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Genistein Induces Cytokinesis Failure Through RhoA Delocalization and Anaphase Chromosome Bridging

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ABSTRACT

Genistein, an isoflavone abundantly present in soybeans, possesses anticancer properties and induces growth inhibition including cell cycle arrest and apoptosis. Although abnormal cell division, such as defects in chromosome segregation and spindle formation, and polyploidization have been described, the mechanisms underlying the induction of abnormal cell division are unknown. In this study, we examined the effect of genistein on cell division in cells that are synchronized in M phase, since genistein treatment delays mitotic entry in asynchronous cells. HeLa S3 cells were arrested at the G2 phase and subsequently released into the M phase in presence of genistein. Immunofluorescence staining showed that genistein treatment delays M phase progression. Time-lapse analysis revealed that the delay occurs until anaphase onset. In addition, genistein treatment induces cleavage furrow regression, resulting in the generation of binucleated cells. Central spindle formation, which is essential for cytokinesis, is partially disrupted in genistein-treated cells. Moreover, aberrant chromosome segregation, such as a chromosome bridge and lagging chromosome, occurs through progression of cytokinesis. RhoA, which plays a role in the assembly and constriction of an actomyosin contractile ring, is delocalized from the cortex of the ingressing cleavage furrow. These results suggest that genistein treatment induces binucleated cell formation through cleavage furrow regression, which is accompanied by chromosome bridge formation and RhoA delocalization. Our results provide the mechanism that underlies genistein-induced polyploidization, which may be involved in genistein-induced growth inhibition. J. Cell. Biochem. 115: 763–771, 2014.

KEY WORDS: GENISTEIN; CHROMOSOME BRIDGE; RhoA; CYTOKINESIS; CLEAVAGE FURROW REGRESSION

Genistein, an isoflavone abundant in soybeans, has been shown to possess anticancer properties as demonstrated by in vitro assays and in vivo animal models [Zhou et al., 1998; Hewitt and Singletary, 2003]. Because of structural similarities with estradiol, genistein binds to the estrogen receptor (ER) and acts as a weak agonist and antagonist of estradiol in a concentration-dependent manner [Fioravanti et al., 1998]. The effects of genistein in human breast cancer were initially studied in ER-positive cells [Peterson and Barnes, 1996; Wang et al., 1996]. However, the effects of genistein are not limited to ER-positive cells [Peterson and Barnes, 1996; Shao et al., 1998]. Indeed, genistein induces G2/M arrest in ER-negative non-neoplastic MCF-10F human breast cells [Frey et al., 2001] and several other cell types [Matsukawa et al., 1993; Davis et al., 1998; Frey et al., 2001; Kobayashi et al., 2002; Li et al., 2008].

Genistein-induced growth inhibition includes induction of cell cycle arrest [Matsukawa et al., 1993] and apoptosis [Constantinou et al., 1998; Davis et al., 1998; Onozawa et al., 1998; Zhou et al., 1998; Li et al., 1999a, b; Lian et al., 1999]. Cell cycle arrest at G2/M occurs in various cancer cells, possibly through a decrease in the protein levels of cyclin B1, Cdk1, and Cdc25C [Davis et al., 1998; Frey et al., 2001; Kobayashi et al., 2002; Li et al., 2008] and an increase in p21 expression levels [Davis et al., 1998; Lian et al., 1998; Frey et al., 2001; Kobayashi et al., 2002]. Genistein may provoke DNA damage response [Tominaga et al., 2007] through the activation of the ataxia–telangiectasia-mutated (ATM) kinase [Chang et al., 2004] and inhibition of Cdk1 activity [Cappelletti et al., 2000]. Alternatively, G2/M arrest may be induced by the decatenation checkpoint during genistein-induced topoisomerase II inhibition [Deming et al., 2001; Luo et al., 2009], since genistein inhibits topoisomerase II activity [Markovits et al., 1989].

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Upon treatment with genistein, abnormal cell division, such as chromosomal abnormalities and polyploid formation, was observed in Brca1-mutated mammary tumor cells [Tominaga et al., 2007]. Polyploidization raises the possibility that cytokinesis failure may be caused in genistein-treated cells. Despite the genistein-induced tubulin depolymerization in vitro through the binding of genistein to tubulin [Mukherjee et al., 2010], the effect of genistein on mitotic spindles, which plays a critical role in cytokinesis, is controversial [Metzler and Pfeiffer, 1995; Webb et al., 2005; Rusin et al., 2009; Gogler-Pigłowska et al., 2012]. Furthermore, the effects of genistein on cytokinesis remain to be unknown. In this study, we wished, therefore, to explore the mechanism that underlies genistein-induced abnormal cell division.

We show here that genistein treatment delays M phase progression. The delay occurs until the anaphase onset. Genistein induces cleavage furrow regression, which is accompanied by chromosome bridge formation and RhoA delocalization, resulting in the generation of binucleated cells. Our findings suggest a mechanism underlying the genistein-induced polyploidization, and this may be one of the mechanisms behind the genistein-induced growth inhibition.

