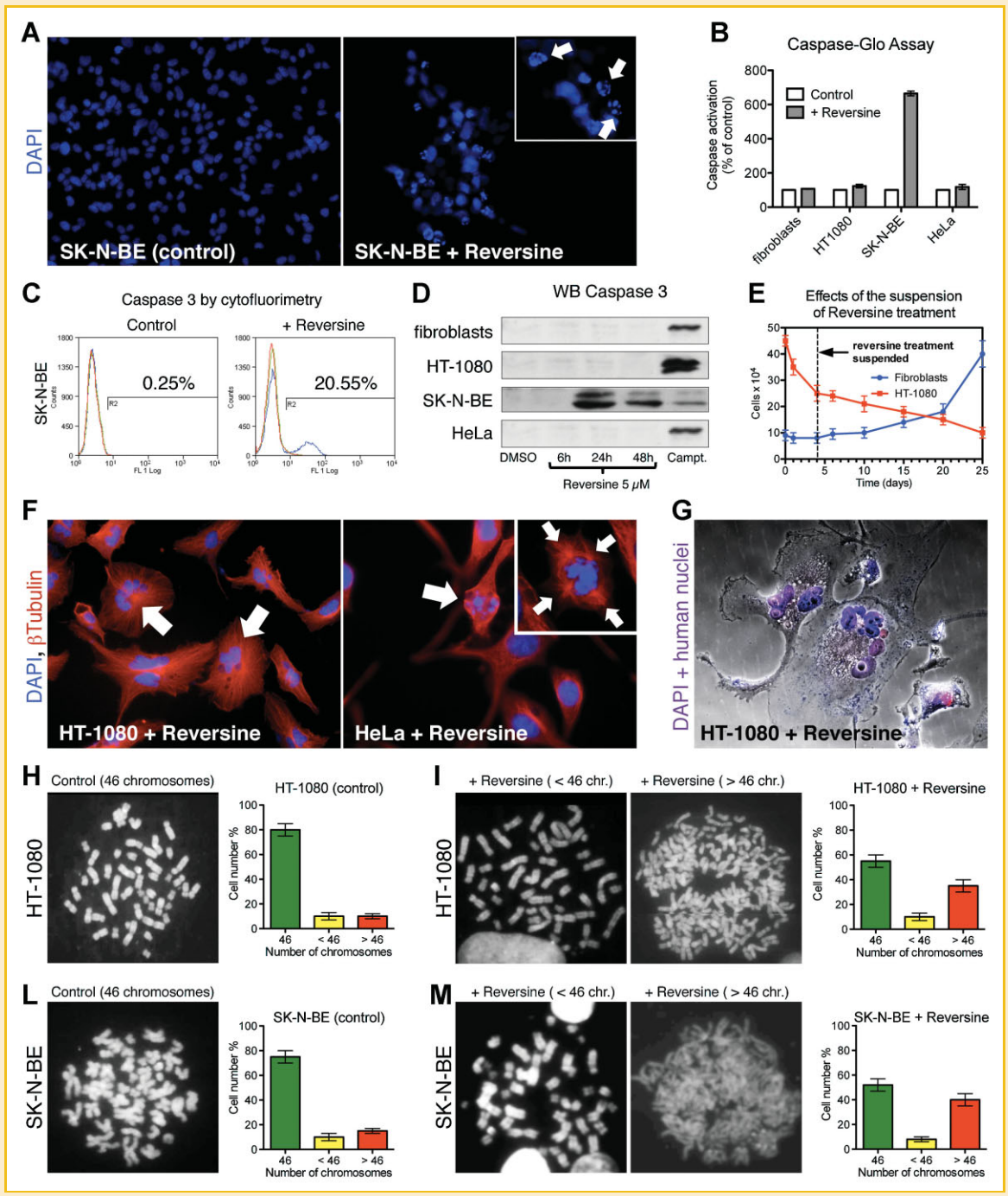

Fig. 3. Reversine causes cell death in cancer cells. A: DAPI staining of DMSO (control) or 5 μM reversine treated SK-N-BE cells reveals the formation of chromatin condensation in reversine-treated cells (white arrows); (B) Caspase-Glo Assay after 72 h reversine treatment reveals activation of caspases 3 and 7 only in SK-N-BE cells; (C) flow cytometry analysis with anti-caspase 3 active fragment performed after 72 h of 0.05% DMSO or 5 μM reversine treatment; (D) immunoblotting of anti-caspase 3 active fragment performed on reversine-treated cells at 6, 24, and 48 h, using camptotecin as positive control for caspase activation; (E) effects of suspension of reversine treatment on cell proliferation: After a 4-day treatment with 5 μM reversine cells were switched to reversine-free medium and cultured for 21 days. Cell count was plotted versus days of culture; (F) immunofluorescence analysis of HT1080 and HeLa cells, staining with antibody against β-Tubulin (red) reveals the formation of multiple centrosomes (white arrows), nuclei were stained blue with DAPI dye; (G) immunofluorescence analysis of HT1080, staining with antibody against human nuclei (red) is consistent with the formation of micronuclei due to aberrant mitoses and endoreplication, nuclei were stained blue with DAPI dye; (H–M) chromosome analysis of untreated or 5 μM reversine-treated HT1080 and SK-N-BE cells. Diploidy and aneuploidy frequencies are shown in histograms, see experimental for details. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

45% of metaphases (of which 11% were hypodiploid and 34% hypertriploid) (Fig. 3I). However, the modal chromosome number of 46 was still present in 55% of reversine-treated cells. Cytogenetic analysis of untreated SK-N-BE cells showed the modal chromosome number of 46 in 75% of metaphases, and chromosomal aneuploidy ranging from 43 to 73 chromosomes in 25% of metaphases (11% hypodiploid and 14% hypertriploid) (Fig. 3L). After 24 h of reversine treatment, cells showed increased chromosomal aneuploidy, ranging from 37 to 92 chromosomes in 46% of cells (9% hypodiploid and 37% hypertriploid); still 54% of metaphases showed the modal chromosome number of 46 (Fig. 3M).

REVERSINE EFFECTS ON CSCs FROM GLIOBLASTOMA

