Chromosomal Instability Induced by Pim-1 Is Passage-dependent and Associated with Dysregulation of Cyclin B1*

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Overexpression of the oncogenic serine/threonine kinase Pim-1 has been shown to induce chromosomal missegregation and polyploidy in prostate epithelial cell lines (1). Here we demonstrated that Pim-1-induced polyploidy develops in a passage-dependent manner in culture consistent with a stochastic mode of progression. Induction of chromosomal instability by Pim-1 was not restricted to prostate cells as it was also observed in telomerase-immortalized normal human mammary epithelial cells. Elevated levels of cyclin B1 protein, but not its messenger RNA, were evident in early passage Pim-1 overexpressing cells, suggesting that increased cyclin B1 levels contribute to the development of polyploidy. Furthermore, regulation of cyclin B1 protein and cyclin B1/CDK1 activity after treatment with anti-microtubule agents was impaired. Small interfering RNA targeting cyclin B1 reversed the cytokinesis delay but not the mitotic checkpoint defect in Pim-1 overexpressing cells. These results indicated that chronic Pim-1 overexpression dysregulates cyclin B1 protein expression, which contributes to the development of polyploidy by delaying cytokinesis.

The serine/threonine kinase Pim-1 is an oncogene first identified as a frequent proviral insertion site in Moloney murine leukemia virus-induced lymphomas (2–4). Several lines of evidence implicate Pim-1 in the tumorigenic process, including lymphoma development in Eμ-Pim-1 transgenic mice, the striking cooperativity of Pim-1 with oncogenes like Myc and Gf1 in T-cell lymphomagenesis (5–8), and the overexpression of Pim-1 in a significant proportion of human prostate tumors (9, 10). Pim-1 is known to be a downstream effector molecule of cytokine signaling pathways since its expression is directly regulated by several cytokines, growth factors, and transcription factors such as STAT (signal transducers and activators of transcription). The diverse nature of its substrates identifies Pim-1 as a regulator of apoptosis, cell cycle, and possibly gene transcription (11).

Considering its oncogenic potential in a number of tumor types (9, 12), the role of Pim-1 as a cell cycle regulator is of particular interest. Proper regulation of the cell cycle is crucial for the maintenance of genomic integrity. aberrant cell cycles can lead to chromosomal instability, which is one of the most common characteristics of various cancers. Pim-1 can bind to and phosphorylate Cdc25A (13), which is involved in regulating both early (G1/S) and late (G2/M) cell cycle transitions (14, 15). The cell cycle inhibitor p21 can also be phosphorylated by Pim-1, which leads to cytoplasmic localization of p21 (16). The nuclear mitotic apparatus molecule (NuMA) and the kinase C-TAK1 can also be phosphorylated by Pim-1 (17, 18). It was proposed that Pim-1 plays a role in promoting complex formation between NuMA, HP1b (heterochromatin protein 1b), dynein, and dynactin, a complex that is necessary for mitosis (17). The phosphorylation of C-TAK1 by Pim-1 renders C-TAK1 inactive. Because C-TAK1 is an inhibitory kinase that phosphorylates Cdc25C, the net result is thought to be the promotion of G1/M cell cycle progression (18). In sum, these results point to a role for Pim-1 in regulating various aspects of the cell cycle.

We previously reported that overexpression of Pim-1 could lead to chromosomal missegregation and polyploidy in prostate epithelial cell lines (1). Here we wished to elucidate the molecular mechanism underlying the chromosomal instability induced by Pim-1. We found that Pim-1-induced polyploidy occurred in a passage-dependent manner with in vitro culture consistent with a stochastic mode of progression and identified dysregulation of cyclin B1 as a likely mediator of this process.

