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Cell cycle progression after cleavage failure: mammalian somatic cells do not possess a “tetraploidy checkpoint”

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Failure of cells to cleave at the end of mitosis is dangerous to the organism because it immediately produces tetraploidy and centrosome amplification, which is thought to produce genetic imbalances. Using normal human and rat cells, we reexamined the basis for the attractive and increasingly accepted proposal that normal mammalian cells have a “tetraploidy checkpoint” that arrests binucleate cells in G1, thereby preventing their propagation. Using 10 μ M cytochalasin to block cleavage, we confirm that most

binucleate cells arrest in G1. However, when we use lower concentrations of cytochalasin, we find that binucleate cells undergo DNA synthesis and later proceed through mitosis in >80% of the cases for the hTERT-RPE1 human cell line, primary human fibroblasts, and the REF52 cell line. These observations provide a functional demonstration that the tetraploidy checkpoint does not exist in normal mammalian somatic cells.

Introduction

The purpose of mitosis is the division of a cell into two genetically identical daughters. However, a number of mitotic defects can abrogate the essential fidelity of the process. Some, such as naturally occurring chromosome monoorientation, can be remediated through the action of a checkpoint that stops mitotic progression until all kinetochores are attached to the spindle. Other defects, such as cleavage failure, are intractable. Cleavage failure can arise from a defective actomyosin cleavage apparatus, defects in the abscission of the midbody, chromosome bridging, merotelically attached chromosomes lodged in the cleavage furrow, and spindle malorientation (for review see Margolis et al., 2003; Storchova and Pellman, 2004). For mammalian somatic cells, cleavage failure is normally a rare event in culture (Piel et al., 2001) and is assumed to be a rare event in vivo. Nevertheless, if and when cleavage failure does occur, it is thought to be particularly dangerous to the organism because both the chromosome complement and centrosome number are doubled. Extra centrosomes greatly increase the chances that the subsequent mitosis will be multipolar and chromosomes will be unequally distributed to multiple daughter cells (for review see Brinkley, 2001; Nigg, 2002; Sluder and Nordberg,

2004). In addition, the extra complement of chromosomes increases the chances that some of the aneuploid daughter cells will have enough genetic information to be viable. Cleavage failure is acknowledged to be a significant (if not primary) source of centrosome amplification (Brinkley, 2001; Borel et al., 2002; Meraldi et al., 2002). Genomic instability brought on by unequal chromosome distribution is thought to be a major driving force in multi-step carcinogenesis (for review see Nigg, 2002; Sluder and Nordberg, 2004). Indeed, tetraploidization often precedes aneuploidy in solid tumors (for review see Nigg, 2002).

Given the perceived dangers of cleavage failure, it has been of interest to determine if there are mechanisms that block the proliferation of cells that fail cleavage and become tetraploid. The notion that cells have a p53-dependent checkpoint that blocks the propagation of cells that failed to divide originated with findings that continuous treatment of normal cells with cytochalasin to block cleavage leads to a cell cycle arrest (Carter, 1967; Wright and Hayflick, 1972; Hirano and Kurimura, 1974; Lohez et al., 2003). In addition, cells treated with microtubule inhibitors eventually adapt to spindle assembly checkpoint, exit mitosis without dividing, and arrest in G1 (Minn et al., 1996; Lanni and Jacks, 1998). Similar experiments on cells with a compromised p53 pathway revealed that they continue cycling (Minn et al., 1996; Lanni and Jacks, 1998; Andreassen et al., 2001). The most clear and most explicit demonstration of this checkpoint came from a report by Andreassen et al. (2001) that used dihydrocytochalasin B to inhibit cytokinesis in REF52 cells,