CSCs from glioblastoma were treated with 5 μM reversine and followed for 72 h (Fig. 4A). Reversine caused a 85% inhibition of cell proliferation by MTT test (Fig. 4B), already after 24 h of treatment, and extensive cell death could also be observed. Analysis of reversine effects on cell cycle by cell cytofluorimetry (Fig. 4C) revealed the formation of tetraploid populations already after 24 h of treatment, resulting in endoreplication and formation of cell debris due to extensive cell death.

CHEMICAL OR GENETIC DEREGULATION OF p53 RENDERS NORMAL FIBROBLASTS SENSITIVE TO REVERSINE-INDUCED ENDOREPLICATION

To test if reversine lethal effect was due to cell cycle check point deregulation, fibroblasts were treated with 10 μM pifithrin-α (PFT-α), a commercially available p53 inhibitor known to deregulate the cell cycle G_1 checkpoint [Komarov et al., 1999]. PFT-α treatment alone did not cause appreciable changes in fibroblast proliferation (Fig. 5A), and in terms of cell morphology (Fig. 5E, left panel). On the contrary, when PFT-α pre-conditioned fibroblasts where subjected to 5 μM reversine treatment, a progressive cell loss was observed, reaching 60% after 6 days (Fig. 5B), revealing chromatin condensation typical of apoptotic cells (Fig. 5E, right panel). Real-time PCR analysis after reversine treatment revealed a 2.5-fold increase of p21, a potent cyclin-dependent kinase inhibitor regulating cell cycle progression at G_1 (Fig. 5C), as previously observed in other reversine-treated cells [Hsieh et al., 2007]. No significant increase in p21 mRNA could be observed when PFT-α pre-conditioned fibroblasts were treated with reversine, as it would have been expected by inhibiting p53, which is known to cause p21 down-regulation [el-Deiry et al., 1993]. Cytofluorimetric analysis showed that 10 μM PFT-α treatment alone did not cause changes in normal fibroblast cell cycle, and no tetraploid populations could be detected (Fig. 5F). On the other hand, when PFT-α pre-treated cells were treated with reversine, the formation of a predominant tetraploid population was observed, partially in the tetraploid G_2 phase (Fig. 5G), supporting that p53-defective PFT-α pre-conditioned fibroblasts underwent endoreplication upon reversine treatment.