MATERIALS AND METHODS

Cell Culture—The human prostate cell, RWPE-1, was obtained from American Type Culture Collection (ATCC) and maintained in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen). Telomerase-immortalized normal human breast epithelial cell (hTERT-HME1) (Clontech) was maintained in serum-free Dulbecco’s modified Eagle’s medium/F12 with bovine pituitary extract, epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml), and insulin (10 μg/ml). This cell line stably expresses human telomerase reverse transcriptase, which allows the cell to divide indefinitely while retaining a normal karyotype (19, 20). RWPE-1 and hTERT-HME1 cells were infected with retroviruses expressing either wild-type Pim-1 or the kinase-dead K67M mutant using Phoenix cells, selected with G418 at 200 μg/ml concentrations for 2 weeks, and resistant cells were pooled and analyzed. Empty vector containing only neomycin-resistant gene was used as a control. K67M mutant was cloned by mutating lysine at position 67 (AAG) into methionine (ATG) using PCR. pMSCV-Pim-1 vector (gift from Andrew Kraft) was used as a template. pMSCV-K67M construct sequence was confirmed by sequencing, and lack of kinase activity relative to that of wild-type Pim-1 toward histone H1 was measured to confirm its inactivity.

The abbreviations used are: NuMA, nuclear mitotic apparatus molecule; siRNA, small interfering RNA; shRNA, short hairpin RNAs; hTERT, human telomerase catalytic subunit; FACS, fluorescence-activated cell sorter; HPV, human papilloma virus; CDK, cyclin-dependent kinase; CIN, chromosomal instability; GFP, green fluorescent protein; RT, reverse transcription; MOPS, 4-morpholinepropanesulfonic acid.
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Cell Cycle Analysis, Synchronization, and Cell Sorting—Cells were synchronized by either double-thymidine or nocodazole treatment to block cells in G1/S and G2/M, respectively. For G1/S synchronization, cells were treated with 2 mM thymidine for 22 h, released for 10 h, and then retarded for 18 h. For G2/M synchronization, cells were treated with 40 ng/ml nocodazole for 18 h, washed one time with phosphate-buffered saline, and then switched to normal medium. Then cells were collected either for cell cycle analysis or for Western blotting analysis at the indicated time points. Cell cycle analysis was done by fluorescence-activated cell sorting (FACS) as described previously (1). At each time point, multiple analyses were performed. Indeed, for the RWPE-1 cells alone, longitudinal cell cycle analyses were performed at least 16 times for each of the three cell lines (Neo, Pim-1, K67M) for the data shown in Fig. 1. For cell sorting, intermediate passage (passage 25) cells were sorted according to DNA content (2N, 4N, and >4N) using a cell sorter. After 3–5 cell passages, cell cycle analysis was carried out to verify the cell cycle profile. The 2N-derived cells from each group were cultured for 25 additional passages, and then the cell cycle profile was analyzed by FACS. Mitotic checkpoint analysis was done as described (1). Briefly, cells were treated with 10 mM Taxol to induce mitosis, collected at the different time points, and stained with anti-phospho-histone H3 antibody.

Immunoblotting—Immunoblotting was performed as described previously (1) using the following antibodies: mouse anti-Pim-1, rabbit anti-cyclin B1, rabbit anti-phospho-histone H3 (serine 10), mouse anti-cdc2 (CDK1), and goat anti-actin (all from Santa Cruz Biotechnology).

CDK1 Kinase Assays—Cells lysates were prepared in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, protease inhibitor mixture). 250 μg of cell lysate was incubated with either anti-CDK1 antibody or normal rabbit serum overnight at 4°C. 40 μl of protein-A/G plus agarose slurry was then added for 3 h to immunoprecipitate cdc2 protein. The immunoprecipitated CDK1 was washed three times in the lysis buffer, and the precipitated beads were incubated with 10 μl of 2 mg/ml histone H1 in 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 10 μl of a non-CDK1 kinase inhibitor mixture (20 μM protein kinase C inhibitor peptide, 2 μM protein kinase A inhibitor peptide, and 20 μM compound R24571). The reaction was started by adding 9 μl of 75 mM magnesium chloride/500 μM ATP containing 1 μl of 100 μCi of [32P]ATP (PerkinElmer Life Sciences) and incubated for 10 min at 30°C. Twenty-five microliters of the reaction mixture was spotted onto P81 phosphocellulose paper and washed three times with 0.75% phosphoric acid and one time with acetone. Paper was drained, scintillation mixture was added, and cpm was measured using scintillation counter.

