Toxicology and Applied Pharmacology 241 (2009) 182–194

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO2 long-term exposure

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ARTICLE INFO

Article history:
Received 9 March 2009
Revised 10 August 2009
Accepted 11 August 2009
Available online 18 August 2009

Keywords:
Nano-particles
Aneuploidy
Cytotoxicity
Genotoxicity
TiO2
PLK1

ABSTRACT

Titanium dioxide (TiO2) nano-particles (<100 nm in diameter) have been of interest in a wide range of applications, such as in cosmetics and pharmaceuticals because of their low toxicity. However, recent studies have shown that TiO2 nano-particles (nano-TiO2) induce cytotoxicity and genotoxicity in various lines of cultured cells as well as tumorigenesis in animal models. The biological roles of nano-TiO2 are shown to be controversial and no comprehensive study paradigm has been developed to investigate their molecular mechanisms. In this study, we showed that short-term exposure to nano-TiO2 enhanced cell proliferation, survival, ERK signaling activation and ROS production in cultured fibroblast cells. Moreover, long-term exposure to nano-TiO2 not only increased cell survival and growth on soft agar but also the numbers of multinucleated cells and micronucleus (MN) as suggested in confocal microscopy analysis. Cell cycle phase analysis showed G2/M delay and slower cell division in long-term exposed cells. Most importantly, long-term TiO2 exposure remarkably affected mitotic progression at anaphase and telophase leading to aberrant multipolar spindles and chromatin alignment/segregation. Moreover, PLK1 was demonstrated as the target for nano-TiO2 in the regulation of mitotic progression and exit. Notably, a higher fraction of sub-G1 phase population appeared in TiO2-exposed cells after releasing from G2/M synchronization. Our results demonstrate that long-term exposure to nano-TiO2 disturbs cell cycle progression and duplicated genome segregation, leading to chromosomal instability and cell transformation.

Introduction

Nanomaterials, defined as particles ranging from 1 to 100 nm in at least one dimension, have become widely utilized because of their unique physicochemical properties (Hood, 2004), especially in biomedical settings (Moghimi et al., 2005; Sanvicens and Marco, 2008). Among those nanoparticles, titanium dioxide (TiO2) is frequently used in the production of paints, paper, plastics, welding rod-coating material, and cosmetics. With increased application of TiO2 nano-products (nano-TiO2), however, comes the inevitable possibility of effects on the health of humans exposed to these products. A number of studies indicate that nano-TiO2 exhibit concerned cytotoxicity in different cultured cell models (Rahman et al., 2002; Wang et al., 2007; Kang et al., 2008b), mainly resulted from the induction of apoptosis in mammalian cells (Rahman et al., 2002). In addition, several studies have shown that cells exposed to various doses of nano-TiO2 have higher frequency of *hypoxanthine phosphoribosyltransferase* (*HPRT*) gene mutation, micronucleated binucleated cells, and sister chromatid exchange, indicating that nano-TiO2 possess significant genotoxic activity in mammalian cells (Lu et al., 1998; Rahman et al., 2002; Gurr et al., 2005; Wang et al., 2007; Kang et al., 2008b). Reactive oxygen species (ROS) generation has been implicated in many cellular processes, for example, modulation of signaling transduction pathways and transcription factors activity. They have also been implicated in the multistage carcinogenic process, including DNA damage and mutation, and tumor promotion, and also in various human diseases. Accumulating evidence has revealed that nano-TiO2 catalyzes the formation of superoxide, H2O2, and hydroxyl radicals resulting in cytotoxicity and genotoxicity in different mammalian cells (Gurr et al., 2005; Singh et al., 2007; Kang et al., 2008a, 2008b). However, the controversial cellular responses to different doses of nano-TiO2 are also observed. Ranging from 5 to 200 μg/ml for 12–72 h exposure, nano-TiO2 displayed significant non-cytotoxic and -genotoxic effect to different cultured cells (Peters et al., 2004; Hussain et al., 2005; Jeng and Swanson, 2006; Park et al., 2007; Veranth et al., 2007). Moreover, nano-TiO2 failed to induce generation of ROS in phagocytic cells (RAW 264.7) (Xia et al., 2006), although the failure of ROS induction by TiO2 particles might be cell-type-dependent (Becker et al., 2002).

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doi:10.1016/j.taap.2009.08.013

A large body of *in vivo* animal model studies has shown that intratracheal exposure to ultrafine-TiO2 (UF-TiO2) causes cytotoxicity, inflammation and fibrosis to pulmonary. After intratracheal instillation of nano-TiO2, the inflammatory responses, for instance, increased numbers of polymorphonuclear neutrophil (PMN) in bronchoalveolar lavage (BAL) fluid, macrophage inflammatory protein-2 (MIP-2) in lavaged cells, and tumor necrosis factor alpha (TNF-α) release, were observed at distinct post-exposure time periods in rats (Oberdorster et al., 1992; Osier et al., 1997; Nemmar et al., 2008). Furthermore, *in vivo* exposure to nano-TiO2 also led to the increased cytotoxicity (Afaq et al., 1998; Warheit et al., 2006, 2007), and oxidative stress might involve in nano-TiO2-mediated lung injury (Afaq et al., 1998; Zhang et al., 1998). Chen et al. (2006) also reported that the lung tissues of adult mice displayed severe pulmonary emphysema, macrophages accumulation, extensive disruption of alveolar septa, type II pneumocyte hyperplasia, and epithelial cell apoptosis when they were exposed to the intratracheal single dose of 0.1 or 0.5 mg nano-TiO2. Other study also demonstrated that overload concentrations of the ultrafine TiO2 particles caused lung inflammation and cytotoxicity in certain species animals when inhaled (Bermudez et al., 2004). In addition to cause persistent inflammation, nano-TiO2 has additional effects that contribute to lung tumor formation (Borm et al., 2004). Chronically exposure to nano-TiO2 caused a higher risk for lung squamous cell and bronchoalveolar tumors in rats (Borm et al., 2004), estimating a 32% of lung tumor risk (Heinrich et al., 1995). Therefore, Knaapen et al. suggested that ROS generation induced by TiO2 particles might directly or indirectly damage DNA to cause genotoxicity and impact on cellular signaling pathways to modulate cell proliferation, resulting irreversible cell transformation (Knaapen et al., 2004). These findings were further supported by microarray gene expression profiles indicating roles of nano-TiO2 in modulating numerous gene expressions involved in cell cycle, apoptosis, and inflammation (Chen et al., 2006), indicating that nano-TiO2 can modulate intracellular physiological processes. Therefore, TiO2 has been recently evaluated as *possibly carcinogenic to humans*, Group 2B by IARC Monographs Working Group (Baan, 2007), although only a few epidemiological studies concerning occupational exposure to TiO2 and suggested that occupational exposure to titanium dioxide did not increase the risk of human lung cancer (Chen and Fayerweather, 1988; Boffetta et al., 2001; Fryzek et al., 2003; Boffetta et al., 2004). Although a large body of research has suggested that the cytotoxic and genotoxic effects of nano-particles are correlated to the particle size and surface area, the opposite observation also has been provided from the study of Warheit et al. (2006, 2007).

In conclusion, the recent studies show that nano-TiO2 can induce significant cytotoxicity and genotoxicity in cultured human cells and rats. However, these studies have yielded limited insight on the molecular mechanism of nano-TiO2 induced biological effects. To characterize the molecular mechanism underlying the nano-TiO2 mediated cytotoxicity and genotoxicity, we employed low dose and long-term exposure of nano-TiO2 in NIH3T3 cells and focused on the deregulation of cell cycle progression mediated by nano-TiO2 particles. Surprisingly, our results demonstrate that TiO2 facilitates cell transformation when NIH 3T3 cells were constantly exposed to low dose (10 μg/ml) of nano-TiO2. Our findings establish an unanswered mechanism of nano-TiO2 in inducing both mitotic aberration and genome instability as well as cell proliferation, leading to genotoxicity, mutagenicity and carcinogenicity. Polo-like kinase 1 (PLK1), at least in part, is one of the major molecular targets for nano-TiO2 induced mitotic aberration. This is the first report in our knowledge to investigate the molecular mechanism on cell transformation induced by nano-TiO2.

Materials and methods

Materials. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) if not otherwise indicated. The commercialized nano-size of TiO2 particles was obtained from local vendor and evaluated by transmission electron microscopy (TEM). The size of TiO2 particles was between 2 and 30 nm with a mean particle size of 15 nm. U0126 was obtained from TOCRIS (Cookson, Bristol, UK). Annexin V-FITC Apoptosis Detection kit was obtained from BD PharMingen (San Jose, CA). The polyclonal antibody specific against phospho-ERK1/2 (Thr202/Tyr204) (#9101) was purchased from Cell Signaling (Beverly, MA). The polyclonal antibodies against ERK2 (sc-154) and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon International Inc. (Temecula, CA), respectively. The polyclonal antibody against human PLK1 (ab12157) and monoclonal antibody against α-tubulin (ab7291) were from Abcam, Inc. (Cambridge, MA).