MATERIALS AND METHODS

REAGENTS

Genistein (546-00171, Wako) and RO-3306 (217699, Calbiochem) were dissolved in DMSO.

CELLS

HeLa S3 cells (Japanese Collection of Research Bioresources, Osaka) were cultured in Iscove′s modified Dulbecco′s medium (IMDM) containing 1% fetal bovine serum and 4% bovine serum (BS). For cell cycle synchronization in M phase, cells were treated with 8–10 μM RO-3306 for 20 h to arrest cells at the G2 phase. Subsequently, cells were washed with phosphate-buffered saline (PBS) supplemented with Ca^{2+} and Mg^{2+} [PBS(+)] at 37°C and released into pre-warmed fresh medium to induce them into the M phase [Nakayama et al., 2012]. To generate the stable cell line expressing histone H2B-mCherry, the pBOS-H2B-mCherry was constructed by replacing the *Bam*HI–*Not*I region in the pBOSH2BGFP-N1 vector (provided by H. Saya [Izumi et al., 1991; Kanda et al., 1998]) with the *Bam*HI–*Eco*RI fragment of the pRSET-B-mCherry vector (provided by R.Y. Tsien [Shaner et al., 2004]). HeLa S3 cells were transfected with the pBOS-H2B-mCherry, and cells were selected in 2 μg/ml blasticidin (Kaken Seiyaku).

ANTIBODIES

Mouse monoclonal anti-RhoA (26C4, Santa Cruz Biotechnology), and rat monoclonal anti-α-tubulin antibodies (MCA78G, Serotec) were used. Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of anti-mouse IgG and IgM (BioSource International) and FITC-$F(ab')_2$ of anti-rat IgG (Sigma) secondary antibodies were used. Alexa Fluor 488 donkey anti-mouse IgG secondary antibodies were obtained from Invitrogen.

IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence staining was performed as previously described [Nakayama and Yamaguchi, 2005; Nakayama et al., 2006]. In brief, cells were fixed in PBS containing 4% paraformaldehyde for 20 min or in PTEMF buffer (20 mM PIPES [pH 6.9], 0.2% Triton X-100, 10 mM EGTA, 1 mM $MgCl_2$, and 4% paraformaldehyde) for 20 min at 37°C. For RhoA staining, cells were fixed in PBS(−) containing 10% trichloroacetic acid on ice for 15 min. Fixed cells were permeabilized and blocked in PBS containing 0.1% saponin and 3% bovine serum albumin, and then sequentially incubated with a primary and secondary antibody for 1 h each. DNA was stained using 20–50 μg/ml propidium iodide (PI) for 30 min after treatment with 200 μg/ml RNase A. Confocal and Nomarski differential–interference–contrast (DIC) images were obtained using an LSM510 laser scanning microscope with a 40× 1.2 NA water- or a 63× 1.4 oil-immersion objectives (Zeiss).

To measure the mitotic index, cells were treated with 100 μM genistein or DMSO for 2 or 4 h, fixed with 4% paraformaldehyde for 20 min, and DNA was stained with 50 μg/ml PI for 30 min after treatment with 200 μg/ml RNase A for 30 min. The number of circular mitotic cells was counted. The mitotic index is defined as the ratio of the number of cells in the M phase to the total number of cells.

TIME-LAPSE RECORDING

Cells were cultured in 10 μM RO-3306 for 20 h to arrest cells at the G2/M border, washed with PBS(+), and released into pre-warmed fresh medium supplemented with 20 mM HEPES (pH 7.3). Glass-bottomed dish was placed on a 37°C preheated stage of an inverted Zeiss Axiovert S100 microscope with 20× 0.50 N.A. objectives and phase contrast images were monitored [Nakayama et al., 2009]. Time-lapse recording was started within 5 min after release from the G2 arrest. The time-lapse images were analyzed using ImageJ software (NIH, USA). Fluorescence time-lapse imaging was performed with the live imaging system (Operetta, Perkin-Elmer).

RESULTS

DELAY IN MITOTIC PROGRESSION BY GENISTEIN

To examine the effect of genistein on mitotic entry, asynchronous cells were treated with 100 μM genistein for 2 or 4 h. While mitotic index showed that mitotic cells were approximately 3% among DMSO-treated cells, that was <1% among genistein-treated cells, which is in agreement with a previous report [Matsukawa et al., 1993]. These results suggest that genistein may delay mitotic entry in asynchronous cells. To minimize the effect of genistein on the mitotic entry, cells were synchronized with the Cdk1 inhibitor, RO-3306, and during the release from the G2 arrest, cells were treated with genistein (Fig. 1B). In this protocol, control cells started cell division within 20 min (see Fig. 2A), and cells progressed through the M phase as previously reported [Nakayama et al., 2012]. Despite the presence of genistein, cells entered the M phase similar to control cells (data not shown, see Fig. 2A), indicating that genistein does not delay mitotic entry in cells synchronized at the G2/M border. Immunofluorescence staining for α-tubulin and DNA showed that at 2 h after the release from G2 arrest, control cells showed the interphase nuclei with condensed microtubule bundle that connects two daughter cells (Fig. 1C, cytokinesis, and D). These results suggest that most cells already exit mitosis after 2 h release from G2 arrest. In contrast, approximately half of the genistein-treated cells failed to align their

