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Report

Cells enter a unique intermediate 4N stage, not 4N-G_1, after aborted mitosis

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Abbreviations: ESC, embryonic stem cell; MS-cells, mitotic slippage cells; ED, early differentiated cell; CDK1, cyclin-dependent kinase-1 (cdc2); G_1MTC, G_1-phase microtubule checkpoint; SAC, spindle assembly checkpoint; Rb, rentinoblastoma protein; pRb, phospho-Rb; pp53, phospho-p53

Key words: embryonic stem cell, cell cycle, cell cycle checkpoint, polyploidy, flow cytometry, mitotic exit

It is widely accepted that mammalian cells enter the next G_1-phase (G_1) with 4N DNA after slippage from prolonged drug-induced mitotic block caused by activation of the transient spindle checkpoint. Understanding cell fate after mitotic slippage (MS) has significant clinical importance. The conclusion the MS cells enter 4N-G_1 is based on morphology and mitotic cyclin destruction. Definitive biochemical evidence for G_1 is scarce or unconvincing, in part because of methods of protein extraction required for immunoblot analysis that cannot take into account the cell cycle heterogeneity of cell cultures. We used single-cell-intracellular-flow-cytometric analysis to further define important factors determining cell fate after MS. Results from human and mouse embryonic stem cells (ESC) that reenter polyploid cell cycles are compared to human somatic cells that die after MS. We conclude that phosphorylation status of pRb, p53, CDK1 and especially cyclin B1 levels are important for cell fate decision in MS cells, which occur in a unique, intervening, non-G_1, tetraploid subphase.

Introduction

Chemotherapeutic strategies for treatment of malignant disease in humans depend on the premise that tumor cells are more sensitive to toxic/death-promoting effects of chemotherapeutic drugs and other modes of treatment, than are normal human cells. A detailed understanding of molecular events causing cell death, especially by apoptosis, in tumor cells and normal cells is vital for development of new drugs and improved strategies for treating malignancy. Molecular underpinnings of apoptosis and cell cycle regulation are mechanistically linked, co-regulated and are sometimes interdependent.[1-8] While much is known about molecules that cause cellular demise, events leading to cell-fate decisions to either proliferate, enter quiescence/differentiation, or initiate apoptosis after cellular stress (i.e., DNA or spindle damage), are still unclear.[5,8-10]

We evaluated molecular events involved in mitotic-spindle checkpoint (SAC) activation, adaptation (mitotic-slippage), mitotic-exit and cell death using multi-parametric permeabilized-cell flow-cytometry of human and mouse model somatic and embryonic stem cells (ESC). We report the unexpected finding that the tumor-suppressor protein, Rb, is phosphorylated on several regulatory sites and is abundant in somatic cells that have exited prolonged, but transient, mitotic block without cytokinesis and 4C DNA content (adaptation/mitotic-slippage). This finding contrasts with reports of Rb re-phosphorylation after mitotic-slippage that used immunoblotting techniques to assess Rb phosphorylation levels.[9] We offer possible reasons for these differences. We also report that these adapted cells maintain elevated levels of cyclin dependent kinase 1 (CDK1) in an activated-like state, while mitotic cyclins (A/B1) are undetectable, G_1-phase cyclin (D1) is considerably reduced, tumor suppressor, p53, is highly phosphorylated. These adapted somatic cells ultimately initiate cell death after prolonged mitotic block and mitotic-slippage. The results are compared to mouse and human ESC, which we recently demonstrated re-initiates DNA synthesis and continues cell cycle progression to aneuploidy/polyploidy without initiating apoptosis.[10] We recently showed that early differentiated cells derived from ESC behave like other normal somatic cells when confronted with prolonged mitotic block and adaptation, in that they initiate apoptosis instead of cell cycle progression to aneuploidy/polyploidy.[9] We now report that cyclin B1 levels increase greatly and earlier in pluripotent ESC compared to these differentiated cells, which have cyclin B1 cell cycle kinetics similar to other somatic cells.

Together, our data suggests a prominent role for cyclin B1 and activation status of CDK1 along with p53 and Rb phosphorylation status in cell-fate decisions to either continue cell cycle progression

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to polyploidy or to initiate cell death programs after prolonged mitotic block, mitotic exit and mitotic-slippage. Previous studies suggested this decision is made in cells that are in a 4C-G_1-phase-like state.[9] Our studies using single-cell flow analysis of cell cycle regulators, simultaneously with DNA content analysis, suggest for the first time, that somatic mitotic-slippage cells enter into a unique cell cycle state, distinct from G_1-phase or any other cell cycle state, which we propose is a sub-phase of M-phase, which we refer to as mitotic-crisis phase (M_c-phase). M_c-phase appears to be terminal in normal somatic cells and ESC-derived differentiated cells, but is not apparently terminal in human or mouse pluripotent ESC. The data suggest there may be an unexpected and uncharacterized "out of sequence" inactivation of Rb in somatic cells after mitotic-slippage that may play an important role in cell-fate decisions in this context. The hyper-phosphorylation of Rb in mitotic-slippage cells indicates these cells do not return to a "4C-G_1-phase-like state", which would contain hypo-phosphorylated Rb. Finally, our data could support a previously proposed notion of non-canonical cyclin A/B1-independent CDK1 activity that may be involved in initiating apoptosis after mitotic stress and failure, a state commonly induced by many chemotherapy drugs.[11]