Cell culture and stimulation with nano-TiO2. NIH 3T3 cells and human fibroblast HFW cells (Lin et al., 2003) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Grand Island, NY), containing 10% fetal bovine serum (Gibco Life Technologies) and antibiotics (penicillin at 100 U/ml and streptomycin at 100 mg/ml), and 4 mM L-glutamine (Gibco Life Technologies). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For experimental treatment, cells in exponential growth were plated 1 day before stimulation with various concentrations of nano-TiO2. Cells were pre-treated with 10 μM U0126 for 1 h and then exposed to nano-TiO2 for an additional 24, 48, or 72 h to study the specific role of ERK1/2. At the end of treatment, medium was removed and cells were washed twice with phosphate-buffered saline (PBS). For short-term exposure, cells were treated with various concentrations of nano-TiO2 for 24–72 h. For long-term treatment, cells were subcultured every 3 days with the medium supplemented with 10 μg/ml nano-TiO2.

Measurement of cell viability. Cells (8×10^3) were seeded in 96-well culture plates and permitted to adhere for overnight at 37°C in medium containing 10% serum. Cells were then treated with various concentrations of nano-TiO2 for 24–72 h. At the end of treatment, cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) (USB Corporation, OH) assay. For MTT assay, cells were incubated with media containing 0.5 mg/ml MTT (100 μl/well) at 37°C for 3 h. The intensity was measured using a reader for enzyme-linked immunosorbent assay and an absorption wavelength of 575 nm. Cell viability was expressed as the percentage of viable cells relative to control. All experiments were performed at least in triplicate on three separate occasions.

Trypan blue exclusion assay. Cells were seeded at a density of 5×10^4 cells/35 mm dish, in triplicate, and were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, cells were treated with various concentrations of nano-TiO2 for the indicated time periods. At the end of treatment, cells were washed with PBS, trypsinized from each sample, and the cells pellet was suspended in 100 μl medium, and stained with 100 μl 0.4% (w/v) trypan blue. Cell mixture was mixed thoroughly and allowed to stand 2 min at room temperature. Total number of viable cells and number of blue-stained cells were counted on a hemeocytometer under a microscope.

Colony formation ability assay. Untreated or long-term exposed cells were trypsinized and plated at a density of 300 cells/60 mm Petri dish in triplicate without or with nano-TiO2. Cells were cultured for 10 days to allow colony formation and then fixed in 1.25% glutaraldehyde at room temperature for 30 min. Cells were then rinsed with distilled water and stained with 0.05% methylene blue solution (Fluka, Buchs, Switzerland). Colony numbers were counted and recorded.

Anchorage independent growth assay. Cells were cultured in the presence of nano-TiO2 for different time periods. After exposure,

1×10^4 cells were mixed with media containing 0.3% agar and plated on top of 0.5% agar coated 60 mm dishes. To maintain cell growth, fresh media containing 10 μg/ml nano-TiO2 were changed every week. After 2–3 weeks, cell colonies were stained with 0.5 mg/ml P-Iodonitrotetrazolium in distilled water overnight.

Cell lysis and Western blot. After treatment, cells were washed with PBS twice and lysed in cold lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 ng/ml leupeptin, and 1 mM Na_3VO_4) at 4°C. 50 μg of total cell lysate was separated on SDS-PAGE gel and transferred onto nitrocellular membrane (GE health care). Membranes were blocked with 5% non-fat milk and then incubated with the indicated primary antibodies followed by secondary antibody conjugated with horseradish peroxidase. ECL Western blotting kit (GE health care) was used to detect signals according to the manufacturer's recommendations.

Determination of intracellular TiO2 level. 2×10^5 cells were treated with 10 μg/ml nano-TiO2 for 1 min or 8 h, washed with PBS twice, trypsinized, and resuspended in PBS. Cells were sonicated at 12 W for 10 s (Sonifier s-450D, Branson, Danbury, CT), digested with 70% nitric acid in a microwave oven for 15 min at 180°C. Titanium concentration in the cell lysates was determined using a graphite furnace atomic absorption spectrophotometer with Argon laser at 328 nm (Z2000, Hitachi, Tokyo, Japan).

Measurement of ROS levels. The ROS production was determined by 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA). Cells were treated with nano-TiO2 for 24 h. 500 nM H_2DCF-DA was added into the media 30 min before harvesting cells. After treatment, cells were collected by trypsinization and centrifugation, washed with PBS and centrifuged at $200\times g$ for 5 min and analyzed immediately using a Beckman Coulter FC500 flow cytometer.

Apoptosis assay. Nano-TiO2 treated cells were trypsinized and collected by centrifugation. The cell pellet was washed, resuspended in 1× binding buffer and stained with annexin V-FITC as recommended by the manufacture. Cells were also stained with propidium iodide (PI) to detect necrosis. Apoptosis was analyzed by flow cytometry using a Beckman Coulter FC500.

Transmission electron microscopy, TEM. The structure of nano-TiO2 particles was examined using a transmission electronic microscope (JEM-2010, JEOL Ltd., Japan). After diluting in purified water, samples were placed over a copper grid coated with carbon film followed by staining with 2% phosphotungstic acid. The samples were air dried and dispersion was monitored.

Confocal microscopy. Cells were seeded at a 1×10^4 cells/cover slip and incubated at 37°C in a humidified 5% CO_2 atmosphere. At the end of treatment, cells were washed with PBS and fixed in 3.7% formaldehyde for 15 min. Coverslips were stained with primary and secondary antibodies. Then cells were treated with 0.02% Triton-X100 for 15 min and stained with 50 ng/ml DAPI for another 15 min before immunofluorescent images were acquired on a confocal microscope (TCS SP5, Leica Microsystems USA, Bannockburn, IL).

Cell cycle assay. Cells were treated with 100 nM nocodazole for 18 h, washed with PBS and trypsinized. Cell pellets were resuspended in PBS and fixed in 75% ethanol at 4°C for overnight. After centrifugation, cells were stained with PI for 30 min and analyzed by flow cytometry.

Cell division assay. Nocodazole-arrested cells were labeled with 5 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, Eugene, OR) in fresh medium for 45 min. After treatment, cells were washed with PBS and trypsinized for further cell division assay by flow cytometry.

Results

Cytotoxicity and cell proliferation of TiO2-exposed cells

A large body of evidence has documented the cytotoxic effect of nano-TiO2 in various lines of mammalian cells. To better understand how cells respond to nano-TiO2, NIH 3T3 cells were exposed to various dosages of nano-TiO2. MTT assay after 24 h exposure was used to characterize cell survival and we found that a significant increase ($P<0.01$) was induced to 31% by 50 μg/ml nano-TiO2 (Fig. 1A). To determine the stimulatory effect of nano-TiO2 was not cell line specific, we performed similar experiments using HFW human diploid fibroblasts. Fig. 1B showed a significant increase to 15.7% ($P<0.01$) in cell viability of HFW cells at 50 μg/ml nano-TiO2. Furthermore, 50 μg/ml nano-TiO2 also enhanced NIH 3T3 and HFW cell viability in time-dependent manners (Fig. 1C and D). Interestingly, remarkable enhancement of cell viability in NIH3T3 cells by 10 μg/ml nano-TiO2 was observed at 48 and 72 h exposure (Fig. 1C), indicating that nano-TiO2 have an unexpected effect on mammalian cell survival and proliferation.

The cytotoxicity of nano-TiO2 was also determined by colony-forming ability assay (CFA). Approximately 20.7% and 41.6% of increase in cell survival after exposure to 10 and 100 μg/ml TiO2, respectively, was observed (Fig. 1E). The increased cell survival rate was consistent with our data in MTT assay. To further investigate the proliferative effect of nano-TiO2, NIH 3T3 cells were exposed to 10 μg/ml nano-TiO2 and total viable cells were measured by trypan blue exclusion assay. Remarkably, nano-TiO2 promoted cell proliferation in a time dependent manner, which was consistent with the results in MTT and CFA. As shown in Fig. 1F, viable cells were gradually increased from 24 to 72 h when cells were exposed to 10 μg/ml nano-TiO2. Approximately 37% ($P<0.01$) and 21.4% ($P<0.05$) of enhancement of viable cells were occurred after exposure to nano-TiO2 for 24 and 48 h, respectively, although no significant increase was observed for 72 h treatment (Fig. 1F). Collectively, our data provided evidence that nano-TiO2 promote cell viability and enhance cell proliferation in time- and dose-dependent manners.