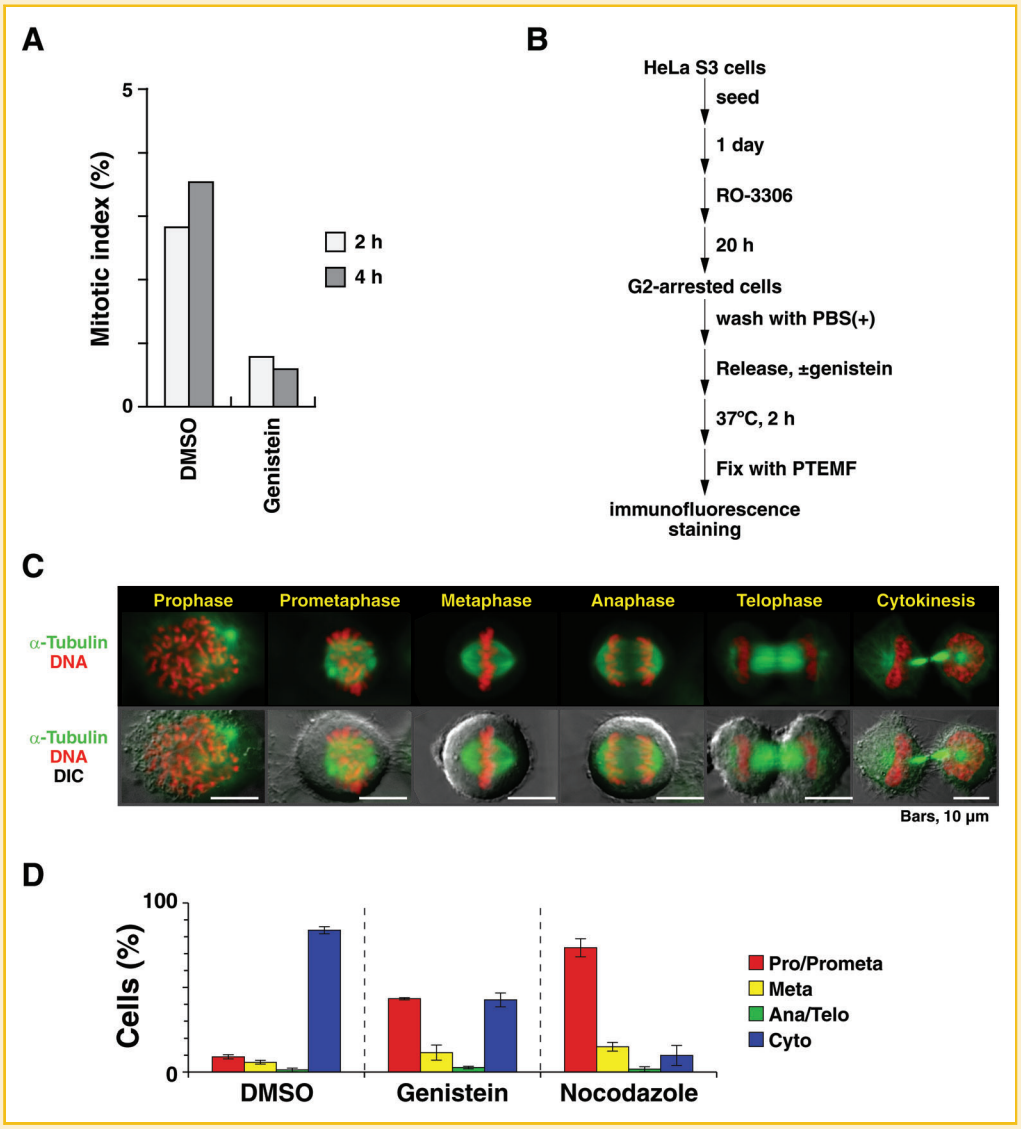

Fig. 1. Genistein inhibits M-phase progression. A: Asynchronous cells were treated with dimethyl sulfoxide (DMSO) or 100 μM genistein for 2 or 4 h, fixed and stained for DNA. Mitotic cells were counted based on their round shape and condensed chromosomes. These data are expressed as the ratio of the number of M-phase cells to the total number of cells ($n > 1600$). B–D: In B, schematic depiction of the synchronization method is shown. Cells were treated with 8 μM RO-3306, and G2-arrested cells were washed with PBS supplemented with Ca^{2+} and Mg^{2+}, released into fresh medium with or without genistein and fixed with PTEMF buffer after 2-h incubation. Fixed cells were stained for α-tubulin and DNA, and representative images of each mitotic phase are shown in C. Ratio of cells at each mitotic step in M phase was quantified (D). Nocodazole was used as a positive control to arrest at prometaphase. Differential–interference–contrast, DIC. Bars, 10 μm.

chromosomes at this time, like nocodazole-treated cells (Fig. 1C, prometaphase, and D), indicating that these cells are in prometaphase. These results suggest that genistein may delay M phase progression via affecting chromosome alignment.