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Table I. Summary of results

Cell type	Cytochalasin D	Fibronectin	BrdU incorporation at indicated time			Mitosis observed within indicated time		
			Mononucleate	Binucleate	Time	Mononucleate	Binucleate	Time
	μM		%	%	<i>h</i>			<i>h</i>
hTERT-RPE1	10	–	81.8 (<i>n</i> = 677)	41.3 (<i>n</i> = 717)	@18	12/20	0/6	72
hTERT-RPE1	5	–	93.3 (<i>n</i> = 986)	44.0 (<i>n</i> = 851)	@18	34/37	0/12	72
hTERT-RPE1	5	+	93.0 (<i>n</i> = 888)	83.0 (<i>n</i> = 817)	@18	31/32	23/32	36
hTERT-RPE1	0.5	–	92.0 (<i>n</i> = 818)	77.0 (<i>n</i> = 861)	@18	40/41	13/14	36
hTERT-RPE1	100 (Blebbistatin)	–	ND	ND	–	30/32	20/20	18
REF52	0.5	–	84.7 (<i>n</i> = 819)	25.0 (<i>n</i> = 996)	@24	8/11	0/6	72
REF52	0.5	+	93.0 (<i>n</i> = 887)	85.3 (<i>n</i> = 874)	@24	23/25	12/13	48
Human primary fibroblast	0.5	–	92.7 (<i>n</i> = 921)	94.3 (<i>n</i> = 788)	@12	20/23	25/33	36
Human primary fibroblast	0.5	+	93.2 (<i>n</i> = 207)	97.5 (<i>n</i> = 200)	@12	24/27	13/13	14

BrdU incorporation: the percentage of mononucleate and binucleate cells that incorporated BrdU are averages of at least three separate experiments; “*n*” denotes the total number of cells counted. The times indicate when the coverslips were fixed after removal of cytochalasin or blebbistatin. ND, not determined. Mitosis: the number of mononucleate and binucleate cells entering mitosis within the duration of the cine records (hours after the removal of cytochalasin or blebbistatin). The number of cells entering mitosis is shown over the total number of cells followed by continuous time-lapse microscopy.

a primary rat fibroblast cell line. After drug removal, they found that the tetraploid cells arrested in G1, whereas many of the mononucleate cells in the same preparations continued cycling. By temporally separating the G1 arrest from the action of the drug, these workers provided evidence that the arrest was specific to the binucleate condition. Further indications that this arrest was due to failed cleavage came from observations that expression of a dominant-negative mutant p53 allowed the binucleates to undergo DNA synthesis.

The notion that normal mammalian somatic cells have a “tetraploidy checkpoint” that arrests binucleate cells in G1 after cleavage failure has been intensely attractive for many, including ourselves, because it provides a logical way for an organism to deal with a potentially dangerous and intractable situation (for review see Margolis et al., 2003). Not surprisingly, this proposed checkpoint has received considerable interest and has been reviewed as an established mechanism. However, the universal applicability of this checkpoint is brought into question by evidence that liver regrowth in living humans and rodents is due in part to the proliferation of multinucleate hepatocytes (for review see Fausto and Campbell, 2003).

Our interest in this checkpoint led us to initiate a series of experiments with telomerase-immortalized normal human cells (hTERT-RPE1) to determine what event or condition this checkpoint monitors. Using cytochalasin D at the concentration used by Andreassen et al. (2001) for dihydrocytochalasin B, we essentially reproduced their results. However, our finding that a significant percentage of the binucleates synthesized DNA prompted us to reexamine the link between cleavage failure and G1 arrest using lower drug concentrations and modification of substrate characteristics.

Results and discussion

hTERT-RPE1 cells

Asynchronous hTERT-RPE1 cells were treated with 10 μM cytochalasin D for 12 h, washed five times with fresh medium, and then put in medium containing BrdU. This concentration of cytochalasin D is same as that was used by An-

dreassen et al. (2001) for dihydrocytochalasin B. After removal of the drug, approximately half the cells were binucleate. To characterize cell cycle progression, some coverslips were fixed at 18 h after removal of the drug and assayed for BrdU incorporation. Other coverslips were mounted on chambers within 30 min of drug removal for continuous observation by time-lapse video microscopy of individual cells to determine what proportion of the mononucleate and binucleate cells entered mitosis. We found that 81.8% of mononucleate cells and 41.3% of binucleate cells incorporated BrdU (Table I). Time-lapse records revealed that 12 of 20 mononucleate cells went through a normal mitosis, whereas none of the six binucleate cells entered mitosis within 72 h of cytochalasin removal (Table I). The same phenomena were observed for REF52 cells even when we used a lower dose of cytochalasin to block cleavage (described later in the REF52 section). The fact that we can readily repeat the previous observations indicates that there is nothing peculiar about our culture conditions or the use of cytochalasin D that would somehow abrogate the proposed tetraploidy checkpoint.