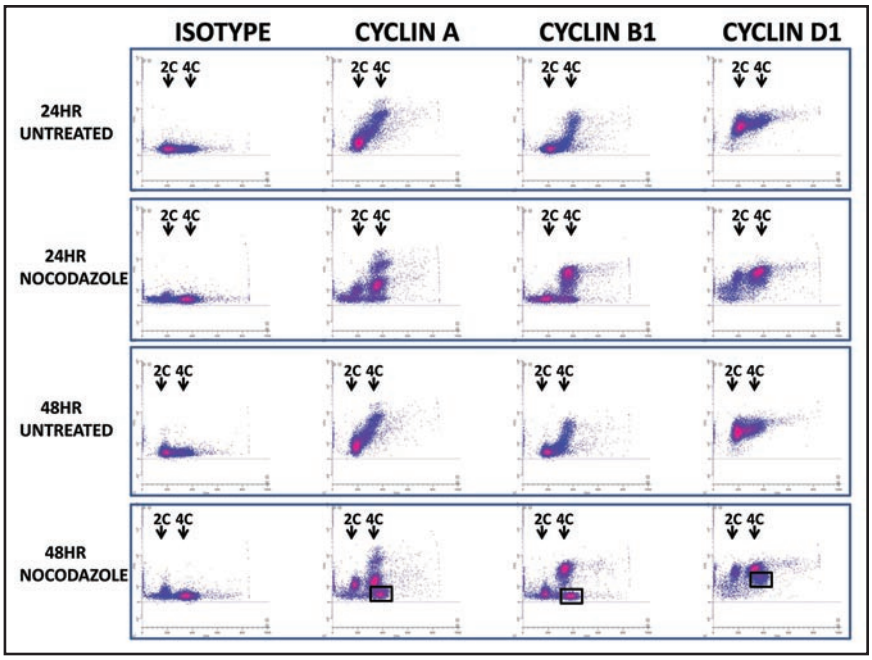

Figure 1. Effects of nocodazole on cell cycle kinetics of cyclins A, B1 and D1 in MO7e cells. Multi-parametric dot-plot/cell cycle analysis performed according to the methods and materials I shown. DNA content (2C and 4C) is on the X-axis and the indicated cyclin on the Y-axis. 24 and 48-hour time points were analyzed after the beginning of nocodazole treatment. Untreated control is treated with vehicle only. The squares in the lower denote the cells with shifted DNA content, or cells that have undergone mitotic slippage. Data represent reproducible results from more than six experiments.

Results

Nocodazole or paclitaxel were used to activate the mitotic spindle assembly checkpoint (SAC). Figure 1 shows cell cycle kinetics of cyclin A, B1 and D1 metabolism in growth factor-dependent human somatic MO7e cell and effects of nocodazole. Cyclin A was initially detected in G_1-phase (2C DNA content), increased during S-phase, and was maximal in G_2/M- phase (4C DNA content). Cyclin B1 was not detected until late S- or G_2-phase, which is consistent with canonical cyclin A, B1 cell cycle kinetics (i.e., cyclin A oscillations precede cyclin B1oscillations).[18] After nocodazole treatment, cells accumulated with 2C DNA content (at microtubule-dependent checkpoint in G_1 phase (G_1MTC) and with 4C DNA content (mitosis), with a pronounced reduction in S-phase cells, which we previously reported.[16,17] Cyclin A levels in 2C cells were low, but detectable, consistent with our previous identification of G_1MTC. Three main 4C populations were visible: (1) cells with maximal cyclin A levels, consistent with late G_2/mitosis; (2) cells with cyclin A levels similar to 2C cells; (3) cells with undetectable cyclin A that had an upward shift in DNA content which is likely due to increased propidium iodide staining when chromatin is decondensed (as opposed to chromosomal condensation).[17] Some cells also began a decline in DNA content to sub-diploidy from this 4C population; these are likely to be apoptosing cells. Cyclin B1 levels after treatment remained undetectable in 2C cells, while two main populations of 4C cells were observed: (1) cells with maximal cyclin B1 (consistent with metaphase arrest); (2) cells with undetectable cyclin B1. The 4C populations with no cyclin A or cyclin B1 that had shifted DNA content are consistent with our previous report indicating that these cells had decondensed chromosomes, allowing greater propidium iodide binding to DNA.[17] Again, there were cells with decreasing DNA content, likely apoptosing cells, which appeared to originate from the 4C-cyclin B1 negative population. Paclitaxel treatment produced nearly identical results (data not shown). Populations with shifted DNA content (decondensed chromosomes) increased in proportion at 48 hours with continuous treatment, compared to 24-hour treatment, suggesting these cells originated from 4C cells and accumulated over time (Fig. 1). The presence of the 4C-DNA-shifted (PI-shifted) cell population also appeared to influence mathematical modeling of cell cycle profiles adversely (Fig. S1). Typical models could be fitted with greater precision when the 4C-DNA-shifted population was excluded from analysis. The presence of 4C cells with decondensed chromatin is also indicated morphologically (Fig. S2). The presence of cells with condensed chromatin was lower at 48 hour nocodazole treatment than at 24 hours (Fig. S2A–C), even though the numbers of 4C cells was approximately the same (Fig. 1). The morphological progression in these non-adherent hematopoietic cells appears analogous to morphologies seen in adherent cells treated with similar agents where 4C cells exit mitosis, decondense chromosomes and become larger, flattened cells.[9]

We interpret these data to indicate expected metaphase arrest after SAC activation, with low cyclin A and high cyclin B1. However, also present were a large number of cells that apparently exited mitosis without cytokinesis (mitotic-slippage, adaptation), and that had decondensed chromatin, degraded cyclin A and B1, and contained 4C DNA content. We refer to these cells as mitotic-slippage (MS) cells. This is consistent with a previous report using mouse embryonic fibroblasts, in which mitotic-slippage is referred to as adaptation.[9] It is important to note that MS-cells do not