Internization of TiO2 and ROS production

The toxicological concern has been raised regarding redox active nanomaterial preparation (Colvin, 2003), and their transportation across cell membranes, especially into mitochondria (Foley et al., 2002). Determination of cellular nano-TiO2 levels by atomic absorption spectrophotometer revealed that nano-TiO2 were intracellular accumulated after 8 h exposure (Fig. 2A). Suzuki et al. have proposed that the cytoplasmic concentration of nanoparticles is reflected in the intensity of light side scatter (Suzuki et al., 2007). Side scatter analysis by flow cytometry also showed a time-dependent increase in cellular uptake of nano-TiO2 (Fig. 2B), suggesting that nano-TiO2 are internalized and accumulated in mammalian NIH 3T3 and HFW cells. Determination of cellular ROS production revealed that nano-TiO2 significantly augmented cellular ROS level after 24 h exposure. Approximately 2.3-fold increase in ROS production was detected at 50 μg/ml nano-TiO2 after 24 h treatment in both lines ($P<0.05$) (Fig. 2C and D).

MEK/ERK signaling pathway activation by TiO2

Mitogen-activated protein kinases (MAPKs) are involved in regulation of cellular responses leading to cell growth, differentiation,

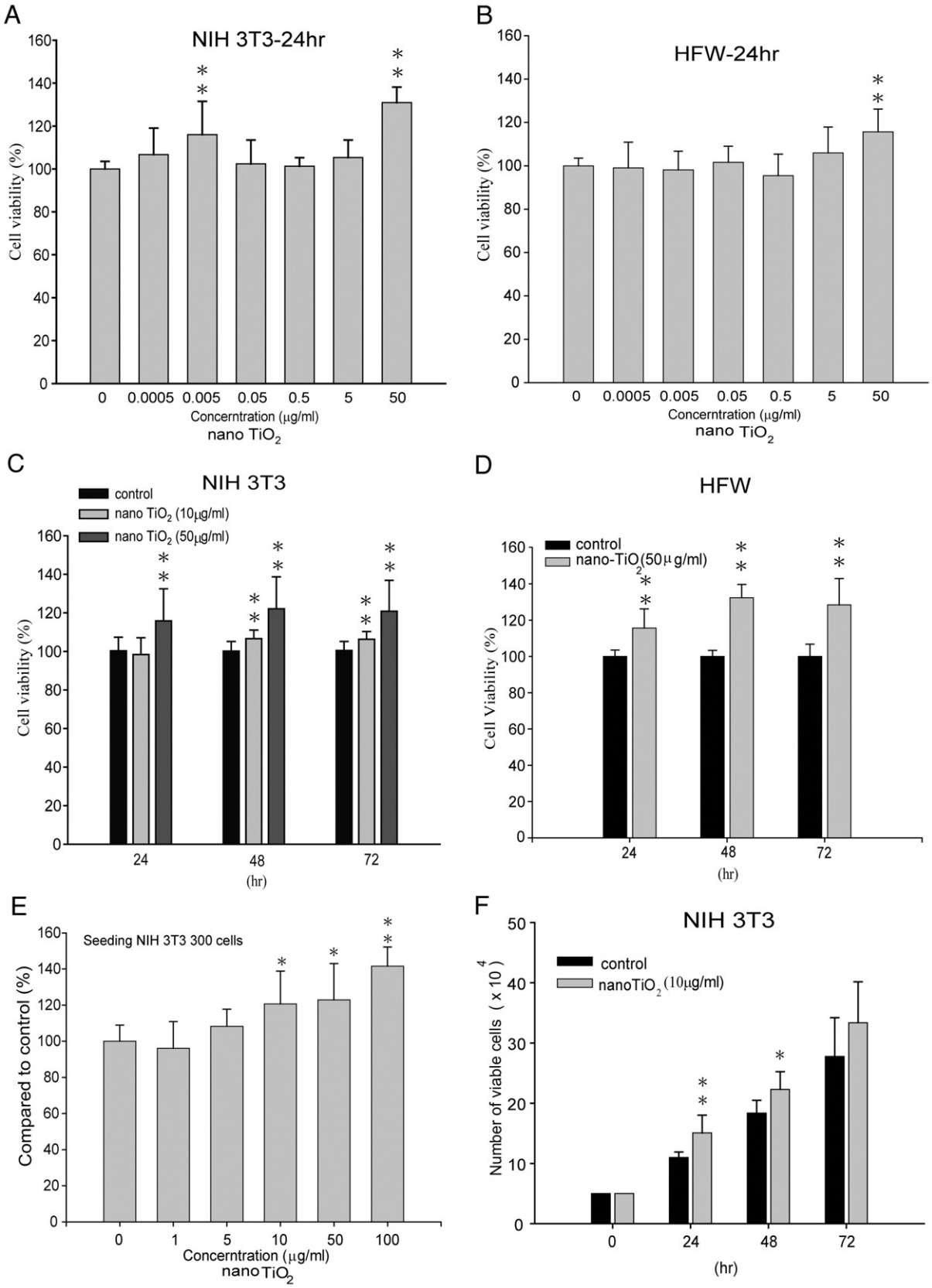

Fig. 1. Dose- and time-dependent increased in cell viability by nano-TiO2 treatment. (A) NIH 3T3 and (B) human diploid fibroblast HFW cells were seeded in 96-well culture plates 1 day prior to treatments and cell viability was determined by MTT assay after 24 h of nano-TiO2 exposure. Exponential growth of (C) NIH 3T3 and (D) HFW cells were exposed to 10 or 50 μg/ml nano-TiO2 for 24–72 h and then cell viability was determined by MTT assay. (E) NIH 3T3 cells were exposed to various concentrations of nano-TiO2 for 24 h and then subjected to colony-forming ability assay. (F) NIH 3T3 cells were grown in complete medium in the absence or presence of 10 μg/ml nano-TiO2. At the end of treatment, cells were washed with PBS and the numbers of viable cells were determined by trypan blue exclusion assay. Values (mean ± S.D.) are from three to six independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ using one-way ANOVA for the comparison between untreated and TiO2-exposed cells.