GENISTEIN INDUCES CYTOKINESIS FAILURE

To measure the duration of M phase, time-lapse recordings were performed. Mitotic index showed that >80% of the cells entered mitosis within 20 min, and there was little difference between control and genistein-treated cells, confirming that genistein does not delay mitotic entry in cells that are synchronized at the G2/M border. In agreement with the results as shown in Figure 1, more than 50% of genistein-treated cells remained in the M phase at 3 h after being released from the G2 arrest, while control cells exited M phase within 2 h (Fig. 2A and B), suggesting that genistein-treated cells delay in M phase progression. Measurement of the duration of each mitotic phase revealed that anaphase onset was delayed in genistein-treated cells (Fig. 2C). In contrast, once cleavage furrow ingressed, genistein-treated cells completed the furrow ingression in kinetics similar to that in control cells (Fig. 2D), suggesting that the duration from anaphase onset to cleavage furrow ingression is not affected by genistein. Interestingly, phase contrast image showed that cleavage furrow was regressed in approximately 60% of genistein-treated cells, resulting in the formation of binucleated

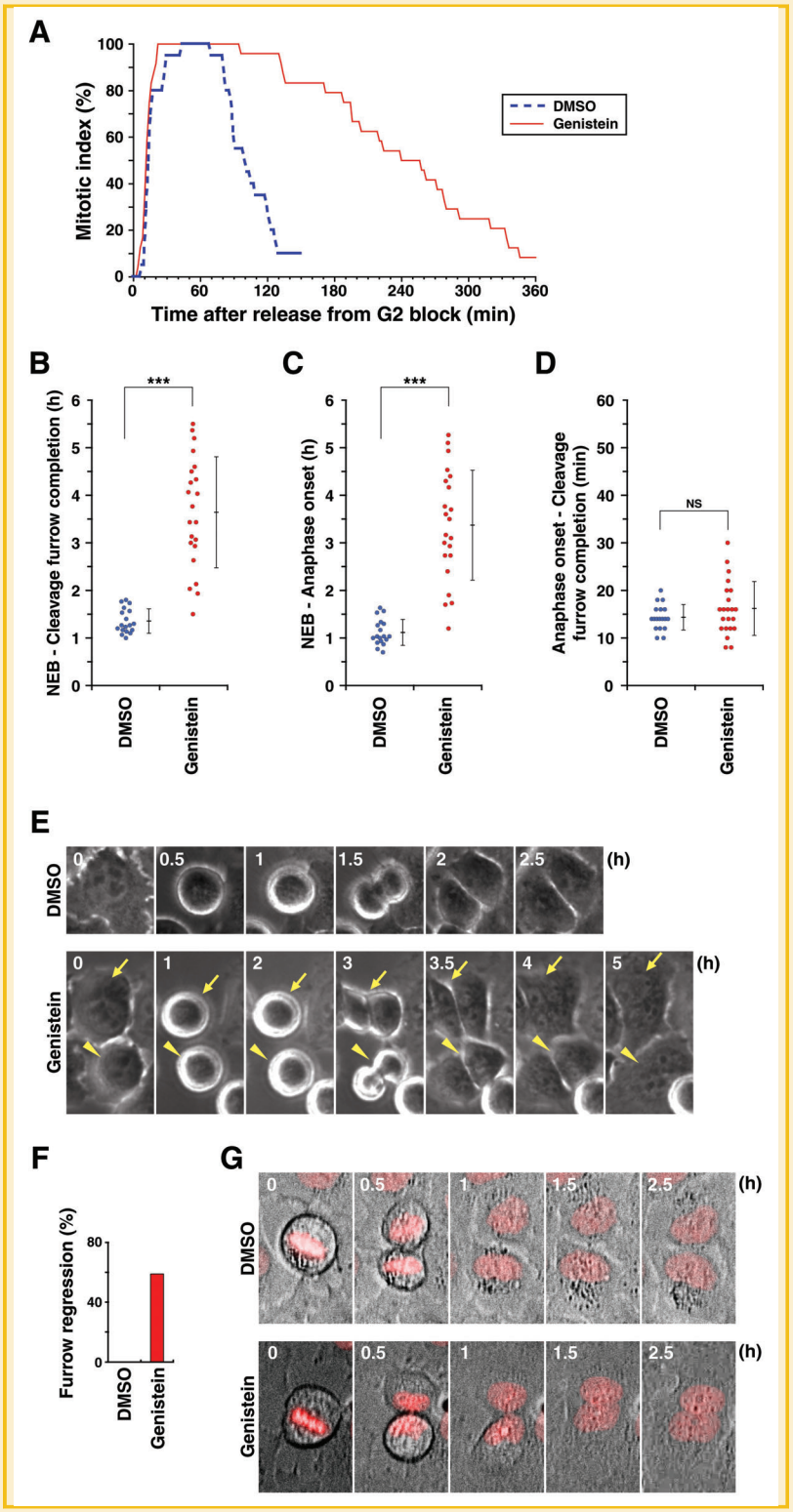

Fig. 2. Genistein delays anaphase onset and induces cytokinesis failure. Cells were arrested at G2 as shown in Figure 1, washed and released into pre-warmed fresh medium. Then, cells were observed under a phase contrast microscope, and mitotic entry and mitotic exit were judged from nuclear envelope breakdown and re-spreading of cells, respectively. A: The percentages of M-phase cells at the indicated times are shown (DMSO: n = 20; genistein: n = 24). B–D: Durations from nuclear envelope breakdown (NEB) to cleavage furrow completion (B), from NEB to anaphase onset (C), and from anaphase onset to cleavage furrow completion (D) were quantified. Error bars represent means ± SD. The asterisks indicate significant differences (***$P < 0.001$; NS, not significant) in Student's two-tailed *t*-test. E: Selected frames from phase-contrast images are shown. Arrows and arrowheads indicate cells in which cleavage furrow was regressed. F: The number of cells with furrow regression was counted, and the data are expressed as percentages to M-phase cells. G: HeLa S3 cells expressing histone H2B-mCherry were treated with genistein, and fluorescence time-lapse imaging was performed. Selected frames from mCherry (red) and bright-field images are shown.

cells (Fig. 2E, F, and G). These results suggest that genistein induces the delay in the anaphase onset via defect in chromosome alignment and results in cytokinesis failure, raising the possibility of a correlation between the cytokinesis failure and the defect in chromosome alignment.

MECHANISMS UNDERLYING THE DELAY IN M PHASE PROGRESSION AND CYTOKINESIS FAILURE