The fact that 41% of the binucleate cells incorporated BrdU prompted us to investigate whether the extent of G1 arrest was influenced by the drug dosage. We halved the concentration of cytochalasin (5 μM) and found that a higher percentage of the mononucleate cells (93.3%) incorporated BrdU and entered mitosis (34/37; Fig. 1 A and Table I). Under these conditions, 44.0% of binucleate cells incorporated BrdU within 36 h, and none of the 12 followed initiated mitosis within 72 h.

Our finding that a higher percentage of the mononucleated RPE1 cells incorporated BrdU and entered mitosis at a lower cytochalasin dose raised the possibility that G1 cell cycle progression might be influenced by persistent actin cytoskeletal disruption that could perturb cell interactions with the substrate. When we cultured these cells on fibronectin-coated coverslips, we observed a dramatically different behavior for the binucleate cells even though the drug treatment (5 μM) was the same. On this substrate, 93.0% of the mononucleate cells and now 83.0% of the bi-

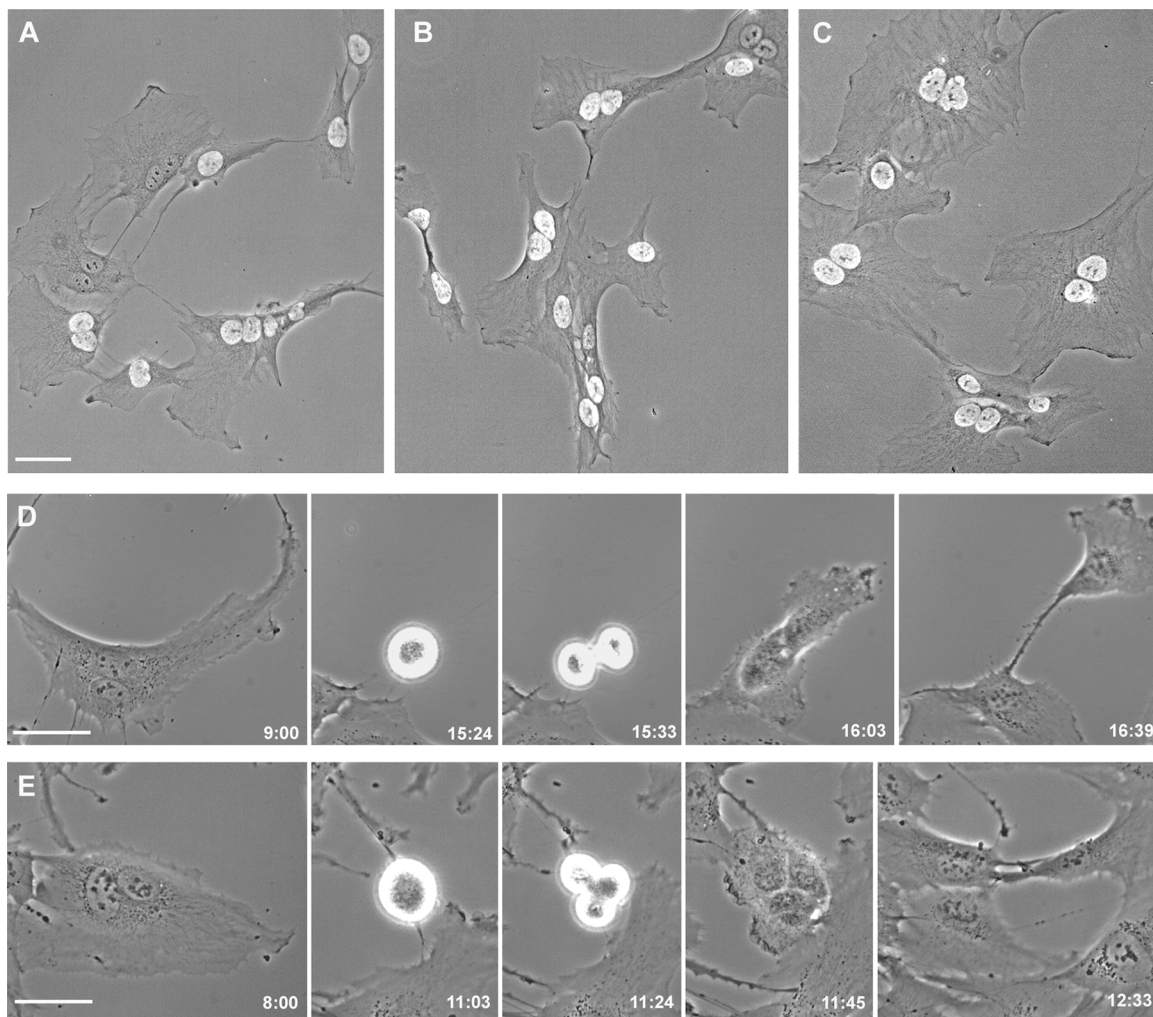


Figure 1. **hTERT-RPE1 cells.** (A–C) Overlaid phase and fluorescence images showing BrdU incorporation in mononucleate and binucleate cells. (A) Cells previously treated with 5 μM cytochalasin D and cultured on bare glass. Some binucleates have incorporated BrdU and others have not. (B) Cells previously treated with 5 μM cytochalasin D and cultured on fibronectin-coated glass. Binucleates have incorporated BrdU. (C) Cells previously treated with 0.5 μM cytochalasin D and cultured on bare glass. Both mononucleate and binucleate cells have incorporated BrdU. (D) Cells previously treated with 0.5 μM cytochalasin D to block cleavage and cultured on bare glass (images taken from Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). Frames from a time-lapse video sequence showing a binucleate cell entering and progressing through mitosis. This cell divides into two despite the presence of four centrosomes. (E) Cells previously treated with blebbistatin and cultured on bare glass (images taken from Video 3). Frames from a video sequence showing a binucleate cell entering and progressing through mitosis. Phase-contrast microscopy. Times are in h:min after drug removal. Bars, 50 μm .