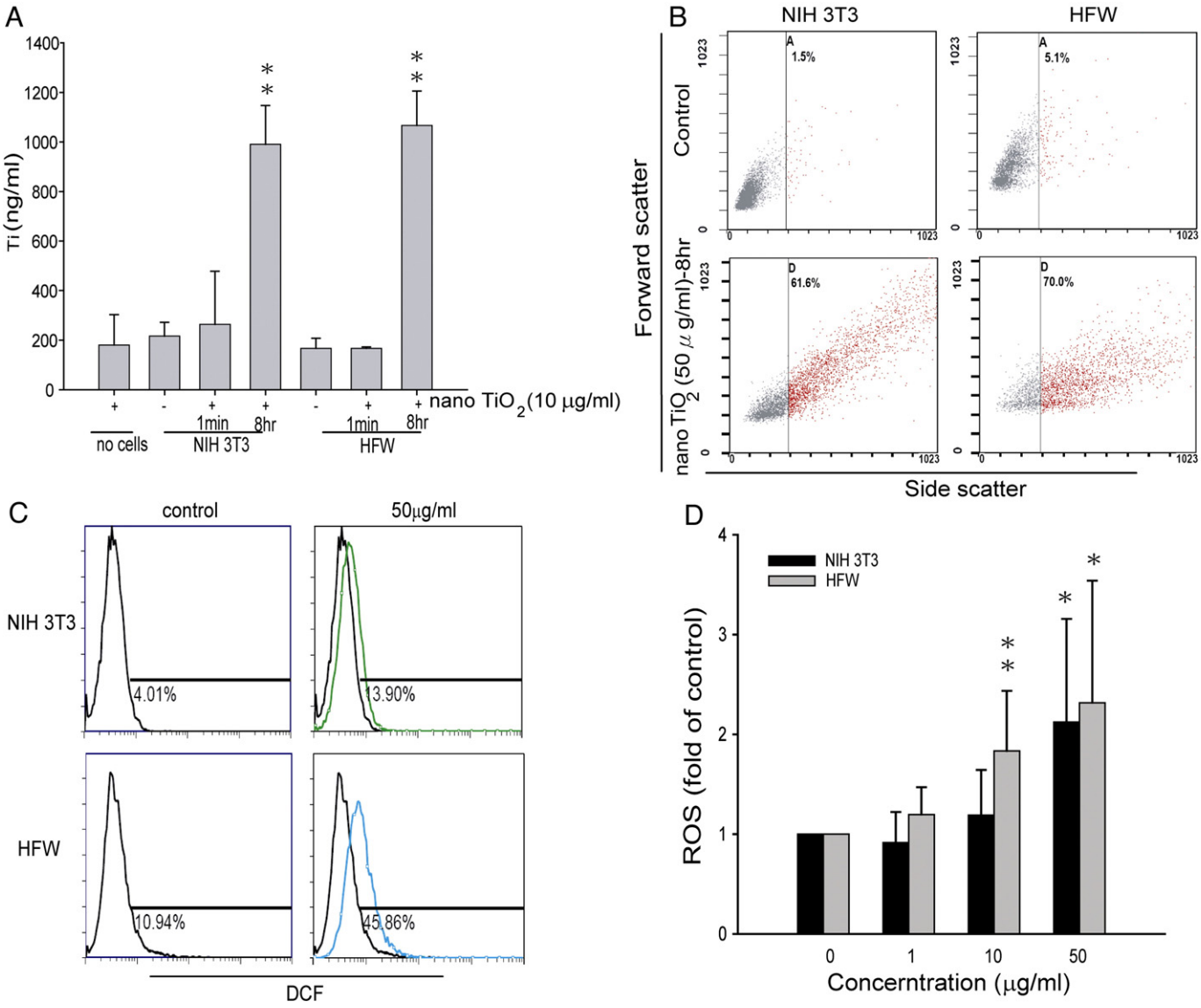

Fig. 2. Nano-TiO2 accumulated in cells and elicited intracellular ROS production. (A) NIH 3T3 and HFW (2×10^5) cells were treated with 10 μg/ml nano-TiO2 for 1 min or 8 h, and the intracellular titanium concentration was determined by atomic absorption as described in Materials and Methods. (B) Nano-TiO2 exposed cells were assayed for light side scatter intensity by flow cytometry. (C, D) Exponential growth of NIH 3T3 and HFW cells were exposed to 50 μg/ml nano-TiO2 and the intracellular DCF fluorescence intensity was measured by flow cytometry (C) and the relative fluorescence intensity was calculated from the average of three independent experiments and was normalized by control cells to 100% (D). Results (mean ± S.D.) are from three to six independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ using one-way ANOVA for the comparison between untreated and TiO2-exposed cells.

cell cycle progression and apoptosis in mammalian cells. In general, extracellular regulated kinases 1/2 (ERK1/2) cascade is a critical pathway for mitogenesis and differentiation. Recent studies have shown that modulation of ERK1/2 signaling pathway also plays a critical role in DNA damage response in human cells (Lin et al., 2003). It is reasonable to postulate that TiO2 induced cell proliferation and tumorigenesis could be, at least in part, through ERK1/2 modulation. To test this idea, NIH 3T3 cells were challenged with indicated TiO2 concentrations in medium containing 10% serum. After 24 and 48 h of exposure, we found that ERK1/2 activation was elevated in a dose-dependent manner, whereas the total ERK2 protein levels remained unchanged (Fig. 3A). With increasing TiO2 concentrations, ERK1/2 was activated initially by 1 μg/ml nano-TiO2 and remained highly activated at 100 μg/ml nano-TiO2 and then sustained to 48 h (Fig. 3A). Similarly, TiO2-induced ERK1/2 activation was observed in other cell types, including HFW and A549 cells (Fig. 3B and data not shown). Consistently, 100 μg/ml nano-TiO2 significantly activated ERK1/2 kinases and high level of activation continued for 48 h. In agreement with cell growth results, dose- and time-dependent activation of ERK1/2 signaling pathway was postulated to contribute in TiO2-facilitated cell proliferation. To determine whether mitogen activated protein kinase/extracellular regulated kinase kinases 1 and 2 (MEK1/2) was involved in TiO2-induced ERK1/2 activation, cells were exposed to nano-TiO2 in the presence or absence of U0126, the most potent MEK1/2 inhibitor. The protein levels of total and activated ERK1/2 were examined by immunoblotting. As expected, nano-TiO2 stimulated ERK1/2 activation whereas 10 μM U0126 dramatically attenuated ERK1/2 activation by nano-TiO2 (Fig. 3C and D).

Increase in cell transformation and chromosomal instability by long-term TiO2 exposure

Considering the tumorigenesis is a long-term process involved with multiple gene mutation and signaling pathways, it is conceivable that long-term exposure to low dosage of nano-TiO2 increases

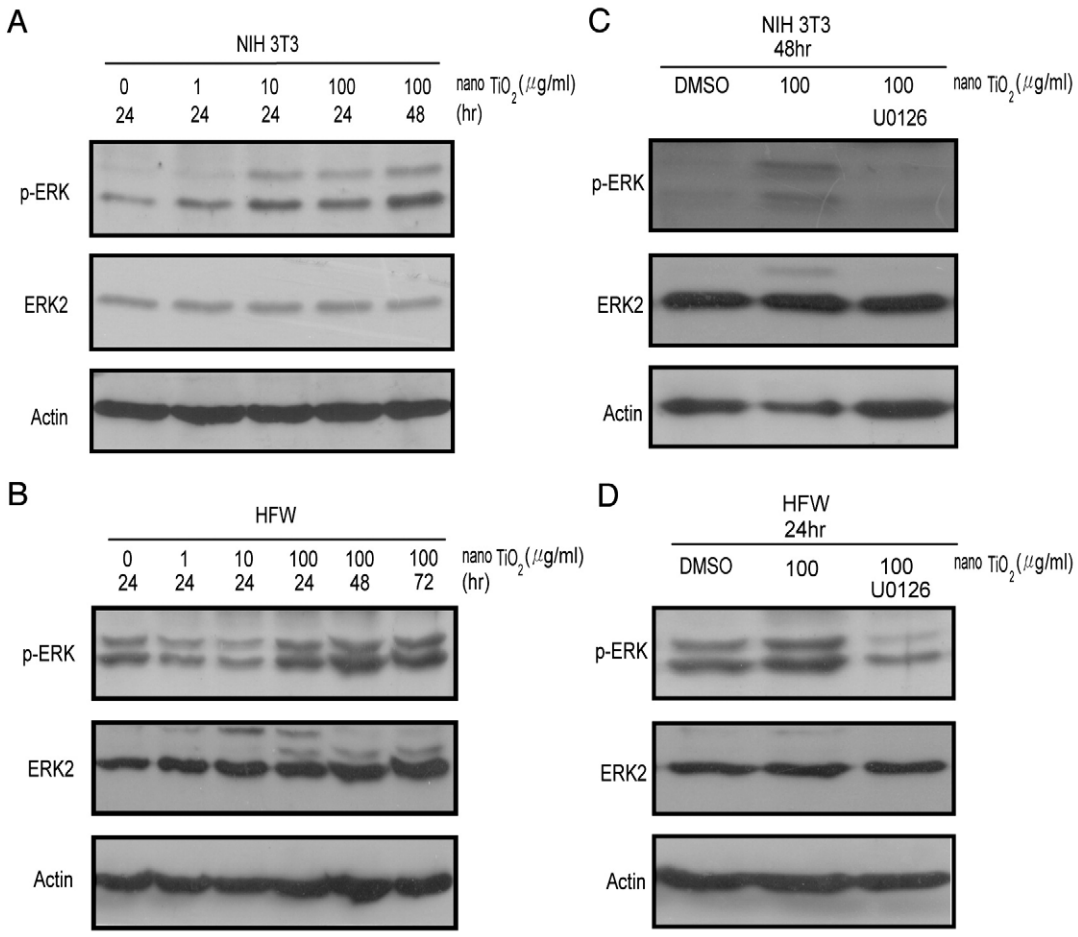

Fig. 3. Nano-TiO2 activated ERK1/2 activity. NIH 3T3 (A) and HFW (B) cells were treated with various concentrations of nano-TiO2. At the end of treatment, the levels of phosph-ERK1/2, ERK2 or β-actin were determined by immunoblotting. (C) NIH 3T3 and (D) HFW cells were pre-treated with 10 μM U0126 1 h before challenged to nano-TiO2 for another 48 and 24 h, respectively, and assayed for activation of ERK1/2. Results are obtained from three independent experiments.

genomic instability leading to cell transformation. To investigate the cellular behavior in response to nano-TiO2, NIH 3T3 cells were exposed to 10 μg/ml nano-TiO2 for various time periods. Fig. 4A showed that long-term nano-TiO2 exposure significantly ($P<0.01$) increased cell proliferation in colony formation assays. Secondly, given nano-TiO2 promoted cell proliferation and viability as mentioned above, we intended to address whether internalized nano-TiO2 promoted anchorage-independent growth on soft agar, a characteristic of cell transformation *in vitro*. Cells were long-term exposed to nano-TiO2 for different time periods and then subjected to anchorage-independent growth assay for 2–3 weeks. As shown in Fig. 4B, a dramatic increase in growth on soft agar was obtained in 12-week nano-TiO2 exposed cells, suggesting a stimulatory effect of nano-TiO2 long-term exposure on cell viability, proliferation, as well as cell transformation in cultured mammalian cells. Interestingly, a change in cell morphology was observed in long-term exposed cells (Fig. 4C). A larger size also was detected in 12-week exposed cells using flow cytometry and confocal microscopy (Fig. 4D–F). There is precedence for the induction of cell morphological change without causing a large extent of cell death by nano-TiO2 as demonstrated in Park's study (Park et al., 2007). Furthermore, the numbers of binucleated and multinucleated cells were gradually increased over time in long-term nano-TiO2 exposed cells after 11 weeks (Fig. 5A–C). Surprisingly, the percentage of micronuclei cells was significantly higher in long-term exposed groups (Fig. 5D). It is conceivable to speculate that the multinucleated cells might be resulted from deregulation of cell cycle progression at mitotic phase. To clarify this idea, 13-week exposed cells were released from nocodazole arrest and subjected to cell cycle phase analysis. Table 1 showed that a significant decrease of G1 and S phase cells was observed after releasing from nocodazole treatment at 1–4 h in nano-TiO2 exposed cells as compared to untreated cells. CMFDA (5-chloromethylfluorescein diacetate) staining, a tracking dye for cell division also revealed that 12-week exposed cells displayed a slower cell division compared to untreated cells (Fig. 6), implying a regulatory effect on mitotic progression of nano-TiO2. Taken together, our data presented here demonstrate that nano-TiO2 enhance cell growth on soft agar accompanied with increased chromosomal instability in cultured mammalian cells.