Because spindle has a critical role in cytokinesis and genistein inhibits tubulin polymerization [Mukherjee et al., 2010], we investigated the effect of genistein on spindle formation. Irrespective of genistein treatment, cells formed apparently normal spindles in metaphase cells (Fig. 3A). However, measurement of the distance between the two poles indicated that the spindle length was shorter in genistein-treated cells than in control cells (Fig. 3A–C). In addition, calculating the difference between the distance of the poles to their respective polar cortex (L_1 and L_2 in Fig. 3B) revealed that the spindles were not centered in genistein-treated cells (Fig. 3A, B, D). These results suggest that genistein treatment disrupts proper organization of the mitotic spindles.

Because central spindle formation is critical for the execution of cytokinesis, we next investigated whether genistein affected the formation of central spindle in anaphase. Synchronized cells in the M phase were cultured in the presence or absence of genistein and stained for α-tubulin. Quantification of fluorescence intensity for α-tubulin staining in anaphase cells showed that fluorescence intensities were lower in genistein-treated cells than in control cells (Fig. 3E), suggesting that the central spindle formation is inhibited by genistein [Raich et al., 1998; Mishima et al., 2002].

In the course of the analysis of anaphase cells, anaphase cells having a chromosome bridge were frequently observed. We thus examined whether genistein treatment induced a defect in chromosome segregation. Genistein-treated cells were stained for α-tubulin and DNA, and abnormal chromosome segregation, such as chromosome bridge (see Fig. 4B) and lagging chromosome, was counted in cells after the anaphase onset. While aberrant chromosome segregation occurred in <5% of control and nocodazole-treated cells, 52% of genistein-treated cells exhibited aberrant chromosome segregation (Fig. 4A, chromosome bridge, 43%; lagging chromosome, 9%), suggesting that genistein causes a failure of chromosome segregation. These data also suggest the possibility that cytokinesis failure upon genistein treatment may occur via the aberrant chromosome segregation.

Cytokinesis requires the contraction of actomyosin ring that is assembled at the equatorial cell cortex in a manner dependent on RhoA activity [White and Glotzer, 2012]. Thus, we examined whether RhoA was deregulated in genistein-treated cells. Immunostaining analysis of control cells showed that RhoA was present at the equatorial cell cortex in early anaphase, and subsequently accumulated at the cleavage furrow (Fig. 4B). In contrast, asymmetrical accumulation of RhoA was observed in genistein-treated cells having a chromosome bridge (Fig. 4B). These results suggest that the delocalization of RhoA may be involved in the cytokinesis failure in the chromosome bridge-containing cells.