To further support this hypothesis, stable silencing of p53 in human fibroblast was achieved by lentiviral transduction with p53 shRNAs, and confirmed by real-time PCR (50-fold decrease, Fig. 5H) and by western Blot (fourfold decrease, Fig. 5I). This caused p21 down-regulation (6.5-fold decrease, Fig. 5J), as expected. Moreover p53 activity was determined in normal and shRNA-fibroblasts, as well as in all used cancer cell lines (Suppl. Fig. 4C–D). Activity of p53 was significantly lower (fivefold decrease) in p53-shRNA fibroblasts as compared to controls. Cancer cells SK-N-BE and HeLa revealed low p53 expression and activity, while HT-1080 revealed a p53 activity higher than normal fibroblasts. However, as expected from the literature [Kuerbitz et al., 1999], HT-1080 showed low levels of p16 (Suppl. Fig. 4A), which also compromise cell cycle control. Silencing of p53 did not cause significant effects on fibroblast cell-growth (Fig. 5K), while cells progressively died upon 5 μM reversine treatment (Fig. 5L). Cytofluorimetric analysis showed that reversine treatment caused the formation of a tetraploid population in p53 silenced fibroblasts (Fig. 5L).

DISCUSSION

The cell cycle effects induced by reversine have parallels in several precedent studies indicating that synthetic purines, analogous to reversine, also possess cell cycle perturbing activities [Chang et al., 1999; Chang et al., 2001]. A successive mechanistic study on reversine activity showed that the molecule is a dual inhibitor of MEK1 and of non-muscle myosin heavy chain (NMMII) [Chen et al., 2007], a cytoskeleton protein that plays a major role in cytokinesis, thus explaining the observed inhibition of cell growth. Concomitant inhibition of both NMMII and MEK1, obtained by other means, also caused growth inhibition and de-differentiation in C2C12 myoblasts [Chen et al., 2007]. These results generated some controversy since they were disputed in a successive independent study [Amabile et al., 2009], reporting that inhibition of NMMII and MEK1 did not cause reprogramming. However, this statement was given without any supporting evidence, as "*data not shown*". Conversely, it was reported that reversine effects are due to its inhibitory activity on several kinases of the Aurora's family [D'Alise et al., 2008; Amabile et al., 2009]. In particular, reversine inhibition of Aurora B was found to be essential for its de-differentiating activity and it further corroborated the observed G2/M accumulation in treated cells, as impaired Aurora B activity is known to result in cytokinesis failure [Vader and Lens, 2008]. Nevertheless, it was shown that inhibition of cytokinesis alone does not explain the de-differentiating effects of reversine. Actually, it was also hypothesized that chromatin remodeling, due to reversine-induced dephosphorylation at Ser10 of histone H3, might be involved in the de-differentiation process [Amabile et al., 2009]. However, the same authors have recently re-evaluated their hypothesis in consideration of their latest results [Santaguida et al., 2010], showing that reversine is also a potent mitotic inhibitor of MPS1 (a kinase which is crucial in the spindle assembly checkpoint and for chromosome bi-orientation on the mitotic spindle), and suggesting that its inhibition is possibly the principal target of reversine in mitosis. Thus, until reversine mechanism of action will be fully clarified, it may be wise to simply endorse that reversine acts on multiple targets, as indicated by our proteomic analysis [Fania et al., 2009], and that they may all contribute to the observed effects. On the other hand, all reported data indicate that cancer cells respond to reversine in a different way than normal cells. In fact, while no significant cell loss has been