nucleate cells incorporated BrdU (Fig. 1 B and Table I). 23 out of 32 binucleate cells followed went through mitosis within 36 h. Two showed tripolar divisions, and the remainder divided in a bipolar fashion despite the presence of four centrosomes.

We note that for 20 of the binucleate cells of this last dataset, we initiated time-lapse observations of individual mitotic cells just after addition of the cytochalasin at the start of the experiments. After 12 h, the cells under observation were circled on the coverslip with a diamond scribe and the drug was washed out before time-lapse observations resumed. We found that none of the 20 cells showed any signs of cleavage furrow formation (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>), yet 13 of these binucleates proceeded into mitosis. These observations indicate that the tetraploidy checkpoint does not monitor the formation of a cleavage furrow.

Next, we lowered the concentration of cytochalasin D to 0.5 μM , the minimum concentration able to inhibit cytokinesis in this cell line, and grew the cells on bare glass. Observations of cells undergoing mitosis in the presence of this lower drug concentration revealed that cleavage furrows often formed, but later regressed (Video 1). We found that 92.0% of mononucleate cells and 77.0% of binucleated cells incorporated BrdU at 18 h (Fig. 1 C and Table I). Time-lapse video analysis revealed that 40 of 41 mononucleate cells proceeded through normal mitosis within 36 h, as expected. Strikingly, 13 of 14 binucleate cells completed mitosis to form two daughter cells within this period (Fig. 1 D and Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). We note that a higher proportion of binucleates entered mitosis (13/14) by 36 h than incorporated BrdU (77%) at 18 h after cytochalasin removal. We think that this is due to some binucleates being slow to come