Disturbed mitosis and cytokinesis by long-term TiO2 exposure

To further characterize the formation of multinuclei, a key characteristic of mitosis deregulation, 11-week TiO2 exposed cells were treated with nocodazole for 18 h, released from nocodazole synchronization, collected at different time points, and analyzed for their physical properties. We showed that the number of multinuclei was increased (Fig. 7A), and the level of polyploidy was increased from 1.9% to 7.3% with nano-TiO2 long-term exposure (Fig. 7B). It is well known that precise regulation of dipolar spindle formation and chromatin alignment ensure accurate distribution of sister chromatids over two daughter cells. Hence, we postulated that multinuclei induced by long-term TiO2 exposure were resulted from mitotic deregulation. Therefore, cells synchronized at G2/M

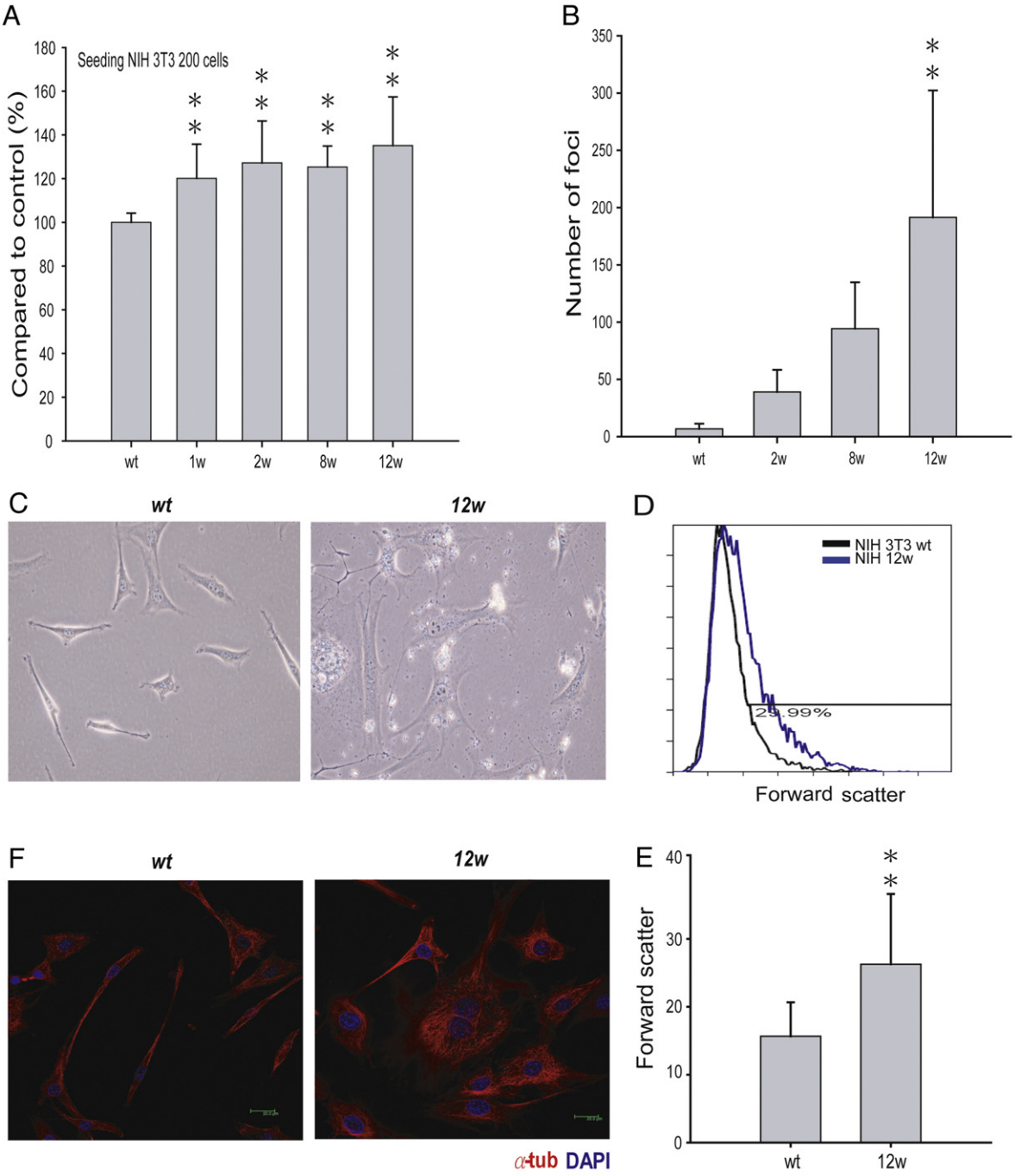

Fig. 4. Long-term exposure to nano-TiO2 promoted cell survival and anchorage-independent growth in cultured mammalian cells. (A) NIH 3T3 cells were exposed to 10 μg/ml TiO2 for 1–12 weeks. Colony-forming ability assay was performed at each time point. (B) Cells were long-term exposed to nano-TiO2 for indicated time periods and then subjected to soft agar growth assay at indicated time points as described in Materials and Methods. Cell morphology was examined by light microscopy (C) and confocal microscopy (F). (D) Cell sizes of 12-week nano-TiO2 exposed cells were determined by flow cytometry analysis. (E) The increased cell size by nano-TiO2 was calculated from the average of three independent experiments and was normalized by control cells to 100%. $^{**}P<0.01$ using independent-sample *t*-test for the comparison between untreated and TiO2-exposed cells and the bar represents ± SD. Results are obtained from three independent experiments.

phase by nocodazole were to test whether mitotic progression interruption was involved in increased multinuclei induced by long-term nano-TiO2 exposure. Interestingly, confocal microscopy revealed an abnormal chromosome alignment and segregation during anaphase and telophase in 12-week exposed cells (Fig. 8A). The image also showed that certain multipolar spindles appeared in 12-week exposed cells, indicating aberrant centrosome amplification with TiO2 long-term treatment (Fig. 8B). Taken together, we provide evidence demonstrating that intracellular nano-TiO2 disturb mammalian cell cycle progression, especially at mitosis and cytokinesis.

Involvement of PLK1 in mitotic deregulation

Genetic and biochemical experiments have indicated that PLK1, serine/threonine kinases, are important regulators of mitotic progression in mammalian cells, including bipolar spindle formation (Hamanaka et al., 1995; Glover et al., 1996), chromosome segregation,