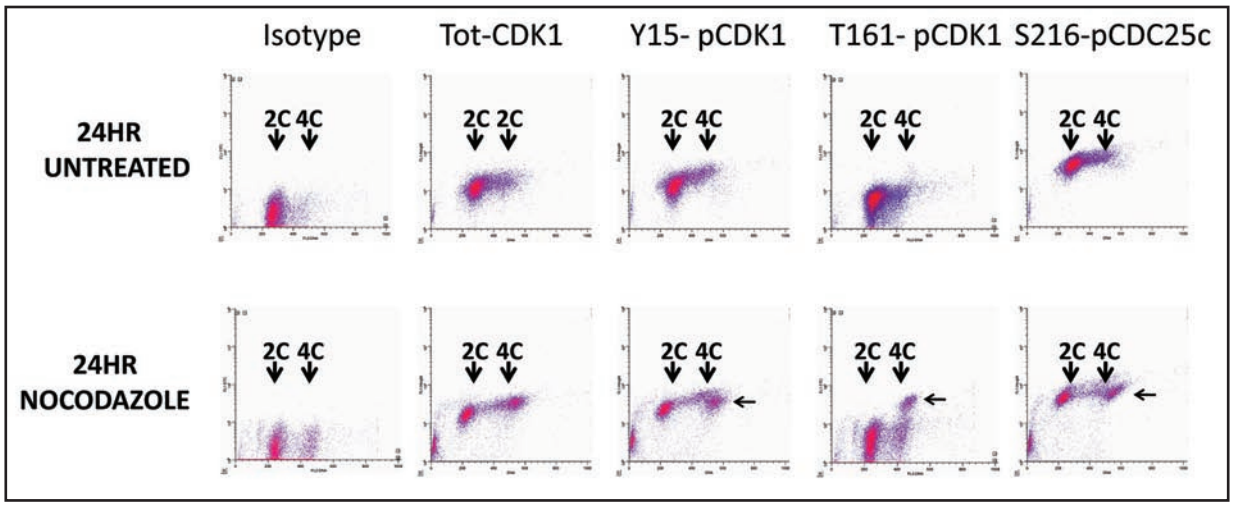

Figure 2. Effects of nocodazole on CDK1, CDC25c and their phosphorylation status in MO7e cells. Experiments were performed as in Figure 1 except antibodies to the indicated forms of CDK1 or CDC25c were used. Dot-plots are displayed the same as Figure 1. Small arrows denote the DNA-shifted (MS-cells) population. Data represent one of three reproducible experiments.

re-initiate cell cycle progression to 8C/polyploidy, but instead initiate cell death as indicated by lack of 8C cells and increase in hypodiploid cell (Figs. 1–3).

Our previous studies and those of others, demonstrated a requirement for a p53/p21$^{cip\text{-}1/waf1}$ axix, but not p27^{Kip1} tumor-suppressor in ensuring apoptosis after mitotic block instead of re-initiating DNA synthesis, cell cycle progression and aneuploidy/polyploidy.[5,9,15-19] P21 is an inhibitor of cyclin-dependent kinases (CDKs), including CDK1 (cdc2), a master regulator of the cell cycle whose expression levels and activation state we previously demonstrated are involved in the G_1-phase-microtubule-checkpoint (G_1MTC), and which is implicated in apoptosis after mitotic stress and mitotic slippage.[16-18,20-22] To gain insight into molecular mechanisms involved in sensing mitotic failure and subsequent cell-fate decisions, either to re-enter cell cycle progression or to initiate apoptosis after mitotic slippage, we investigated the levels and activation status of CDK1 in individual cells after nocodazole treatment.

MS-cells maintain CDK1 in an activated-like state. CDK1 is required for proper cell cycle progression to mitosis.[18] Extinguishing its activity and its destruction is required for regulating mitotic exit, proper chromosome segregation, cytokinesis and return to G_1-phase during normal cell cycles. The activation status of CDK1 depends on cyclin B1 binding, dephosphorylation of inhibitory phosphorylation sites at tyrosine-15 (Y15) and threonine-14, which blocks the ATP-binding/catalytic cleft, and also depends on activity-promoting phosphorylation at threonine-161 (T161), which is located in the "T-loop" and is involved in cyclin binding and substrate binding/selection.[23,24] T161 phosphorylation is regulated by CDK-activating kinase (CAK; CDC7/cyclin H).[25] Y15 phosphorylation is controlled by the CDC25 phosphatase family of enzymes, which are regulated by ATM-CHK1/2-CDC25c pathways in DNA damage checkpoints.[26-28] We previously provided evidence implicating p21$^{cip\text{-}1/waf1}$ in this pathway, and linked it to G_1MTC.[17] During those studies, we investigated levels and phosphorylation status of CDK1 and CDC25 in the context of G_1MTC, but not in the context of SAC or in MS-cells. CDK1 is also implicated in apoptosis regulation and is suggested to lie at a "crossroads" of cell cycle and apoptosis regulation.[20-22] This prompted us to investigate CDK1 phosphorylation status in the context of the SAC, mitotic slippage and MS-cells.

Figure 2 shows the effects of nocodazole treatment on CDK1 levels and phosphorylation state in individual MO7e cells. Total CDK1 levels increase beginning in late G_1-phase (at the G_1MTC) and in S-phase, and is maximum in G_2/M-phase in untreated cells.[16,17] After nocodazole treatment, total CDK1 levels increased slightly in MS-cells (as indicated by the upward DNA shift described previously), indicating that mitotic slippage does not result in destruction of CDK1 as it does in normal cell cycles during return to G_1-phase, or as it does for cyclin B1 oscillations in normal cell cycles, or in MS-cells (Fig. 1). Y15 phosphorylation of CDK1 increased in G_1-phase and was maximal in G_2/M-phase cells, but declined in MS-cells. T161 phosphorylated CDK1 was greatly increased in MS-cells compared to G_1 and G_2/M-phase cells. Moreover, pCDC25C was reduced in MS-cells compared to G_2/M phase cells, consistent with reduced Y15-CDK1. We interpret these results to indicate that CDK1 is in an "activated-like" state (T161phosphorylated CDK1 without Y15 phosphorylation) in MS-cells even though cyclins A and B1 are undetectable (Fig. 1). This is unusual because T161 phosphorylation and Y15 dephosphorylation of CDK1 during mitotic progression in normal cell cycles is usually associated with detectable levels of cyclin A and or B1. In this analysis, activated-like CDK1 is present in the absence of cyclins A/B1 in MS-cells, which are cells apparently destined for death.