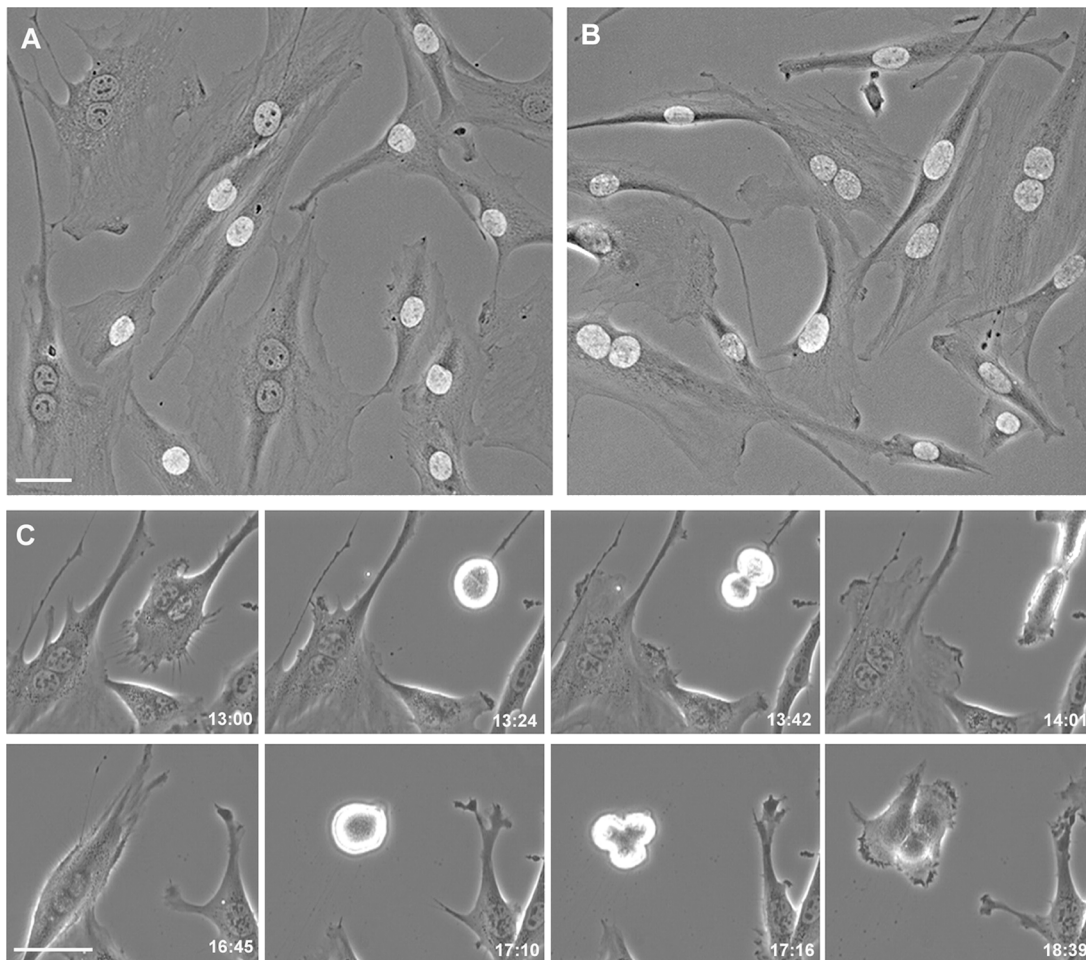


Figure 2. REF52 cells. (A and B) Overlaid phase and fluorescence images showing BrdU incorporation in mononucleate and binucleate cells. (A) Cells were previously treated with 0.5 μM cytochalasin D and cultured on bare glass. Mononucleate cells have incorporated BrdU, whereas the binucleates have not. (B) Cells treated with 0.5 μM cytochalasin D and cultured on fibronectin-coated glass. Both the mononucleate and binucleate cells have incorporated BrdU. (C) Cells previously treated with 0.5 μM cytochalasin D and cultured on fibronectin-coated glass (images taken from Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). Frames from a video sequence of two binucleate cells in the same field progressing through mitosis. The first to enter mitosis (top row) divides into two, whereas the second (bottom row) divides into three. Phase-contrast microscopy. Times are in h:min after cytochalasin D removal. Bars, 50 μm .

into S phase and thus not being counted as BrdU positive by the time the coverslips were fixed. The time-lapse records were run twice as long, and this extra time presumably allowed the slower binucleates to go through S phase and enter mitosis by the time the cine records were terminated. For BrdU incorporation assays, coverslips were fixed at 18 h because thereafter the fastest cycling binucleates undergo mitosis to form mononucleate cells. Together, these results indicate that the ability of binucleate cells to progress through interphase is influenced by the concentration of cytochalasin used to block cleavage, and progression through G1 is not dependent on the presence of fibronectin on the substrate.