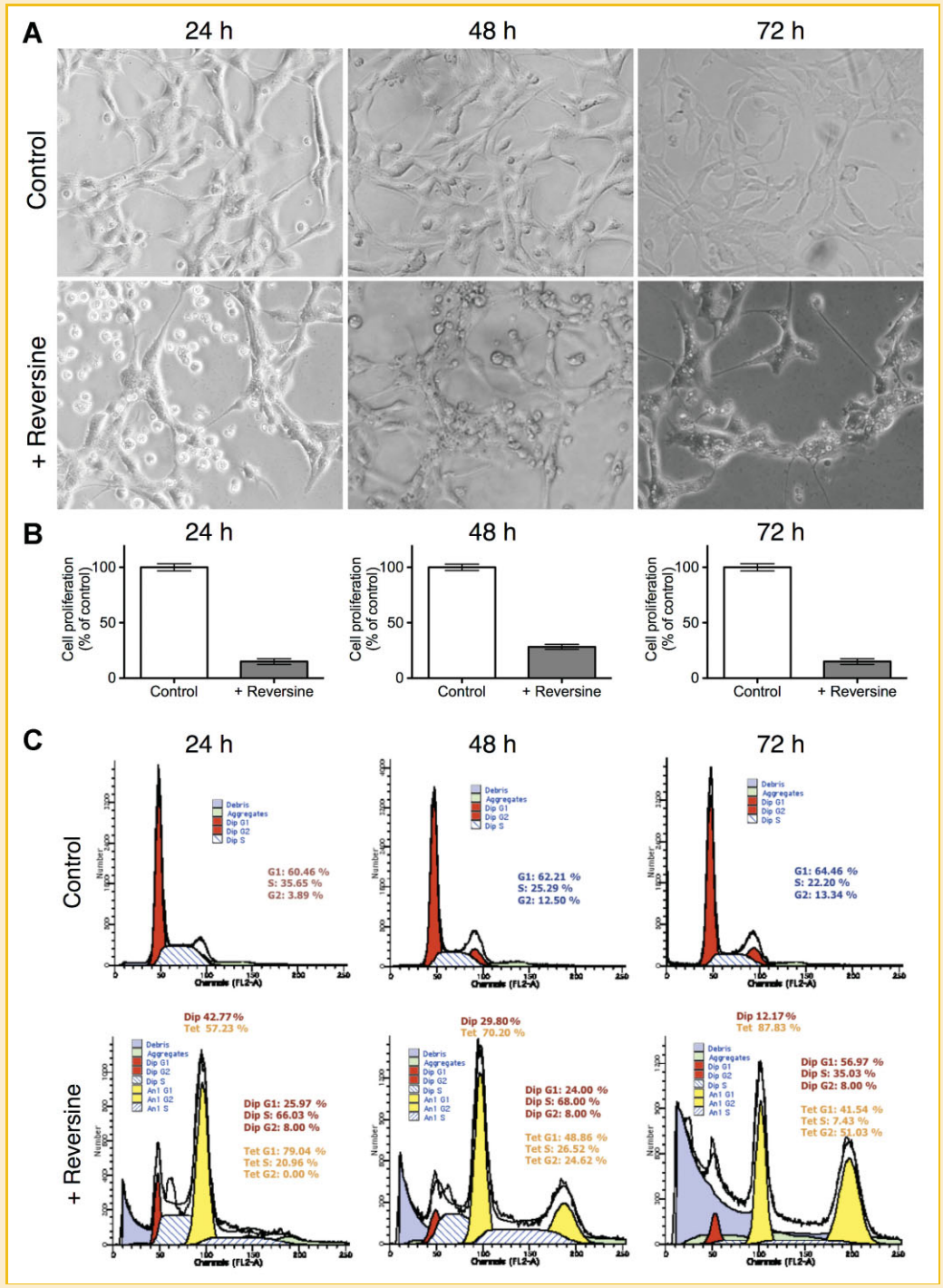

Fig. 4. Effect of 24, 48, and 72 h treatment with DMSO 0.05% (Control) and 5μM reversine (+Reversine) on cancer stem cells from human glioblastoma (L0627): (A) Phase-contrast microphotographs; (B) MTT proliferation test; (C) flow cytometry analysis reveals the formation of tetraploid populations (yellow) undergoing endoreplication and forming tetraploid G2 peaks, together with the presence of cell debris consistent with extensive cell death caused by reversine treatment. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

observed when treating normal cells for de-differentiation (that is up to 5 μM concentration), reversine induces cell death in cancer cells under the same experimental conditions. These findings support the hypothesis that reversine causes, at least in the critical 5 μM concentration, peculiar and irreparable damages to cancer cells. Cell cycle analysis clearly demonstrated that reversine treatment caused the formation of a mixed diploid/tetraploid population in normal fibroblasts, both populations being blocked in their respective G_1 phases, as long as reversine was present in the culture medium. Reversine, by interacting with several proteins involved in cell mitosis, inhibits cell cytokinesis and induce endoreplication. Moreover, our data show that cell cycle checkpoints, which are