Quantitative RT-PCR Analysis—RNA isolation and quantitative RT-PCR (TaqMan, Applied Biosystems) using SYBR-GREEN dye were performed as described (1). PCR reactions were performed in triplicate. Primers used are: cyclin B1 forward primer, 5’-CGCAAAGCGCCTTCT-3’; cyclin B1 reverse primer, 5’-AATGACTTTTCCAGTAGCTGAAGGT-3’; 18 S forward primer, 5’-CGCCGCTAGAGGTGAAATCT-3’; 18 S reverse primer, 5’-CGAACCTCCGACTTTGTTT-3’.

RNA Interference—Mouse Pim-1 short hairpin RNAs (shRNAs) constructs (catalog number RMM1273) and control vector (catalog number RHS 1763) were obtained from Open Biosystems. Late passage RWPE-Pim-1 cells were infected with Pim-1 shRNA retroviral constructs (four constructs) and selected in puromycin (1 mg/ml). A total of three stable clones were established and analyzed. siRNA sequence tar-
targeting human cyclin B1, 5′-AAGAAAUGUACCCUCCAGAAA-3′ (21), was synthesized by Dharmacon Research Inc. Control targeting GFP, 5′-GGC TAC GTC CAG GAG CGC ACC-3′, was purchased from Dharmacon. Briefly, cells were transfected with siRNAs at a concentration of 50 nM using TransIT-TKO transfection reagent (Mirus), treated with 40 ng/ml nocodazole 24 h after siRNA treatment, and harvested 18 h after nocodazole treatment for Western blot.

**Cell Division Tracking**—RWPE cells were transfected with either control GFP or human cyclin B1 siRNA and stained with PKH67 green fluorescent dye (Sigma) the following day. Mean fluorescent intensity was determined by flow cytometry for several days as indicated.

**RESULTS**

**Pim-1 Induces Genomic Instability (Polyploidy) in a Passage-dependent Manner**—Our previous study demonstrated that overexpression of Pim-1 in prostate cell lines induced chromosomal missegregation and genomic instability (tetraploids and polyploids) (1). The cells lines used in our previous study have been carried in culture for over 25 passages...
when analyzed. During the course of analyzing additional Pim-1 over-
expressing cell lines, we observed that Pim-1-induced polyploidy
appeared in a passage-dependent manner; polyploid cells having ≥4N
DNA content emerged gradually with continued in vitro culture. To
examine this phenomenon in more detail, we established independent
stable cell lines transfected with wild-type Pim-1, a kinase-dead mutant
Pim-1 with a lysine-to-methionine mutation at position 67 (K67M) (22),
or vector control (Neo). We used RWPE-1 cells, which are immortal-
ized, non-tumorigenic prostate epithelial cells that we used previously
(1). In wild-type Pim-1 overexpressing cells, polyploidy began to appear
by about passage 20 and gradually increased such that after passage 50,
essentially all the cells had become polyploid (Fig. 1A). Neo control cells
showed no evidence of development of polyploidy even after more than
70 passages. RWPE-1 cells expressing the mutant Pim-1 (K67M), on the
other hand, showed a curious phenotype. Initially, the cells showed
intermediate levels of polyploidy, but this decreased after about passage
50 to levels seen in control cells (Fig. 1A). This reversion of K67M
expressing cells may be due to loss of Pim-1 expression in the late pas-
Sage cells (Fig. 1A, inset, Western blot P63). Pim-1 expressing cells can be
classified as early passage (majority diploid population, passages 1–20),
intermediate passage (mixed diploid and polyploid population, passages
25–40), and late passage (majority polyploid population, passages more
than 40).