Inhibition of cleavage by blebbistatin

As an alternative agent to block cleavage, we used the myosin II inhibitor (–)-blebbistatin (Straight et al., 2003). Asynchronous RPE1 cells, grown on bare glass, were treated with 100 μM (–)-blebbistatin and individual mitotic cells were observed by time-lapse cinematography for the 45-min duration of the treatment. After each cell was circled on the

coverslip with a diamond scribe, the drug was washed out and time-lapse observations were resumed. None of the cells exiting mitosis in the presence of the drug showed any furrowing activity (Video 1). Nevertheless, all 20 binucleates followed went through mitosis by 18 h after drug removal (Fig. 1 E and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). These results indicate that the previously observed progression of binucleate RPE1 cells through G1 was not due to unexpected side effects of cytochalasin that could putatively abrogate the tetraploidy checkpoint.

REF52 cells

Next, we wanted to address the theoretical concern that the hTERT-RPE1 cell line has a diminished response to cleavage failure or has somehow acquired properties that could conceivably abrogate the proposed tetraploidy checkpoint. Thus, we turned to the REF52 cell line originally used by Andreasen et al. (2001). Asynchronous REF52 cells were treated with 0.5 μM cytochalasin D for 4–12 h and were fixed for

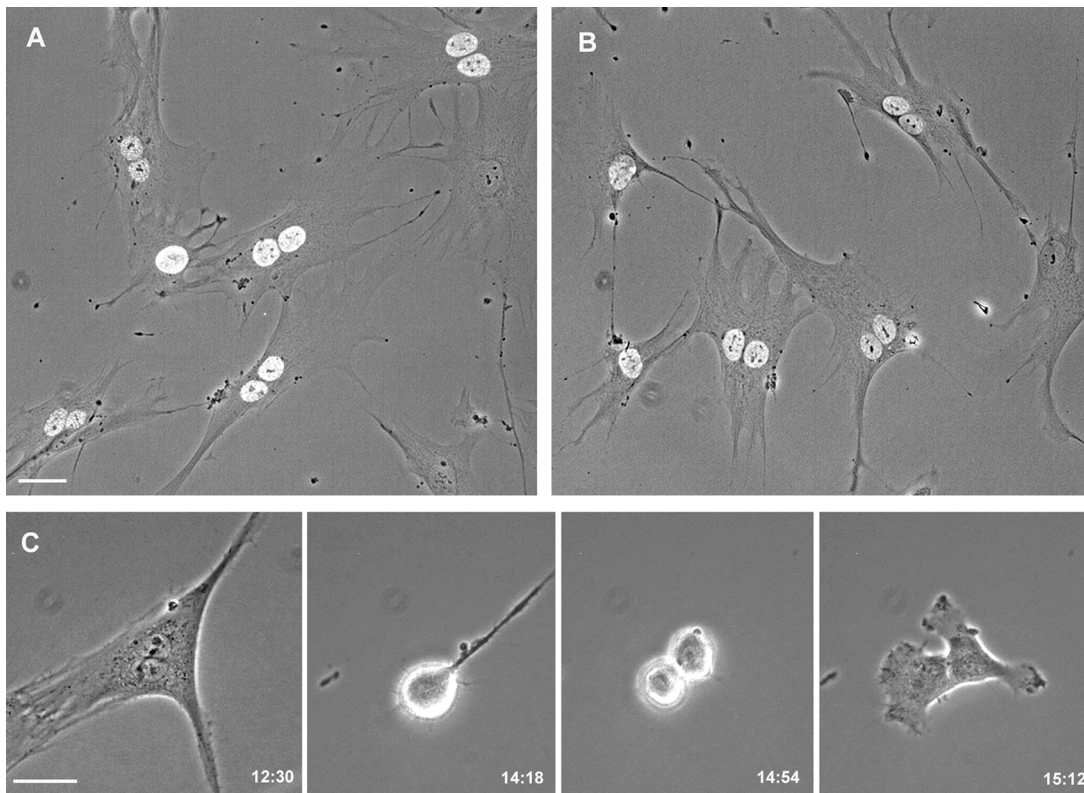


Figure 3. Primary human fibroblasts. (A and B) Overlaid phase and fluorescence images showing BrdU incorporation in mononucleate and binucleate cells. (A) Cells previously treated with 0.5 μ M cytochalasin D and cultured on bare glass. Binucleate cells incorporate BrdU. (B) Cells previously treated with 0.5 μ M cytochalasin D and cultured on fibronectin-coated glass. Binucleate cells incorporate BrdU. (C) Cells previously treated with 0.5 μ M cytochalasin D and cultured on bare glass (images taken from Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). Frames from a video sequence of a binucleate cell progressing through mitosis. Phase-contrast microscopy. Times are in h:min after cytochalasin D removal. Bars, 50 μ m.