MS-cells contain abundant levels of highly phosphorylated Rb. The retinoblastoma tumor-suppressor Rb, binds to and suppresses the transcriptional regulator E2F in G_1-phase cells.[29,30] During growth-factor stimulation and induction of proliferation, Rb is highly phosphorylated which prevents its suppression of E2F, allowing transcriptional activation of S-phase cyclins and numerous other cell cycle related genes associated with restriction-point transition in G_1-phase.[30] In our previous studies, we noted that Rb was highly phosphorylated in 4C cells after nocodazole treatment, but the significance of this was unknown at that time.[17] This finding was unusual, as Rb function is usually associated with G_1-phase to

S-phase transition.[29,30] Because E2F also transcriptionally regulates apoptosis related genes, we hypothesized this somewhat anomalous behavior might be involved in the decision to initiate apoptosis after failed mitosis in MS-cells. We asked if phospho-Rb (pRb) was present in MS-cells or in metaphase cells after nocodazole treatment. The data in Figure 3 demonstrate that pRb (phosphorylated on several sites) increases during G_1-phase and further increases slightly during S-phase and G_2/M-phases where it is maximal, consistent with its known role in normal cell cycle.[30] This is also consistent with our previous study showing that phosphorylation of Rb occurs after the G_1MTC.[17] After nocodazole treatment, pRb was highly increased in the MS-cell population, much more so than in the G_2/M-phase population. We conclude that Rb is highly phosphorylated in MO7e cells destined for cell death after mitotic failure and mitotic slippage due to prolonged mitotic block.

MS-cells contain high levels of phosphorylated p53. The tumor suppressor, p53, is implicated in apoptosis and cell death decisions.[31] The p53-p21 axis has also been implicated in regulating apoptosis or quiescence after failed mitosis.[5,7] Thus, we evaluated p53 and phospho-p53 (pp53) in MS-cells after nocodazole treatment. Figure 4 shows that total p53 levels begin increasing in G_1 phase and reach maximum levels in G_2/M phase during normal cell cycles. After nocodazole treatment, total p53 levels were elevated in 4C cells (likely metaphase-arrested cells), but then drop in MS-cells to levels below that observed in untreated 4C cells. Levels of phospho-(serine15)-p53 (S15pp53), which is located in the MDM2/4-binding domain, increased during G_1 phase and was maximum in 4C cells.[31] After nocodazole treatment, some 4C cells had reduced levels of S15pp53, which also contained decreased DNA content, which may represent apoptosing cells. Importantly, S15pp53 was highly elevated in MS-cells (in squares). We also investigated S20pp53 and it's levels appeared to decrease substantially during G_1 phase in untreated cells and this level was maintained during S and G_2/M phases but was increased dramatically in MS-cells.

S37pp53 was investigated, and behaved similarly to S15pp53 in untreated cells. However, after nocodazole treatment, MS-cells had reduced levels of S37pp53, but these levels were not reduced to those observed in G_1 phase cells. Altogether, we interpret these data to suggest that, even though total p53 levels were decreased in MS-cells compared to 4C/G_2/M cells, pp53 levels, especially S15/20-pp53were remarkably increased in MS-cells. Thus, p53 phosphorylation status, as well as total p53 levels, could be involved in the decision to initiate cell death in MO7e cells after mitotic slippage.[5,7,31]

Human MS-embryonic stem cells express high levels of cyclin B1, while ESC-derived, somatic-like early-differentiated cells do not. We recently demonstrated that human and mouse ESC fail to initiate apoptosis after failed mitosis, manifest aberrant mitotic exit in response to nocodazole treatment, and then re-initiate cell cycle progression to become polyploid.[10] In contrast, both differentiated mouse embryoid body cells and human ESC-derived, early differentiated (ED) cells, which behave like other somatic cells, including MO7e, do initiate apoptosis after nocodazole treatment, do not re-initiate cell cycle progression, and do not become polyploid. We proposed that apoptosis and the SAC were "uncoupled" in ESC.

Using human ESC as a model of a cell that has a different fate in the post-mitotic exit population other than apoptosis (i.e., polyploidy), we asked if somatic cell-like, differentiated human ED

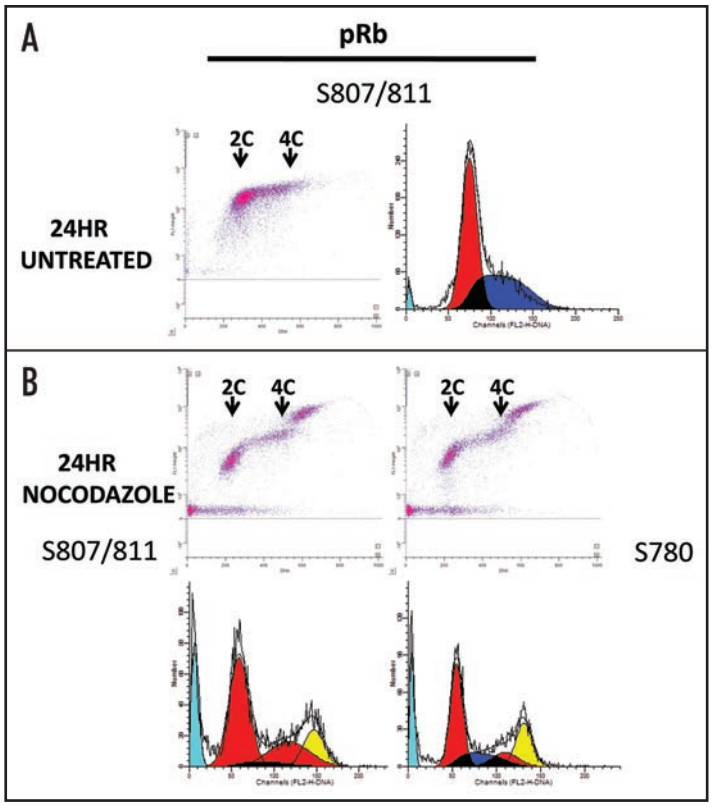

Figure 3. Effects of nocodazole on Rb phosphorylation in MO7e cells. Experiments were performed and data displayed as in Figure 1, except antibodies to various forms of Rb were used. Cell cycle profiles are also shown. Data represent reproducible results from more than three experiments.