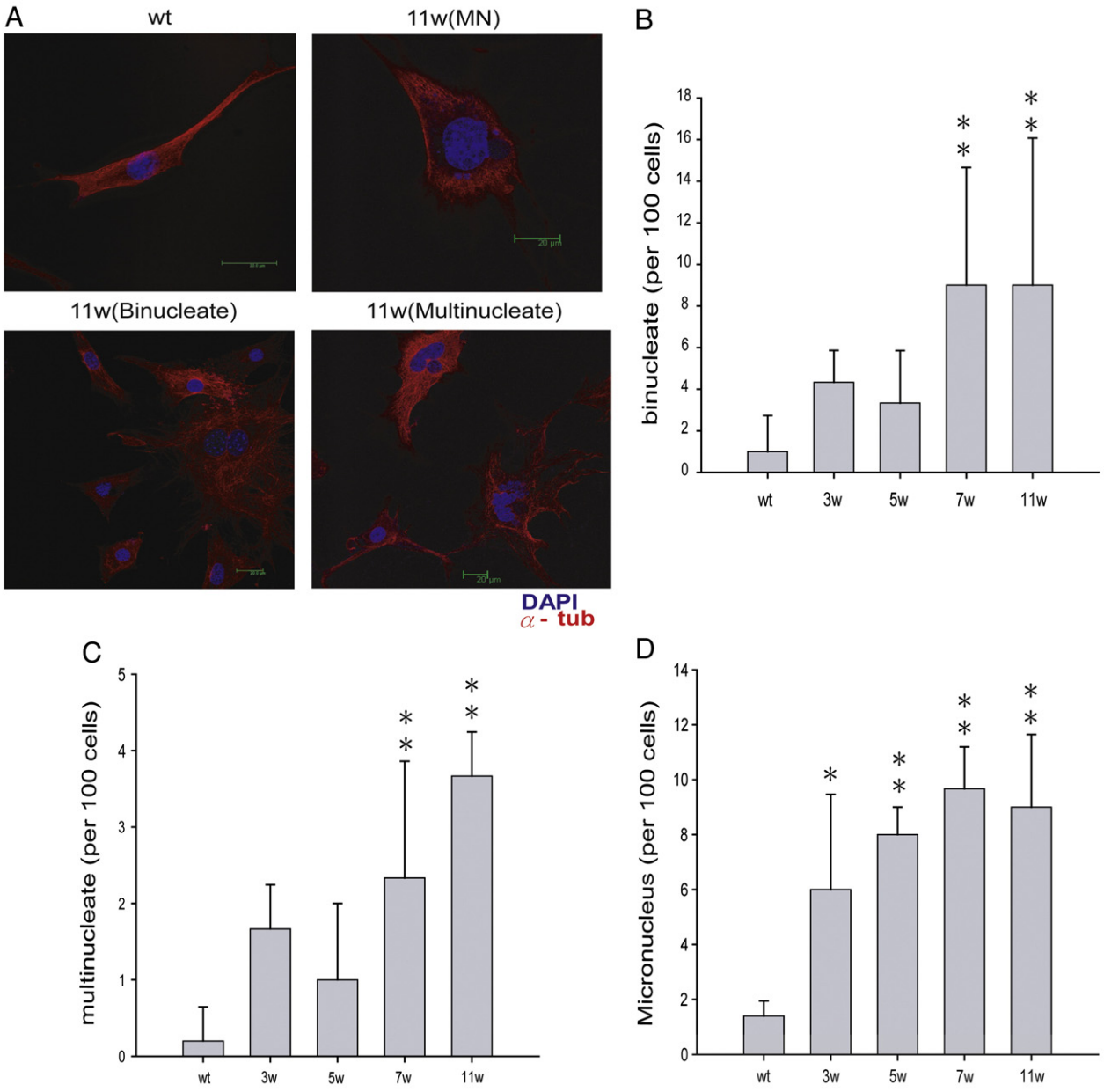

Fig. 5. Long-term exposure to nano-TiO2 increased the numbers of multinucleated cells and micronuclei. (A) Untreated or 11-week exposed cells were stained with DAPI and anti-tubulin antibody and then subjected to confocal microscopy analysis. The numbers of binucleated (B) and multinucleated (C) cells were counted per 100 cells. (D) The amount of micronuclei (MN) was counted per 100 binucleated cells. Results are from at least three independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ using one-way ANOVA for the comparison between untreated and TiO2-exposed cells and the bar represents ± SD.

Table 1
Effect of long-term exposure to nano-TiO2 in cell cycle progression

Noco. release (hr)	G0/G1	S	G2/M
1 h			
Wt	48.4 ± 7.3	17.4 ± 3.0	34.2 ± 4.2
13w	37.0 ± 5.4[a]	7.3 ± 3.7*	55.7 ± 8.3*
2 h			
Wt	50.6 ± 6.5	19.7 ± 2.9	29.6 ± 4.2
13w	38.8 ± 5.4*	11.5 ± 0.8*	49.7 ± 6.2**
4 h			
Wt	56.8 ± 5.3	15.3 ± 3.6	27.9 ± 7.6
13w	43.3 ± 5.3*	7.1 ± 4.6*	49.6 ± 10.6*

[a] $P=0.05$.
* $P<0.05$.
** $P<0.01$.

execution of cytokinesis (Lane and Nigg, 1996; Glover et al., 1998; Nigg, 1998) and centrosome maturation (Lane and Nigg, 1996). Given that the requirement of PLK1 in mitotic progression, it is conceivable that nano-TiO2 might have an effect on PLK1 function. To address this idea, untreated and 12-week exposed cells were immunostained with anti-PLK1 antibody, and we showed that PLK1 was localized around mid-zone during telophase in untreated cells whereas a distributed PLK1 over the nuclei in 12-week exposed cells (Fig. 9A). Notably, after releasing from nocodazole for 24 h, there was approximately 16.2% of cells at sub-G1 phase population in 12-week exposed cells compared to 2.7% of cells in untreated cells (Fig. 9B), suggesting that these cells continually progressed through mitotic phase and possibly died of apoptosis in next cell cycle. Based on our results, we conclude that nano-TiO2 interfered with spindle assembling and centrosome maturation through, at least in part, deregulating PLK1 function on mitotic exit (cytokinesis).

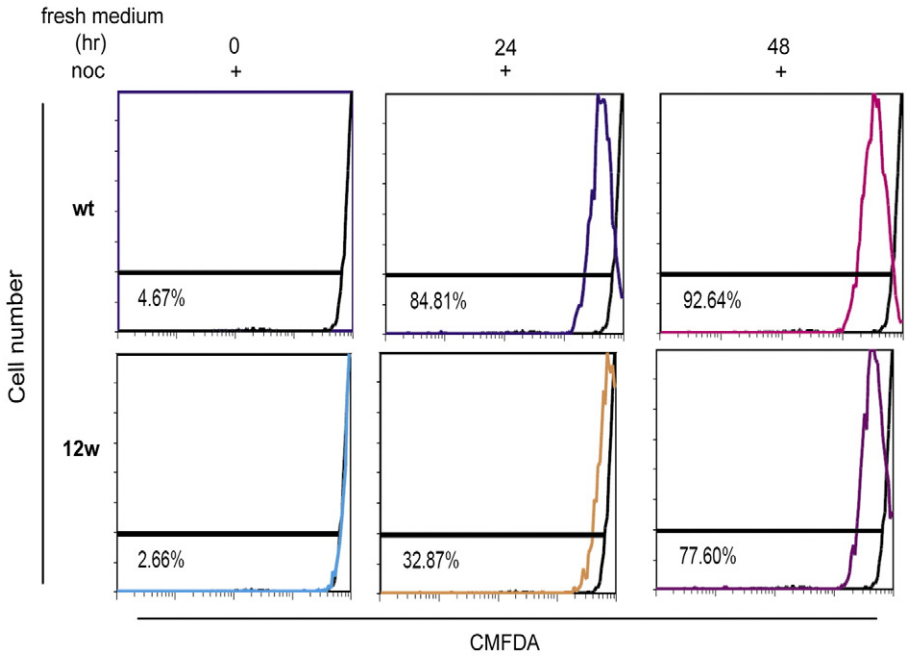

Fig. 6. Long-term exposure to TiO2 caused a slower cell division. After nocodazole arrest, cells were stained with CMFDA followed by flow cytometry analysis. Long-term exposed cells displayed slower cell division profiles after releasing 24–48 h when compared to untreated counterpart. Results are from at least three independent experiments.

Discussion

Nano-TiO2 particles receive considerable attention in recent years, as they exhibit inflammatory, proliferative, and genotoxic effects on mammalian cells and animal systems. Despite extensive interest in cyto- and genotoxicity, no comprehensive study paradigm has been developed to investigate the molecular mechanisms in tumorigenicity of nano-TiO2. In this study, we unraveled that nanoscale TiO2 particles (nano-TiO2) enhanced cell proliferation and growth on soft agar, as well as multinuclei, micronuclei and polyploidy formations. Moreover, cell cycle progression was disturbed at G2/M phase and abnormal chromosome segregation and centrosome amplification were significantly enhanced, therefore, resulting in chromosomal instability and multipolar spindles. These cellular events might be mediated through nano-TiO2-induced oxidative stresses, consistent to the study from Knaapen et al. (2004). Additionally, we further demonstrated that the mitotic checkpoint protein PLK1 was involved in nano-TiO2-mediated mitotic deregulation. However, a fraction of deregulated cells might continue cell cycle progression and undergo apoptotic cell death (Fig. 9C). Studying the genotoxic molecular mechanism of nano-TiO2 has helped elucidate pathways related to its tumerigenesis. Our data provide strong evidence that long-term exposure to nano-TiO2 might interfere with cell cycle progression at mitosis. The central hypothesis based on our studies is that genotoxic events and sustained signaling pathway stimulation drive deregulated cell proliferation and anchorage-independent growth, the processes both required for mutations and progression towards neoplastic lesions, play a role in nano-TiO2 particles induced mutagenesis and carcinogenicity.