analysis of BrdU incorporation at 24 h after cytochalasin removal; others were observed by continuous time-lapse microscopy. When cultured on bare glass, 84.7% of mononucleate cells and 25.0% of binucleate cells incorporated BrdU (Fig. 2 A and Table I). Time-lapse observations revealed that 8 of 11 mononucleate cells and 0 of 6 binucleate cells went through mitosis within 72 h. However, our results were quite different when the cytochalasin-treated cells were grown on fibronectin-coated glass. 93.0% of the mononucleate cells and now 85.3% of the binucleate cells incorporated BrdU by 24 h after cytochalasin removal (Fig. 2 B and Table I). Also, 23 of 25 mononucleate cells and all but one (12/13) of the binucleate cells completed mitosis within 48 h (Fig. 2 C, Table I; Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). As before, we think that a 48-h duration of the time-lapse records reveals the interphase progression of binucleates that were slow to enter S phase and thus were not counted as BrdU positive. Nine of the binucleates divided into two, and three divided into three daughters. The finding that almost all binucleate REF52 cells, under such conditions, enter mitosis indicates that rat cells and RPE1 cells have an equivalent response to cleavage failure.

Human primary fibroblasts

To avoid any further concerns about the peculiarities of immortalized cell lines, we turned to presenescent primary hu-

man foreskin fibroblasts that are normal in every respect, including p53 (Di Leonardo et al., 1994). These cells, obtained at population doubling 22, are competent for a total of 74 population doublings before the onset of senescence (American Type Culture Collection data sheet supplied with the cells). Asynchronous cultures (population doublings 23–50) were treated with 0.5 μ M cytochalasin D for 4–12 h to block cleavage. Some coverslips were fixed at 12 h after cytochalasin removal and other coverslips were used for time-lapse video microscopy. When grown on bare glass, 92.7% of mononucleate cells and 94.3% of binucleate cells incorporated BrdU within 12 h of cytochalasin removal (Fig. 3 A and Table I). Time-lapse observations revealed that 20 of 23 mononucleate cells and 25 of 33 binucleate cells entered mitosis within 36 h of cytochalasin removal (Fig. 3 C, Table I; Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>). 19 binucleates divided into 2, and the remaining 6 divided into 3 daughters. When these cells were cultured on fibronectin-coated coverslips, 93.2% of mononucleate cells and 97.5% of binucleate cells incorporated BrdU within 12 h of cytochalasin removal (Fig. 3 B and Table I). All 13 binucleates followed went through mitosis within 14 h (Table I). Six showed bipolar and seven showed tripolar divisions.

To determine if primary human cells have a transient but significant tetraploidy checkpoint, we analyzed video records

to compare interphase durations for binucleate and mononucleate cells. We defined interphase for binucleates as the time from removal of the cytochalasin to nuclear envelope breakdown because cells, regardless of nuclear number, are arrested in G1 while exposed to cytochalasin (Hirano and Kurimura, 1974; Bohmer et al., 1996; Lohez et al., 2003). For mononucleates, we used the time from nuclear envelope reformation to nuclear envelope breakdown as the duration of interphase. For fibroblasts cultured on bare glass, interphase averaged 18.5 h ($n = 18$) for the binucleates and 16.6 h ($n = 10$) for the mononucleates. For binucleates cultured on fibronectin-coated glass, interphase averaged 12.1 h ($n = 13$). Because almost all cells progress into mitosis, we conclude that even a 2-h prolongation of interphase is functionally insignificant and cannot represent a realistic mechanism to prevent the propagation of tetraploid cells.

Possible causes for binucleate cells arresting in G1

Our results with lowered cytochalasin D concentrations suggest that the G1 arrest in binucleate cells observed by a number of investigators (Carter, 1967; Wright and Hayflick, 1972; Hirano and Kurimura, 1974; Andreassen et al., 2001; Lohez et al., 2003), and ourselves for higher drug doses, may be due to subtle disorganization of the actin cytoskeleton that persists even when the drug is washed out. Such disorganization may induce the p38 and the SAPK/JNK stress-activated protein kinase pathways (Ailenberg and Silverman, 2003). The observation that the G1 progression of mononucleate cells is seemingly less sensitive than that of binucleate cells to higher doses of cytochalasin is a possibly informative phenomenon for which we do not have a definitive explanation. Whatever the difference may be, it must be subtle because immunofluorescence did not reveal any obvious differences in F-actin distribution between mononucleate and binucleate cells (unpublished data). Also, vinculin and phosphotyrosine immunofluorescence did not reveal any obvious differences in the number per unit area, size, or distribution of focal adhesions between mononucleate and binucleate cells (unpublished data).