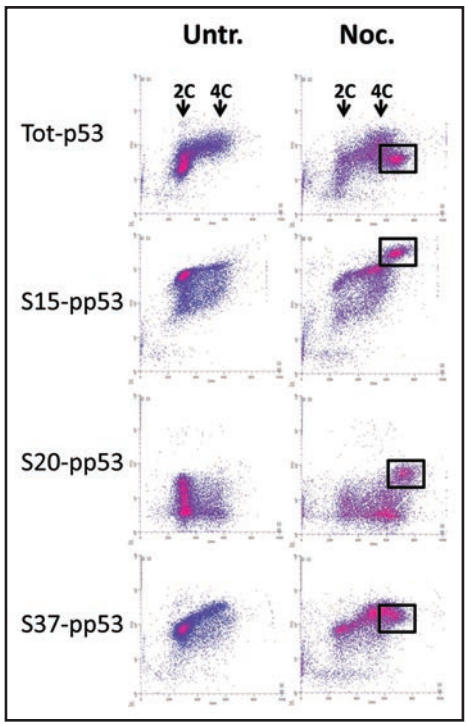

Figure 4. Effects of nocodazole on total and various forms of phosphorylated p53 in MO7e-MS-cells. Experiments were performed and data displayed as in Figure 1, except antibodies to various forms of p53 were used. Small boxes denote the population with shifted DNA content or the MS-cell population. Data represent one of 2 reproducible experiments.

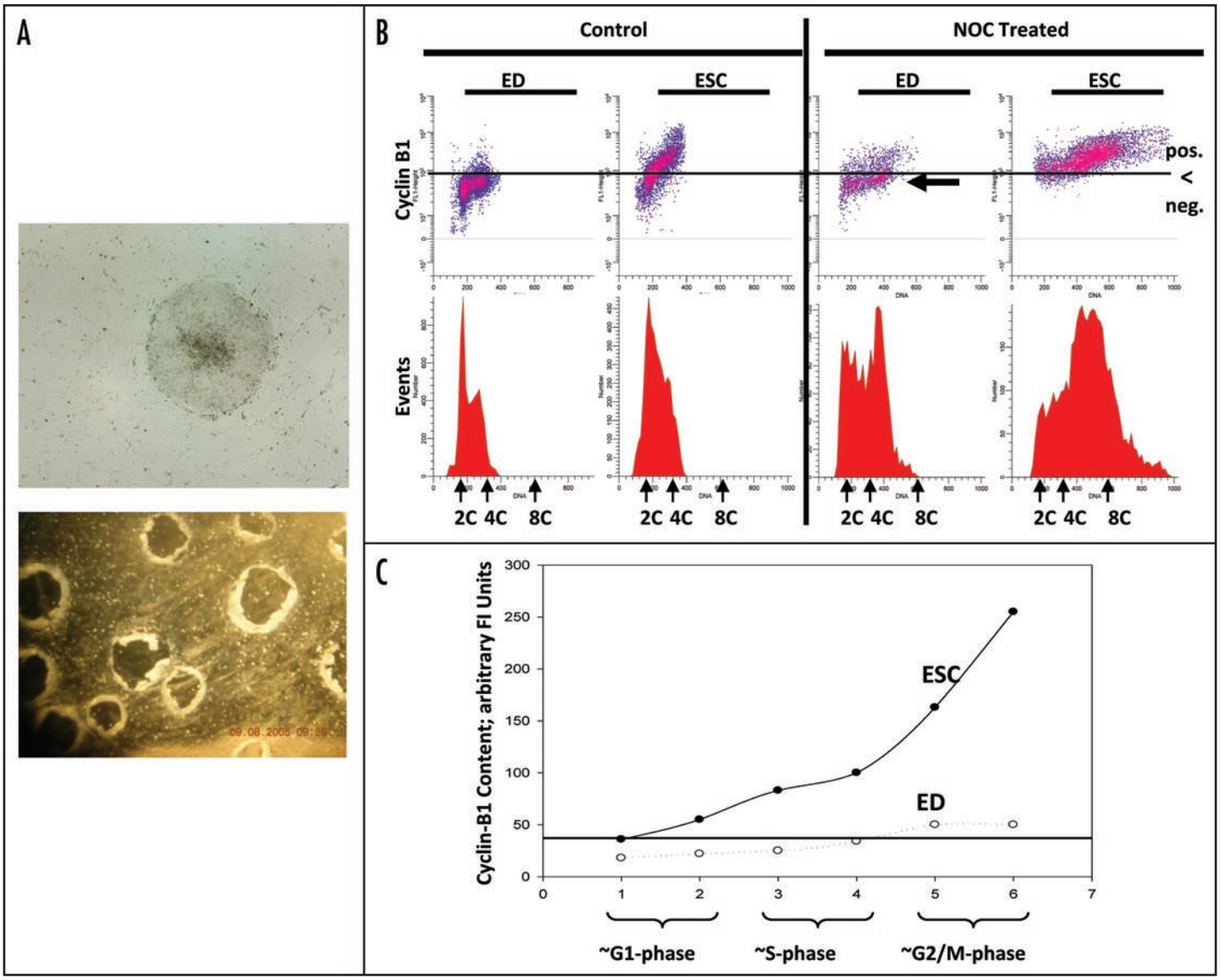

Figure 5. Effects of nocodazole on cell cycle kinetics of cyclin B1 in human ESC and ESC-derived early-differentiated cells. Typical colony appearance of MI01 human ESC and micro-dissection harvest of individual colonies (A). Colony cells were disrupted to single cell suspensions for flow cytometric staining was done according to materials and methods and flow cytometry was done as in Figure 1 (B, upper). Gating of two populations ED (differentiated cells) and ESC was done using laser light scatter properties as we have previously described (9). Arrow denotes somatic MS-cells (see text). Cell cycle profile of the same data in shown in the lower. Cyclin B1 data in (B) was subdivided and gated into a 6 equal region array based on DNA content. The average cyclin B1 level in each region (corresponding to different parts of the cell cycle) are plotted against DNA content (C). The data represent one of two reproducible experiments.

initiated cell death from mitotic-slippage.[10] This would be similar to human MO7e somatic cells. We also asked if pluripotent human ESC pass through mitotic slippage state en-route to polyploidy. We used cyclin B1 analysis to address this question because of its ability to differentiate subpopulations of 4C cells in nocodazole-induced mitotic block and exit, which is an important defining characteristic of the MS state.