The well-known biological mechanisms, such as the alteration of cell-signaling pathways and induction of DNA damage, play a vital role in neoplasia induction. MEK/ERK signaling is a major signaling pathway promoting cell proliferation and its deregulation often leads to uncontrolled cell proliferation and tumorigenesis. Nano-TiO2 activated MEK/ERK signaling pathway in a dose- and time-dependent fashion indicating that the particles affect cell cycle progression through modulating cellular signaling processes. Based on our results, we conclude that stimulated intracellular ROS production by nano-TiO2 might, at least in part, mediated MEK/ERK signaling transduction. Reactive oxygen species (ROS) are now considered to be the modulators underlying and regulating several cellular processes, specifically cellular signaling, inflammatory, genotoxic and proliferative responses.

The initiation stage of carcinogenesis is mainly characterized by genotoxic processes, which may lead to irreversible changes in the structure of cellular genetic materials. Although DNA repair pathways exist for DNA restoration, however, erroneous repair and extensive DNA damage may cause mutations and ultimately lead to cell transformation. In agreement with previous studies, we showed that nano-TiO2 remarkably induce micronuclei in mammalian cells and enhance cell proliferation on soft agar. As discussed above, we postulate that long-term exposure to nano-TiO2 persistently activated MEK/ERK signaling pathway and forced cells to proceed into mitosis despite genetic defects, accumulating genetic alterations followed by oncogenic transformation. In regard to effective induction of ROS by TiO2, we also speculate that chromosome instability augmented by long-term exposure to nano-TiO2 is partly resulted from significant ROS production. Our results are agreed with the previous reports (Knaapen et al., 2004). However, we did not observe the increased *HPRT* mutation rate by 6-thioguanine (6-TG) assay, implying that nano-TiO2 may not effectively cause gene mutation in our system (data not shown).

Accurate distribution of a duplicated genome over two daughter cells requires condensed chromosomes, a perfect spindle, and a mechanism called spindle assembly checkpoint that ensures correct attachment of these chromosomes to the spindle prior to cell division. Therefore, any regulatory molecules defected during mitotic phase might drive cells toward chromosomal instability. Aneuploidy is defined as an abnormal number of chromosomes and is a characteristic of tumerigenesis. Aneuploidy can be resulted from abnormal centrosomal amplification, deregulated spindle assembly checkpoint, or the failure of cytokinesis (King, 2008). PLK1, one of the most important regulatory molecules, has been demonstrated to participate in mitotic phase progression from mitotic entry to cytokinesis (Archambault and Glover, 2009). PLK1 is shown to translocate to the central spindle and the mid-zone in anaphase and telophase, respectively, to control contractile ring formation and mitotic exit (cytokinesis) (Golsteyn et al., 1995; Archambault and Glover, 2009).

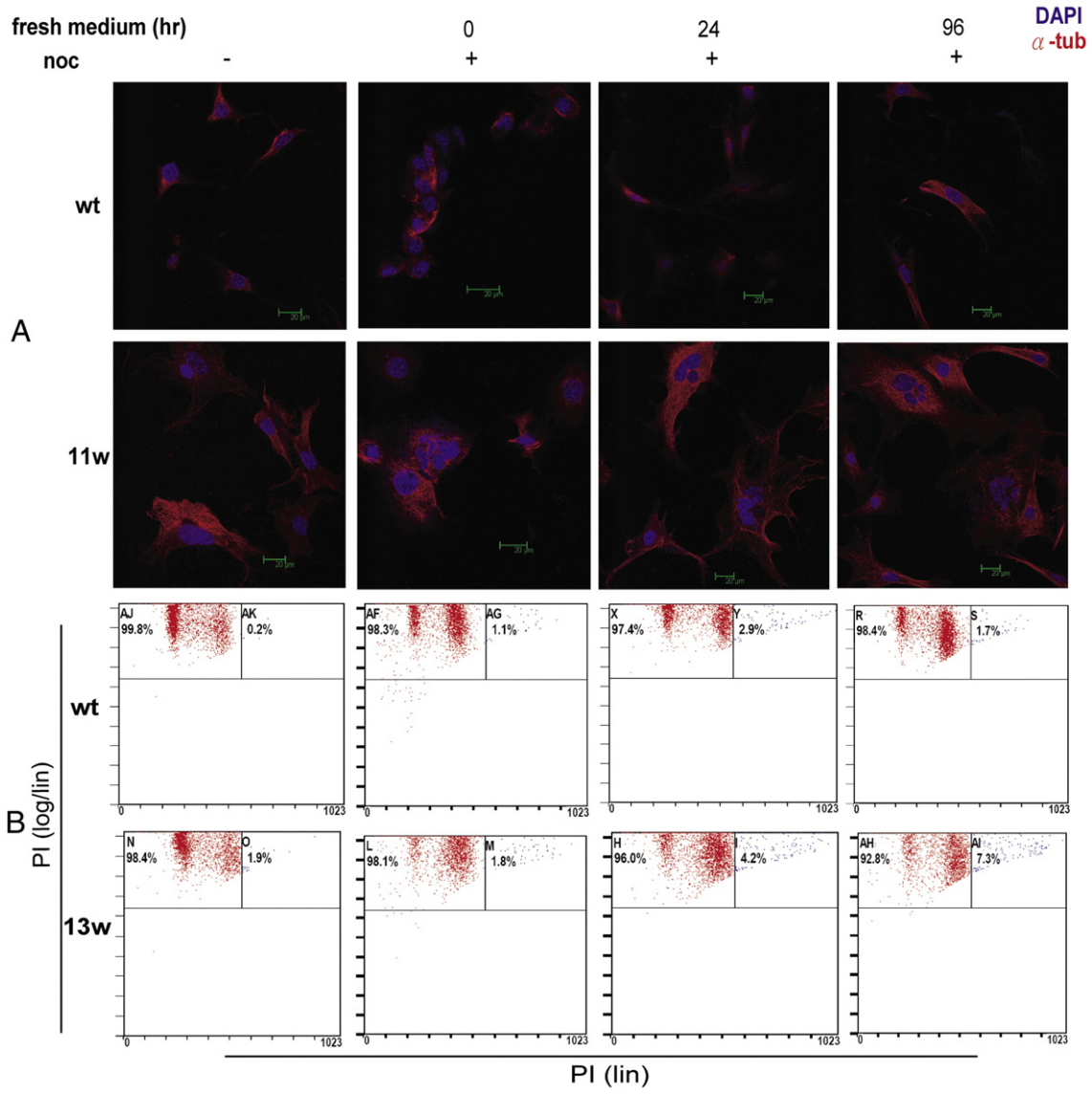

Fig. 7. Long-exposure to TiO2 caused an increase in multinuclei. (A) TiO2-treated or untreated cells were synchronized at G2/M by nocodazole and cells were harvested at 0, 24, 48, 72, and 96 h after releasing. Cells were stained with DAPI and tubulin antibody. (B) Untreated or 13-week exposed cells were subjected to flow cytometry and analyzed for polyploidy. Results are from at least three independent experiments.

Moreover, one striking consequence of deregulated PLK1 function is the formation of impaired centrosomes size/number, which is tightly correlated with aneuploidy and genomic instability in tumor development. Here, we showed that the numbers of binucleated and multinucleated cells were gradually increased over time in long-term nano-TiO2 exposed cells (Figs. 5A–C and 7). Moreover, long-term nano-TiO2 exposure also led to multipolar spindles formation (Fig. 8B). We further provide evidence indicating PLK1 was not localized at central spindles and mid-zone during mitotic exit in nano-TiO2 long-term exposed cells (Fig. 9A), suggesting a stimulatory effect of nano-TiO2 on deregulated mitotic progression is possibly by interfering with PLK1 function. Furthermore, enhanced multinucleation induced by long-term nano-TiO2 exposure can be also resulted from the failure of cytokinesis. Apparently, deregulation of PLK1 is involved in TiO2-modulated chromosome instability, mitotic deregulation, mitotic exit, and cytokinesis. These subsequences potentially contribute to *de novo* tumor formation stimulated by long-term nano-TiO2 exposure. Further studies will shed more light on those regulatory proteins and possible crosstalk between PLK1 and other kinases (for example, Aurora kinases, PLK2, PLK4, or Cdk1) in the context of the nano-TiO2-mediated deregulation of centrosome maturation, spindle assembly checkpoint, and cytokinesis.

This study adds our understanding of nano-TiO2-mediated genotoxicity and tumerigenicity that have not yet been explored before. Our results provide the first clear demonstration that nano-TiO2 might interfere with chromosome segregation, centrosome duplication, and cytokinesis. Furthermore, nano-TiO2 also regulates mitotic checkpoint protein PLK1 function, which controls several processes during mitotic entry and exit. It remains to be determined the detailed molecular mechanisms of nano-TiO2 mediated mitotic progression through PLK1 modulation.