DISCUSSION

In the present study, we show that genistein treatment causes aberrant cell division. Genistein causes the delay in anaphase onset and cleavage furrow regression. Cytokinesis failure is accompanied by the chromosome bridge formation and delocalization of the central regulator of contractile ring formation, RhoA. Our results provide the mechanism underlying genistein-induced polyploidization and one of the mechanisms for genistein-induced growth inhibition.

Mitotic entry was delayed upon genistein treatment of asynchronous cells (Fig. 1A). In contrast, after the release from RO-3306-induced G2 arrest, genistein treatment did not delay mitotic entry. Given that the inhibition of topoisomerase II provokes decatenation checkpoint and delays mitotic entry, decatenation would be completed during RO-3306-induced arrest at the G2/M border. In this case, genistein would not activate the decatenation checkpoint. In addition, it is unlikely that genistein treatment would suppress the protein levels of cyclin B1, Cdk1 and Cdc25, or increase the levels of p21 within the period between the release from G2 arrest and mitotic entry, while the changes in theses protein levels have been observed in genistein-treated cells using different experimental protocols [Davis et al., 1998; Frey et al., 2001; Kobayashi et al., 2002; Li et al., 2008]. Moreover, caffeine treatment is capable of overcoming genistein-induced G2/M arrest, suggesting that the activation of ATM pathway may be involved in the arrest through DNA damage response [Chang et al., 2004]. However, genistein treatment may be incapable of causing DNA damage in cells arrested at G2/M border.

After mitotic entry, proper organization of spindles was impaired in genistein-treated cells (Fig. 3), consistent with the report that describes the inhibition of tubulin polymerization [Mukherjee et al., 2010]. Although metaphase spindles appear to be normal in genistein-treated cells, the astral microtubules may be affected and this may lead to the shortening of spindle length and the aberrant positioning of spindles. Investigating the effect on the dynamics of astral microtubules will be required to understand the effect of genistein on spindle formation.

During the late phase of mitosis, chromosome bridge formation was observed in genistein-treated cells. The chromosome bridge could be a major mutational mechanism in colorectal cancer through cytokinesis inhibition, thereby inducing polyploidization [Mullins and Biesele, 1977; Shi and King, 2005; Stewénius et al., 2005; Weaver et al., 2006]. Chromosome bridge provokes the NoCut pathway that delays abscission timing [Norden et al., 2006]. During the delay, Aurora B prevents the cleavage furrow regression in chromosome bridge-containing cells until the chromosome bridges are resolved [Steigemann et al., 2009]. However, it has been reported that in topoisomerase II-depleted cells, Aurora B fails to relocate from the centromeres to the central spindle in late mitosis [Coelho et al., 2008], raising the possibility that genistein-induced inhibition of topoisomerase II activity [Markovits et al., 1989] may cause Aurora B delocalization. Aurora B is known to be required for Mklp1 localization, a component of the centralspindlin complex, at the spindle midzone [Severson et al., 2000; Giet and Glover, 2001; Hauf et al., 2003; Douglas et al., 2010]. Centralspindlin promotes the activation of small GTPase RhoA [White and Glotzer, 2012]. RhoA plays a role in the assembly and constriction of an actomyosin