Summary

Our results provide a functional demonstration that normal human somatic cells and rat cells do not possess a tetraploidy checkpoint that arrests the cell cycle in G1 after a mitosis in which cleavage fails. Thus, cleavage failure is still, in principle, a potentially dangerous event for the organism. Given the perceived dangers of cleavage failure, it is interesting that neoplastic transformation does not occur at a higher rate than it does during the many mitoses that take place during development, growth, and adult life of humans. This suggests that in the living organism, cleavage failure is either essentially nonexistent or not sufficient by itself to cause problems.

Materials and methods

Cells and cell culture

Telomerase-immortalized normal human cells (hTERT-RPE1) were obtained from CLONTECH Laboratories and were cultured in 1:1 DME and Ham's F12 media (Sigma-Aldrich). REF52 cells (a rat embryo fibroblast line) were obtained from Dr. Yu-Li Wang (University of Massachusetts, Worcester, MA) and were cultured in DME (Sigma-Aldrich). Human pri-

mary foreskin fibroblasts (BJ strain) were obtained from American Type Culture Collection (Manassas, VA) and cultured at <50 doublings in MEM (Sigma-Aldrich). All media also contained 12–25 mM Hepes, 10% FCS (Invitrogen), 100 U/ml penicillin G, and 100 μ g/ml streptomycin (Invitrogen). Time-lapse video analysis revealed that cell doubling times were 14–24 h for hTERT-RPE1 cells, 19–27 h for REF52 cells, and 16–21 h for human primary fibroblasts. Cytochalasin D (Sigma-Aldrich) and (–)-blebbistatin (Toronto Research Chemicals, Inc.) were used at the indicated concentrations by dilution of DMSO stocks. To terminate drug treatments, cells were washed with drug-free medium more than five times over a period of 30 min. 1 ml of 20 μ g/ml fibronectin solution (F1141; Sigma-Aldrich) was applied for 30 min at 37°C to cleaned 22 \times 22-mm coverslips previously treated with poly-L-lysine.

BrdU incorporation

After cytochalasin D removal, BrdU (Sigma-Aldrich) was added to a final concentration of 5 μ g/ml. After fixation in –20°C methanol, the cells were treated with 2N HCL at 22°C for 30 min, incubated with 1:500 mouse anti-BrdU antibody (Becton Dickinson), and labeled with 1:1,000 goat FITC-mouse antibody (Molecular Probes, Inc.). Observations were made with a microscope (DMR series; Leica) equipped for phase contrast and fluorescence. Images were recorded with a camera (Retiga 1300; Qimaging Corp.) and with QCapture software (Qimaging Corp.).

Time-lapse video analysis

Coverslips bearing cells were assembled into chambers (Hinchcliffe et al., 2001) containing cytochalasin D or (–)-blebbistatin at the indicated concentrations. Individual binucleate cells were circled on the coverslip with a diamond scribe and then followed at 37°C with Universal (Carl Zeiss MicroImaging, Inc.) or BH-2 (Olympus) microscopes equipped with phase-contrast or differential interference contrast optics. After 4 h cytochalasin D or 45 min (–)-blebbistatin exposure, the coverslips were washed with medium and time-lapse recordings were resumed. Some time-lapse observations began just after removal of the cytochalasin D. Images were recorded with Orca ER, Orca 100 (Hamamatsu Corporation), and Retiga EX or EXi (Qimaging Corp.) cameras; sequences were written to the hard drives of PC computers using C-imaging software (Compix, Inc.) or Slide-Book software (Intelligent Imaging Innovations) and were exported as QuickTime movies.

Online supplemental material

Time-lapse sequences of cells failing cleavage and binucleate cells progressing through mitosis are available at <http://www.jcb.org/cgi/content/full/jcb.200403014/DC1>.

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