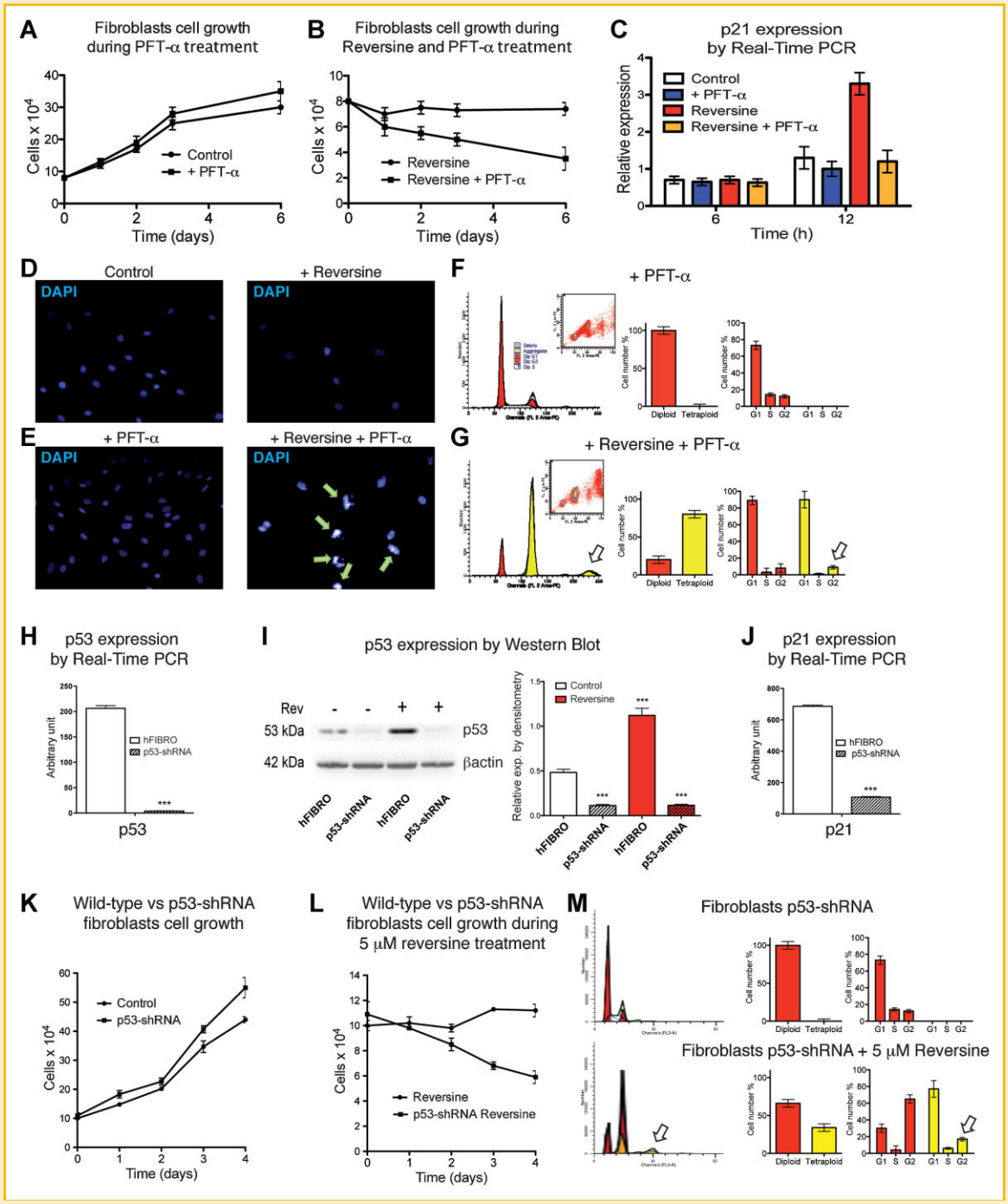

Fig. 5. Incubation of fibroblasts with PFT-α or p53 silencing render them sensitive to reversine treatment. Effect of PFT-α treatment alone (A) or in combination with reversine (B) on cell proliferation, cell count was plotted versus days of culture. C: Expression of p21 in human fibroblasts by real-time PCR after 6 and 12 h of treatment with DMSO, PFT-α, 5 μM reversine, 5 μM reversine + PFT-α; (D–E) DAPI staining of human fibroblasts after 72 h of treatment with DMSO, PFT-α, 5 μM reversine, 5 μM reversine + PFT-α reveals chromatin condensation only in cells treated with reversine + PFT-α (green arrows); (F–G) flow cytometry analysis performed after 72 h of PFT-α alone or in combination with 5 μM reversine. After combined reversine + PFT-α treatment, fibroblasts form a mixed diploid/tetraploid population undergoing endoreplication and forming tetraploid G2 peaks (white arrow). Human p53 silencing by shRNA in dermal fibroblasts increase their sensitivity to reversine treatment. Down-regulation of p53 upon viral transduction was checked by real-time PCR (H) and western-blot (I) and caused p21 down-regulation, checked by real-time PCR (J). Effect of p53 silencing and reversine treatment on cell proliferation, cell count was plotted versus days of culture (K–L). Flow cytometry analysis performed after 72 h of 5 μM reversine (M) reveals the formation of a mixed diploid/tetraploid population undergoing endoreplication. Three experiments were performed. Error bars show mean and SD. The statistical significance ($P < 0{,}001$) is referred to human dermal fibroblasts used as control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