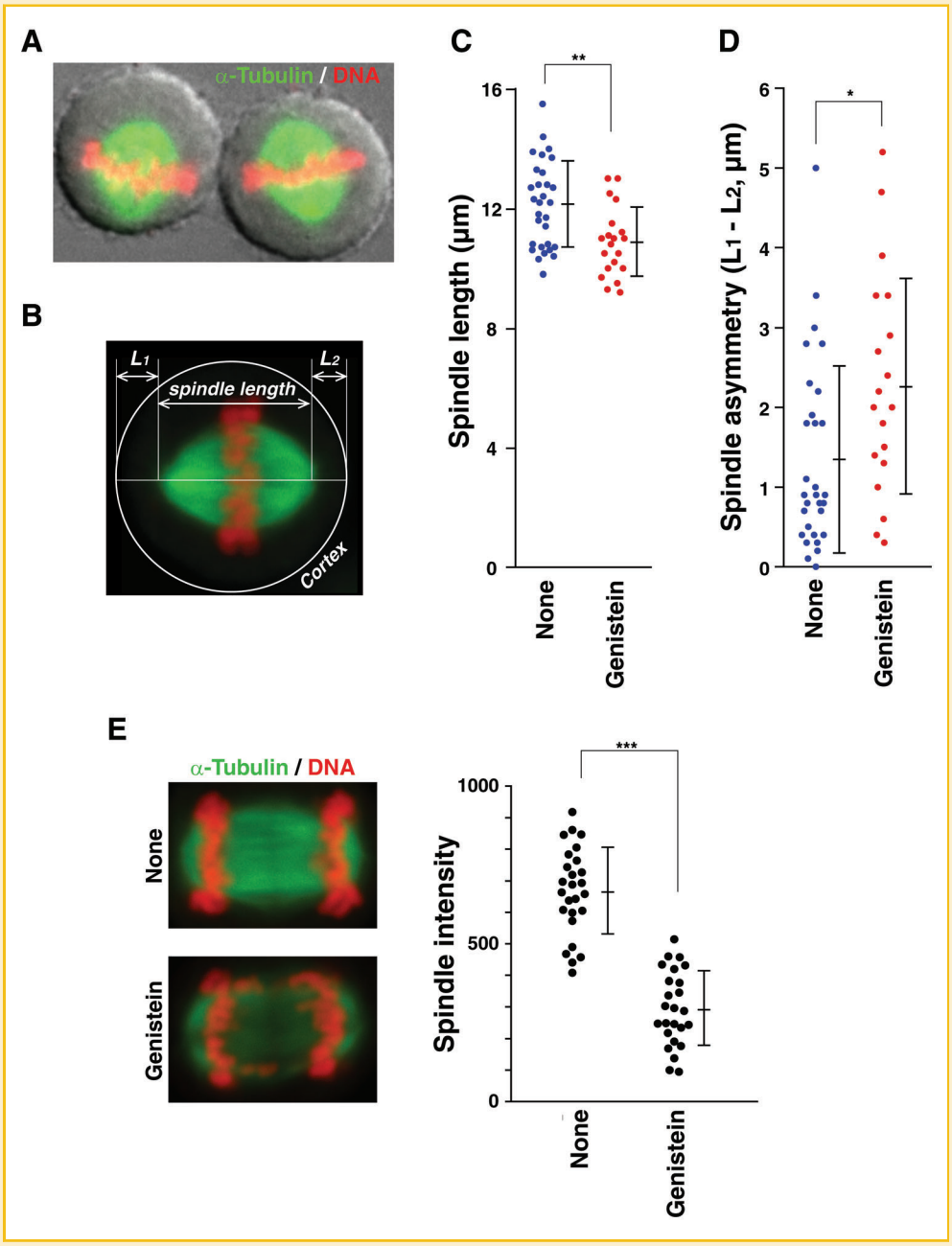

Fig. 3. Defects in spindle formation. A–D: Cells were arrested at G2 by incubation with 10 μM RO-3306, washed with PBS(+) and then incubated in fresh, pre-warmed medium at 37°C for 1 h (None) or 3 h (Genistein). Subsequently, cells were fixed and stained for α-tubulin and DNA, and the representative images are shown in A. In B, the method of quantification of spindle defects in metaphase is shown. L1 and L2 are distances of the poles to their respective polar cortex. The spindle length and the difference between L1 and L2 are shown in C and D, respectively. Error bars represent means ± SD. The asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$; DMSO, n = 30; genistein, n = 20) in Student's two-tailed *t*-test. E: Cells were arrested at G2 by incubation with 10 μM RO-3306, washed with PBS(+) and incubated for 45 min (DMSO) or 2 h (100 μM genistein) in fresh, pre-warmed medium at 37°C. Then, cells were fixed and stained for α-tubulin and DNA, and the representative images are shown left. The integrated fluorescence intensities of α-tubulin staining were measured and plotted right. Error bars represent means ± SD. The asterisk indicates significant differences ($^{***}P < 0.001$; none, n = 26; genistein, n = 25) in Student's two-tailed *t*-test.

contractile ring during cytokinesis, and thus RhoA is clearly required for cytokinesis [Jordan and Canman, 2012]. Therefore, genistein-induced topoisomerase II inhibition may disrupt the Aurora B–centralspindlin–RhoA pathway and cause the cleavage furrow regression. It is noteworthy that spindle microtubules dictate the site of cleavage furrow ingression by specifying RhoA activation [Bement et al., 2005; Murthy and Wadsworth, 2008]. Therefore, genistein-induced microtubule aberration may be involved in the RhoA delocalization (Figs. 3 and 4) and cleavage furrow regression. Taken together, genistein treatment may cause cytokinesis failure