Figure 5A shows an example of a human ESC colony (upper) maintained as we previously reported.[10] The lower is a picture of colonies harvested by manual micro-dissection as we reported. Figure 5B demonstrates that in untreated ED cells, cyclin B1 behaves similarly to that in MO7e cells, and is reduced to undetectable levels in 4C, nocodazole-treated cells before initiating cell death. Surprisingly, cyclin B1 in pluripotent ESC behaved more like cyclin A during untreated cell cycles in somatic cells; i.e., beginning its increase in late G_1 phase. Remarkably, cyclin B1 levels dropped little in nocodazole-treated 4C ESC and continued to rise as cells reinitiated aberrant cell cycle progression to become polyploid. We compared the kinetics of cyclin B1 oscillation in ESC to that in ED cells. Figure 5C demonstrates that cyclin B1 remained undetectable during ED cell cycles until late G_2-phase, while it increased sharply in late G_1 phase in ESC, and rose to levels greater than 4 times those observed in ED cells even when in G_2/M phase.

Together, these data raise the possibility that the differences in cell cycle regulation between human pluripotent ESC compared to somatic-like human ED cells could, in part, be credited to the expression and timing of cyclin B1.[32] The data also suggest that failure of human ESC to degrade cyclin B1 totally during mitotic slippage

and transition to aneuploidy/polyploidy could be linked to their failure to initiate cell death and continue aberrant cell cycle progression.

Polyploid mouse ESC express high levels of p53 after nocodazole treatment, but without subsequent increase in S15pp53 in MS-cells. MS-MO7e cells had a pronounced increase in pp53 (Fig. 4), especially that of p53 which was phosphorylated on serine-15. Because human ESC cells had very different patterns of cyclin B1 oscillations compared to MO7e and ED (somatic) cells, we evaluated if mouse pluripotent ESC also had a different pattern of pp53 expression compared to MO7e cells. Figure 6, upper left shows the typical cell cycle pattern of asynchronous mouse ESC in culture (almost all either G_1- or S-phase). The lower confirms the aneuploid/polyploid nature of nocodazole-treated mouse ESC. Total p53 is abundant in unperturbed mouse ESC cell cycles and is maximal in G_2/M phase. In contrast to nocodazole-treated MO7e MS-cells, which had reduced levels of p53 compared to 4C-G_2/M phase cells (Fig. 4), there was no reduction of total p53 in mouse ESC in 4C cells, nor while cells were in transit to polyploidy. Overall, there was an increase in total p53 after nocodazole treatment. Importantly, 4C mouse ESC do not have an abrupt increase in Ser15pp53, in marked contrast to MO7e MS-cells after nocodazole treatment. This suggests that, both, total intracellular levels of p53 as well as phosphorylation on ser15 are very different in 4C mouse ESC, which are not activated towards cell death, but which will become polyploid, compared to MO7e MS-cells which do activate cell death and do not become polyploid, thus implicating p53 and its phosphorylation status in post-mitotic-slippage cell fate decisions.

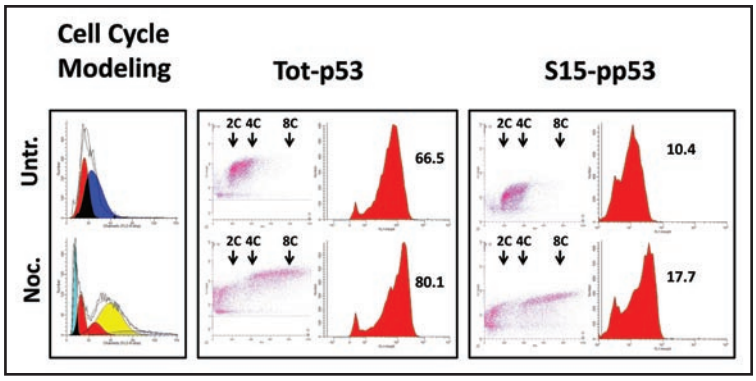

Figure 6. Effects of nocodazole on cell cycle kinetics of total or S15 phosphorylated p53 in mouse ESC. The influence of nocodazole treatment on mouse Esc cell cycle profile is shown in the left. Red is G_1 or G_2/M populations, blue is S-phase populations, yellow and hatched are aneuploid/polyploid populations respectively. Cell cycle kinetics of total or S15-pp53 is shown in middle and right. Dot-plots were obtained the same as in Figure 1. Relative frequency histograms indicating overall expression levels are to the right of the dot-plots with the average in bold text. Data represent one of 2 reproducible experiments.

Discussion

Understanding molecular processes that occur during cellular fate choices after stress, such as occurs during chemotherapy, is vital for improving current therapeutic strategies and developing new drugs. Many of the most effective chemotherapeutic drugs induce mitotic stress in rapidly proliferating cells like those found in tumors and this frequently results in mitotic failure, forcing tumor cells to choose between cell death or entering quiescence/differentiation, or polyploid cell cycles. The latter can ultimately lead to aneuploidy, a hallmark of human tumor cells.[33] A small percentage of cells in rapidly dividing cell populations is believed to experience spontaneous mitotic failure resulting in abnormal tetraploidy.[34] If not eliminated by cell death (apoptosis), these cells have an increased potential to undergo other tumorigenic changes. Whether aneuploidy results from tumorigenesis or plays a more causative role is still a matter of great debate.[34]

In this report, we investigated the behavior of some cell cycle related proteins involved in cell fate decisions after mitotic stress induced by prolonged microtubule disruption that results in mitotic slippage. We applied single-cell flow-cytometric techniques to quantitate these behaviors, especially as it relates to decisions to induce cell death or aneuploidy/polyploidy. A combination of model cell systems allowed us to investigate molecular differences between model somatic cells, which usually initiate cell death after mitotic failure, and that of pluripotent embryonic stem cells, which we showed evades cell death for a number of cell culture passages and continues polyploid cell cycles under such conditions.