Acknowledgments

We thank Dr. Jia-Ling Yang for providing us human diploid fibroblast cell HFW and Dr. Ping-Shan Lai, Dr. Duen-Yau Chuang and Dr. Jeremy-JW Chen for technical assistances in confocal microscopy and spectrophotometer. This work was supported by grants from the National Health Research Institute (NHRI-EX96-9411BC), the Institute of Occupational Safety and Health Council of Labor Affairs,

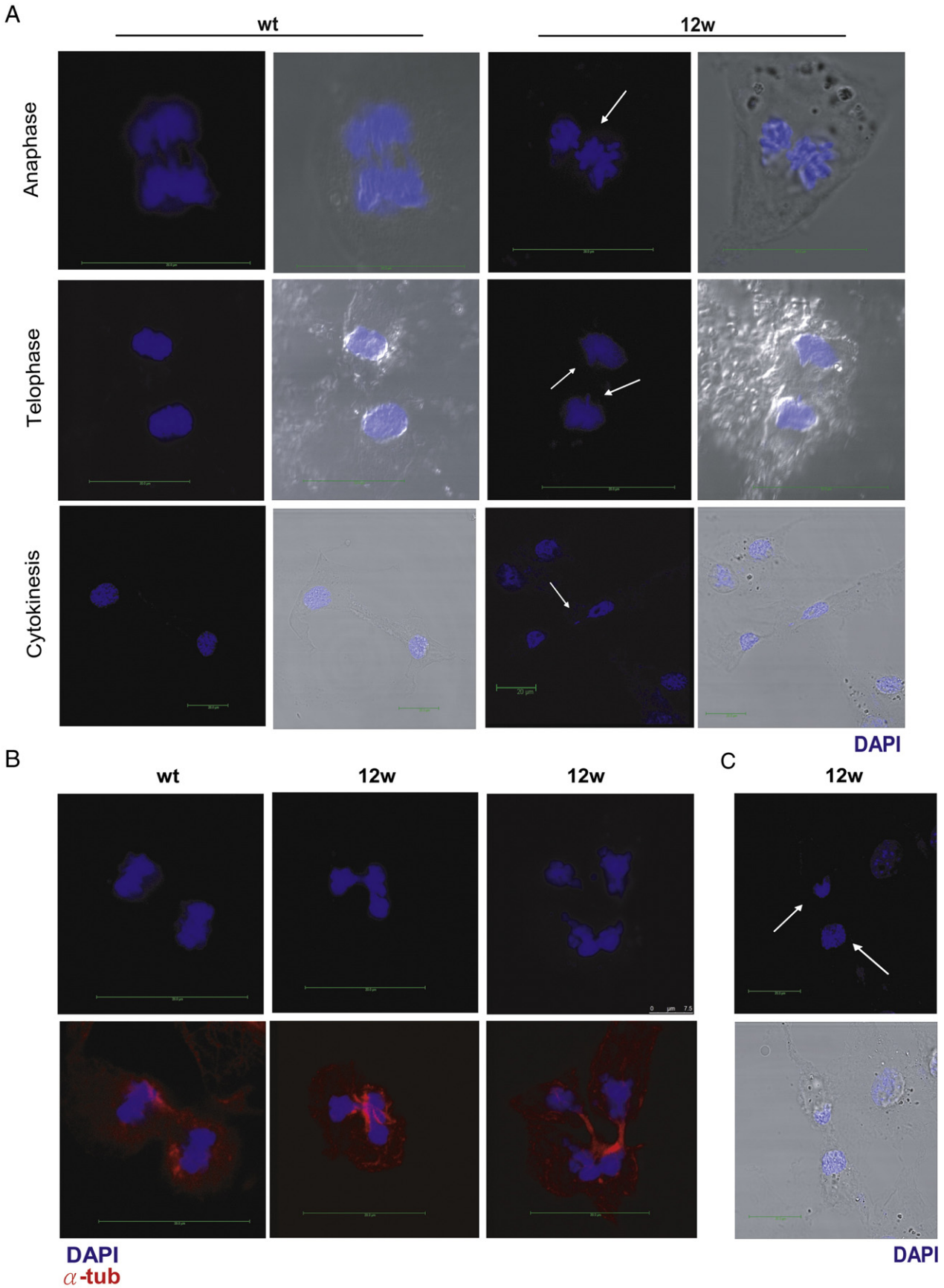

Fig. 8. TiO2 long-term treatment disturbed mitotic progression. Untreated or 12-week exposed cells were arrested by nocodazole treatment. After washing with PBS and releasing into fresh medium to continue cell cycle progression, cells at anaphase, telophase, cytokinesis phase (A), or multipolar spindles (B) were analyzed by confocal microscopy, or aneuploidy (C) were observed by confocal microscopy. Results are from three independent experiments.

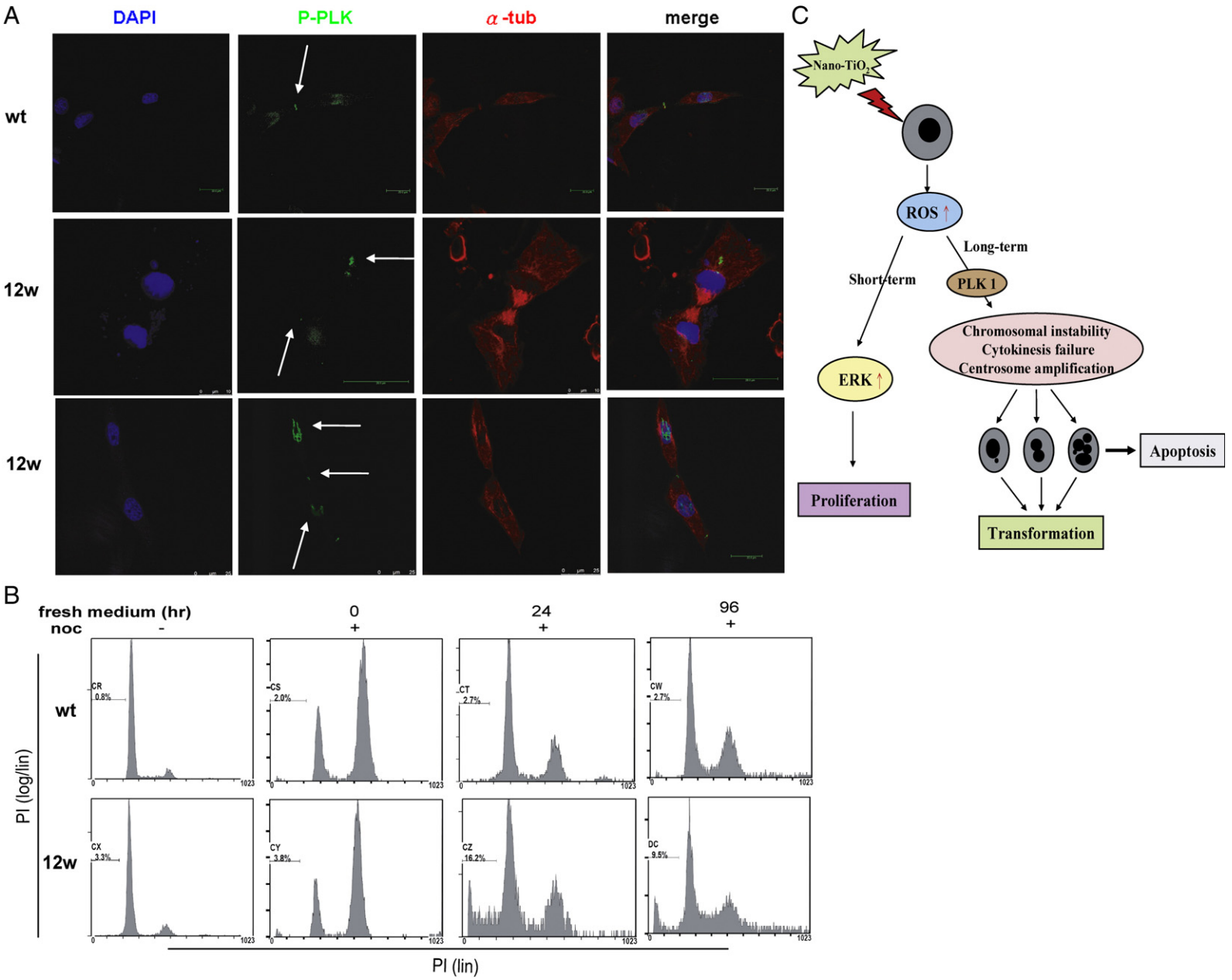

Fig. 9. PLK1 dislocalization during mitosis in TiO2 long-term exposed cells. (A) Untreated or 12-week exposed cells were released from G2/M arrest and fixed during cytokinesis. PLK1 and tubulin were immunostained and observed by confocal microscopy. (B) After releasing from nocodazole arrest, cells were collected and fixed at 0–96 h, and then subjected to flow cytometry. Results are obtained from three independent experiments. (C) A diagram for the hypothesis of proliferative signaling regulation by short-term exposure and cell transformation by long-term exposure of nano-TiO2.

Executive Yuan, and the Ministry of Education, Taiwan, under the ATU plan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2009.08.013.

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