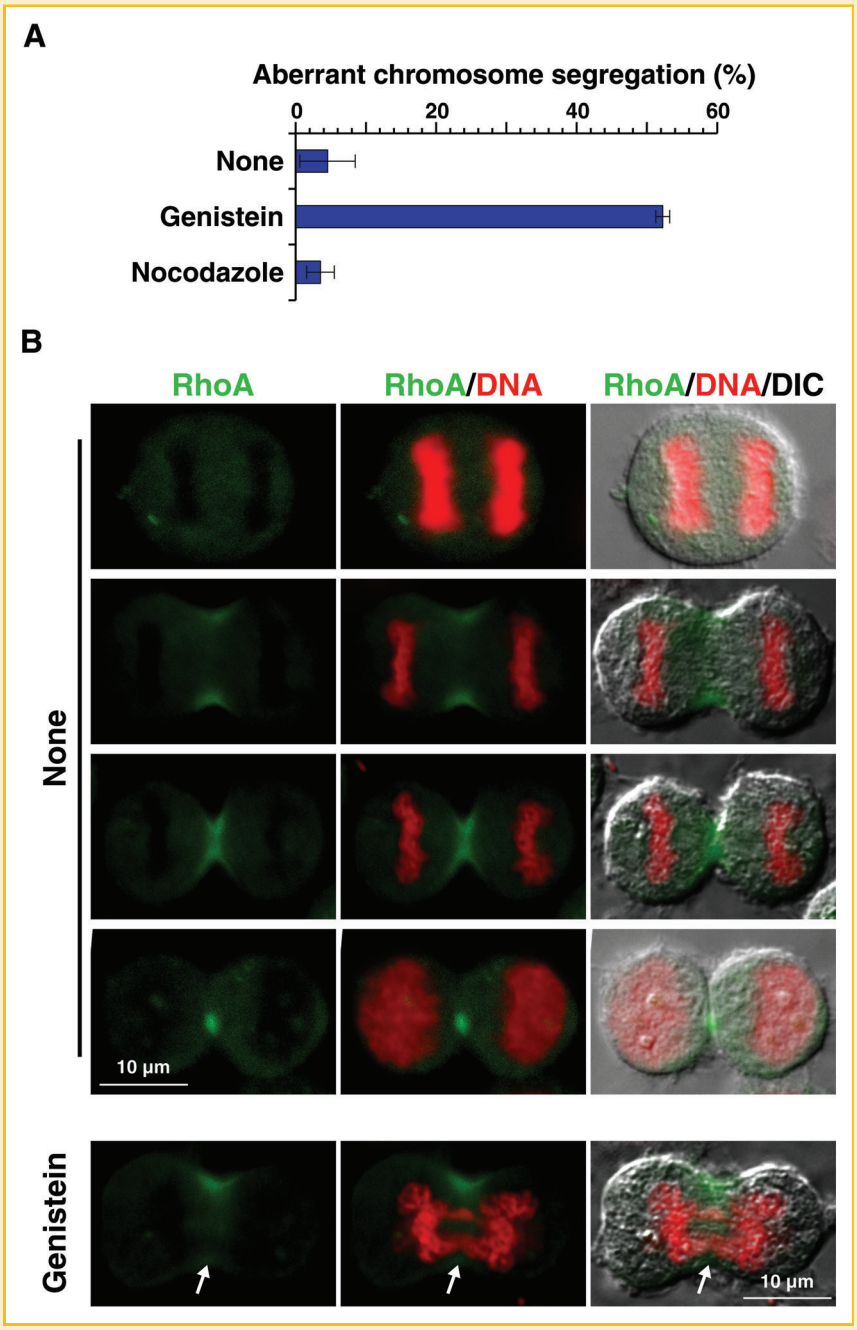

Fig. 4. Genistein induces aberrant chromosome segregation. A: Cells were treated with 8 μM RO-3306, and G2-arrested cells were released into fresh medium with or without genistein and fixed with PTEMF buffer after 2-h incubation at 37°C. Fixed cells were stained for α-tubulin and DNA, and the number of cells having aberrant chromosome segregation was counted. These data are expressed as percentages to cells in anaphase, telophase, and cytokinesis. B: Cells were treated with 10 μM RO-3306 for 20 h and G2-arrested cells were washed and released into fresh, pre-warmed medium. After 60 min or 200 min for control or 100 μM genistein-treated cells, respectively, cells were fixed with 10% trichloroacetic acid in PBS on ice for 15 min and stained for RhoA and DNA. Representative images of cells are shown. Arrows indicate a cleavage furrow where RhoA was not localized. Differential–interference–contrast, DIC. Bars, 10 μm.

through the induction of chromosome bridge formation that is accompanied by RhoA delocalization.

In conclusion, genistein causes cytokinesis failure through chromosome bridge formation and RhoA delocalization, leading to the generation of binucleated cells. Cutting of the chromosome bridge at abscission will result in DNA damage. Even if chromosome bridges were not cut, prematurely triggering abscission would fail and lead to cleavage furrow regression, generating binucleated cells. Binucleated, polyploid cells are usually eliminated by the p53-dependent pathway [Ganem et al., 2007]. Thus, genistein-induced defect of M phase progression may be one of the mechanisms behind the genistein-induced inhibition of cell proliferation via provoking DNA

damage response. Several derivatives have been generated from genistein as a candidate for anti-cancer drugs [Rusin et al., 2010]. Further investigation into the mechanisms of genistein-induced inhibition of cell proliferation may lead to more potent derivatives and more appropriate clinical use.

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