We present evidence that model human somatic cells in culture enter a state with decondensed chromosomes and 4C DNA content after failed cytokinesis and mitotic slippage, but which also lacks some of the hallmarks of G_1-phase cells. This supports and extends similar observations made in somatic embryonic fibroblasts.[9] We observed that it is from this state of decondensed chromosomes with 4C DNA content that most somatic cells die, and not from metaphase arrest caused by microtubule disruption. This unique cellular state may represent a fundamental nexus in a cell's fate choice to initiate cell death, or continue in aberrant cell cycles or differentiate. This uniqueness is attributable, in part, to the unusual abundance of CDK1 in an activated-like state (reduced Y15 phosphorylation and highly increased T161 phosphorylation) with no detectable activating mitotic cyclins A or B1. Cells in this state also contain unique patterns of p53 levels and phosphorylation status along with hyperphosphorylated (inactivated) Rb, which differs from typical G_1-phase cells. To our knowledge, this is the first report of such a cellular condition. We would like to introduce the term, mitotic crisis sub-phase or M_c-phase, which differs from a 4C-G_1-phase cell. Just as G_0-phase refers to a 2C cell status outside of active cell cycle that can be induced into one of three fates (progress into cell cycle, differentiate or die), M_c-phase refers to a 4C cell outside active cell cycle that can be induced into one of the same three fates. While G_0-phase may be considered a "normal" part of a somatic cell's life, M_c-phase would be an abnormal part, requiring differentiation or death as a normal outcome, but with cell cycle progression as an abnormal outcome. Mutation or deregulation of several tumor-suppressor and checkpoint proteins commonly found in tumor cells have been shown to cause this kind of abnormal outcome in response to chemically-induced mitotic stress.[5,7] Apparently, very primitive embryonic stem cells do not follow the same constraints as model somatic cells with respect to mitotic slippage. They appear naturally to behave more like cells with mutant tumor-suppressor/checkpoint proteins and tumor cells. Further experiments investigating CDK1 enzymatic activity in purified (sorted) MS-cells as well as confirmatory studies using immunoblotting, will shed light on this situation, but it could be suggested from our data that MS-cells contain a "non-canonical",

cyclin A/B1-independent CDK1 kinase activity. Other, non-mitotic cyclins (not cyclin A or B1) have been reported, which appear to shift the substrate repertoire of CDK1 (11). For example, the Speedy/Ringo family of non-cyclin CDK binding proteins, which can activate CDK1 and CDK2, causes CDK2 to phosphorylate a non-canonical subset of substrates, including non-cyclin A/CDK2 sites in CDC25.[11,32] The exact function of these non-cyclin CDK activators is unknown, but recent evidence implicates them in cellular responses to DNA damage, including apoptosis and checkpoints.[36] Detecting the presence/absence of these non-cyclin CDK1 binding partners in MS-cells will be important future experiments. A potentially new and different set of CDK1 substrates, ones related to apoptosis initiation in which CDK1 is already implicated, might be the preferred substrate set in MS-cells.[20-22] This hypothetical substrate repertoire shift could be facilitated by the absence of cyclin B1 in favor of binding alternative pro-apoptotic binding partners. This could also be related to another unique observation, which is the highly phosphorylated status of Rb in MS-cells. We previously reported a population of cells with 4C DNA content and very high hyperphosphorylated Rb, which we believed at that time to be M-phase cells.[17] We now show that these are MS-cells. To our knowledge, this is the first report of Rb phosphorylation specifically during mitotic slippage. Rb is generally not considered a substrate for CDK1 during M-phase, but considering the unusual status of MS-cells, it is possible that Rb could act as a substrate under these conditions. Phosphorylation-dependent inactivation of Rb suppression of E2F family transcription factors would be consistent with mitotic slippage and decondensation of chromosomes, thus allowing transcription to be re-initiated, as already poignantly stated which is an important fact often overlooked.[5,7] While interesting, the specific function of Rb inactivation in MS-cells is unknown. However, apoptosis-mediating genes such as caspaces can be activated by E2F.[37,38] Release of Rb suppression of E2F could be important in the decision to initiate cell death in MS-cells. Additionally, there is strong evidence suggesting that cooperation in the Rb and p53 pathways (see below) is very important for cell-fate determination after cell cycle checkpoint arrest.[39]

Our results are not entirely consistent with those reported for NIH 3T3 cells.[9] In that study, 3T3 cells were synchronized and released in the presence of nocodazole, which was then associated with chromosome decondensation, 4C DNA content, cellular adhesion changes and cyclin B1 decreases; these cells were referred to as adapted cells, which appears to be similar, if not identical, to our MS-cells. However, immunoblot analysis showed adapted cultures contained increased levels of hypo-phosphorylated Rb, which is consistent with a return of the adapted and adherent cells to a G_1-phase-like state, and which is different from our findings (Figs. 1, 3 and 5). The reasons for this disparity are unclear, but we offer several potential explanations for these differences. Besides the obvious differences in model cells used (i.e., human vs. mouse; fibroblast vs. hematopoietic cells), we note an important difference in the details of techniques that were used compared to our methods. First, such studies utilize adherent cells in contrast to our MO7e cells that are non-adherent. Adherent MS-cells require trypsinization for detachment and biochemical analysis, after nocodazole treatment. It is very common to remove culture medium and wash adherent cells with PBS to remove serum proteins, which would prevent efficient trypsinization and detachment. Cells arrested in mitosis by nocodazole are rounded and weakly attached or even detached completely (which can be used to advantage in these experimental strategies; i.e., mitotic shake off). The washing could easily remove these weakly adherent cells, and if not collected and combined with trypsinized cells, could easily introduce unnoticed bias by depleting mitotic cells from the harvest, and influence the total amount of measured proteins from batch lysis followed by immunoblotting. Our model obviously avoids such problems. Second, even "synchronized" cell cultures are rarely fully synchronized, and often contain complex mixtures of important sub-populations of cells that cannot be distinguished after batch lysis prior to immunoblot analysis, (i.e., adherent G_1-phase cells are also still attached along with adherent MS-cells). Synchronization techniques often rely on various cell cycle checkpoints that can introduce artifacts that may confuse interpretations, in part because cells often emerge from checkpoint release asynchronously (i.e., from G_1 phase). In the 3T3 study, this issue was partially addressed by using micro-videography of individual cells, but very few cells were actually analyzed. Our intracellular flow-cytometry based "individual" cell analysis avoids such problems. Also, contributions of Rb to bulk cell lysates from dying or apoptotic cells could be significant, a caveat circumvented by single cell methods. All of these issues could help explain the differences we observe in Rb phosphorylation status in MO7e MS-cells compared to that described using NIH3T3 cells. It should also be noted that quiescence as a potential outcome in nocodazole-treated MO7e cells was not addressed in our study and could also contribute to our different observations. However, all our observations of various protein content can be directly linked to DNA content and cell cycle phase in the same individual cell, which is a great advantage.