fully functional in *normal* cells like fibroblasts, can rescue treated cells from aberrant mitosis by reversibly blocking them in the G_1 phase, as demonstrated by cytofluorimetry (Fig. 2). It is still unclear whether this effect on the cell cycle is mandatory for reversine de-differentiation activity, since reversine has been reported to induce de-differentiation also at sub-micromolar concentrations, where the inhibitory effect on cell growth is very limited [Chen et al., 2007]. Conversely, cell cycle analysis of all reversine-treated cancer cells revealed the formation of cycling polyploid cells (Fig. 2), leading to endoreplication and formation of gigantic polynucleated cells. Moreover, cytogenetic analysis performed on reversine-treated cancer cells, revealed the formation of substantial chromosomal aneuploidy (Fig. 3). To further validate our results, we decided to test reversine effect on human CSCs, isolated from a human glioblastoma multiforme, i.e., the most malignant brain tumor of adults [Galli et al., 2004; Lee et al., 2006]. Importantly, among tumor cells, CSCs have been envisioned as the elective target of therapy [Reya et al., 2001]. Surprisingly, reversine activity on GBM CSCs was even higher than on the traditional serum-dependent cancer cell lines we studied, both in terms of reduced proliferation and cell death (Fig. 4).

Overall, these evidences support the initial hypothesis that reversine treatment could become lethal in cells with deregulated cell-cycle checkpoints. This hypothesis was tested in normal fibroblasts by chemically or genetically blocking p53, a known G_1 checkpoint regulator. In fact p53, possibly the most studied tumor suppressor protein, is also known as the "*Guardian of the Genome*", as its up-regulation upon DNA damage, induces growth arrest, preventing replication of damaged DNA [Alarcon-Vargas and Ronai, 2002]. Thus, upon p53 inhibition or silencing, cells became sensitive to reversine, started to endoreplicate (as demonstrated by the appearance of tetraploid cells in G_2 phase), and gradually died, as in the case of cancer cells (Fig. 5). These data support the hypothesis that inhibition of p53 results in fibroblast impossibility to arrest reversine-induced endoreplication. On the other hand, wild-type fibroblasts, upon reversine treatment, can block cells in a quiescent state, as it was observed in all de-differentiation studies. Cell cycle analysis confirmed that p53 inhibition or silencing causes fibroblasts to re-enter cell cycle without dividing, similarly to what was observed in cancer cells. However, the lethal effect of reversine is not limited to p53 deregulated cells. In fact, while SK-N-BE and HeLa cells have low p53 activity, HT1080 possess normal p53 but are sensitive to reversine as they lack of p16, another key cell cycle checkpoint regulator (Suppl. Fig. 4). Interestingly, upon reversine treatment, we observed activation of apoptosis only in neuroblastoma SK-N-BE cells (Fig. 3). However, this is in line with the hypothesis that the endoreplication effect caused by reversine in cancer cells leads to cell death by "mitotic catastrophe", which is not necessarily accompanied by apoptosis [Ivanov et al., 2003; Castedo et al., 2004; Golstein and Kroemer, 2007].

These data give further support to the concept that reversine, at a relatively low concentration, is able to block the proliferation of human fibroblasts and to induce their de-differentiation, thus providing a new source of easily obtainable *stem-cell-like* progenitors, which can be successively oriented toward a specific cell type (skeletal or cardiac muscle cells, osteoblasts, neural cells, adipocytes, etc.) and engaged in tissue-regeneration approaches. In parallel, these data encourage to suggest that reversine, or some of its analogues, could become good candidates for a new class of anti-cancer drugs, with the urgent requirement to test both their toxicity and anti-tumoral effects in vivo, as no studies in this field have been reported till date. Investigations in this direction are currently undergoing in our laboratories.

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