We also demonstrate, for the first time, that human ESC contain very high levels of cyclin B1 compared to early differentiated ED cells within the same colonies. While cyclin B1 levels remain high in ESC after nocodazole treatment, it is degraded to undetectable levels in ED cells. This is similar to that observed in MO7e cells (Fig. 1). Because human (and mouse) ESC do not initiate apoptosis after failed mitosis and subsequently become polyploid, differences in cyclin B1 in pluripotent ESC compared to somatic-like ED cells (and somatic MO7e MS-cells) could be important in regulating these two different cell fates after failed mitosis.[10] This possibility is bolstered by studies demonstrating potent anti-apoptosis properties of overexpressed cyclin B1 and pro-apoptosis properties of cyclin B1 suppression.[40] It is possible that high cyclin B1 in ESC maintains CDK1 (and it's substrate repertoire) "poised" for a rapid return to mitotic progression (as is typical in ESC) and helps maintain ESC in an apoptosis-checkpoint "uncoupled" state as we have already proposed.[10] This also suggests that cyclin B1 does not need to be completely degraded for mitotic exit and return to G_1-phase in ESC. On the other hand, complete cyclin B1 degradation in MO7e- and ED-MS-cells could allow the previously mentioned "unconventional" CDK1 activity (alternative CDK-binding partners) to contribute to apoptosis initiation, and may help explain these two very different cell fate outcomes in response to mitotic-slippage. Analysis of CDK1 enzyme activity is usually done using "mitosis-related" substrates, like histone-H1. It now appears important to determine if CDK1 has a shift in substrate repertoire in MS-cells destined for cell death and when bound to non-cyclin partners, perhaps including pro-apoptotic proteins like BAD.[22]

The tumor suppressor, p53, is one of the most intensively studied proteins because it is mutated or otherwise dysfunctional in most human tumor cells. P53 plays an extremely important role in decisions to initiate apoptosis after cell cycle checkpoint activation, including failed mitosis after prolonged, but transient, SAC activation.[5,7] We now report that p53 levels are reduced in MS-cells compared to those in mitosis during unperturbed cell cycles, but the levels of phosphorylated p53, especially those of Ser15 and Ser20, are extremely elevated in MS-cells. Ser 15/20 phosphorylation inhibits MDM2/4 binding, which occurs in the TAD domain N-terminal regions.[31] Phosphorylation in this domain (S15, 20) is thought to be important for p53 stability and should lead to increased levels of cellular p53. Our findings would appear in to be conflict with this pattern, but much is still unknown about the regulation of cellular p53 levels and what role different levels have in tumor suppression, apoptosis and cell cycle control. Paradoxical interpretations of how phosphorylation and MDM2/4 binding influences p53 stability/levels in cells is not without precedence, especially when comparing in-vitro based predictions of in-vivo behavior.[31] For example, a conundrum was revealed when it was found that reducing MDM2 levels stabilized total p53 levels, but did not increase p53 activities. New evidence emerged potentially resolving this paradox; MDM-2 auto-degradation and MDM2-dependent MDM4 degradation has now been described, which supports a new model of p53 regulation where total p53 levels may not necessarily reflect various p53 functions at all.[31] It is not yet known how these very complex and entangled regulatory pathways of p53 relate to our observation that p53 phosphorylation in the TAD/MDM2/4 binding domain is temporally linked to heightened cell death, while its abundance is reduced. However, there is one clue to this. Cells from mice expressing p53 with targeted mutation at residues Ser18 and 23 (Ser 15, 20 in humans) display a nearly normal phenotype with respect to p53 stability, transactivating activity and cell cycle control, but have very little or no pro-apoptotic capacity.[31] In addition, tumor suppression capacity appears to be substantially diminished in these mice as they develop a wide spectrum of tumors. Therefore, these post-translationally modifiable residues appear extremely important for p53-mediated apoptosis, but in a way separate from its other functions. This could have important implications for our observations that abundant phospho-p53 (in the TAD domain) exists in cells destined to induce cell death (MS-MO7e cells), while it is not found in the same abundance in cells (pluripotent ESCs) that do not induce apoptosis and reenter polyploid cell cycles under the same conditions.

Methods and Materials

Cells and treatments. MO7e is a factor-dependent human myeloid leukemic cell line which is used as a model human somatic cell. MI01 cell line is a human ESC.[10,12] Within MI01 colonies, there are spontaneous "early differentiated" (ED) cells (in previous paper, named 'ESC-A') identified by flow cytometry.[10] ED cells have a phenotype similar to other primitive differentiated somatic cells and are used as a model of primitive human somatic cells. E14 is a mouse ESC line and is used as model of mouse ESC.[10,13] All cell lines were cultured and manipulated as previously described and were treated with either nocodazole (Sigma Chemical Co. St. Louis, MO) or Paclitaxel (Taxol; Sigma).[10,12,14-17]

Flow cytometry. Flow cytometry analysis was done using FACScan (Becton-Dickenson, Inc.; San Jose, CA) or LSRII (Becton-Dickenson) flow cytometers.[10,15-17] Data was analyzed with WinList and ModFit programs (Verity Software House; Topsham, ME).[10] All primary antibodies were from Cell Signaling Technology (Danvers, MA) unless otherwise stated. Fluorescent-labeled (FITC) secondary antibodies were from Pharmingen (San Jose, CA) and were used as described.[10,15-17] FITC-labeled antibodies to cyclin A, cyclin B1 or cyclin D1 were from Pharmingen.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/MantelCC7-4-Sup.pdf

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