The RWPE-1 cell line used in this study was generated by immortal-
izing normal human prostate epithelial cells with human papilloma
virus 18 (HPV 18) (23). Therefore, in this cell line, the p53 and pRb
pathways are expected to be impaired due to expression of HPV viral
proteins. To exclude these confounding factors, we examined the effect
of Pim-1 expression in a telomerase-immortalized normal human
mammary epithelial cell line (hTERT-HME1). This cell line stably
expresses human telomerase reverse transcriptase, which allows the cell
to divide indefinitely while retaining a normal karyotype and does not

FIGURE 4. Pim-1 overexpressing diploid cells
have potential to become polyploid. A, interme-
tiate passage cells (P-25) containing mixed dip-
loid/polyploid cells were sorted based on DNA
content by FACS, cultured for 3–5 passages, and
analyzed for DNA content by FACS after pro-
pidium iodide stain. B, Western blotting showing
Pim-1 expression in the sorted cells. C, 2N-derived
cells were cultured for an additional 25 passages
and then analyzed by FACS for DNA content. D, the
percentage of cells with 8N DNA content before
and after nocodazole treatment in early passage
RWPE-1 (P16) and hTERT-HME1 (P6) cells. Means
and standard deviations are shown. *, p < 0.05. E,
Western blotting showing Pim-1 expression in the
same cells as in panel D.
induce changes associated with a transformed phenotype (19, 20). As shown in Fig. 1B, the proportion of polyploid cells increased with increasing cell passage number in hTERT-HME1-Pim-1 and hTERT-HME1-K67M cells, indicating that induction of polyploidy by Pim-1 overexpression is neither specific to prostate cells nor dependent on the impairment of major cell cycle regulatory pathways such as p53 and pRb.

Early and Late Passage Pim-1 Overexpressing Cells Have Different Cell Cycles—To characterize the alterations in cell cycle caused by Pim-1 overexpression further, we synchronized cells in G1/S and M phase. Cells were treated with nocodazole for 18 h to arrest cells in mitotic stage and released, and then DNA content was analyzed by FACS. After nocodazole synchronization, 63.4 and 17.8% of the Neo control cells had 4N and 8N DNA content, respectively. For Pim-1 expressing cells, the proportion of cells with 4N DNA content was 55.4%, and that with 8N DNA content was 26.8%, implying a mitotic checkpoint defect. Similar to Pim-1 cells, K67M expressing cells had 55.4% of the cells with 4N DNA content and 25.1% with 8N DNA content (Fig. 2A and B). Examination of the cell cycle kinetics after release from nocodazole arrest showed that progression to G1 phase in Pim-1 and K67M expressing cells was delayed (Fig. 2A and B). At 6 h, 34.5 and 29.4% of Pim-1 and K67M cells are in G1, respectively, when compared with 46.4% of controls. These data were consistent with a cytokinesis defect in Pim-1 and K67M expressing cells. For late passage cells, nocodazole synchronization revealed that RWPE-Pim-1 cells cycled between 4N and 8N DNA content cell populations, whereas RWPE-Neo cells cycled between 2N and 4N populations (Fig. 2C and D). In addition, the rate of progression to G1 or G1-like state in Pim-1 expressing cells was also delayed (35% by 2 h in Pim-1 cells when compared with 55% by 2 h in Neo cells), again consistent with a cytokinesis delay in Pim-1 overexpressing cells as shown previously (1).

To analyze progression of Pim-1 expressing polyploid cells through the G1-to-S transition, we synchronized the late passage cells at G1/S using a double thymidine block (Fig. 3A and B). Although most Neo cells (80%) arrested with 2N DNA content, 70% of Pim-1 expressing cells arrested with 4N DNA content. The latter represent tetraploid cells in a G1-like state, which upon release progress through "S-phase" between 4N and 8N DNA content populations. Analysis of intermediate passage cells synchronized by double thymidine block revealed the presence of a mixed population in Pim-1 and K67M expressing cells, consisting of cells that cycle between 2N and 4N and those that cycle between 4N and 8N (Fig. 3C and D).

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Pim-1 Overexpressing Diploid Cells Have Potential to Become Polyploid—A key question related to Pim-1-induced genomic instability is why it takes a long period of the cell culture to develop polyploidy. Two distinct models may be invoked to explain this phenomenon: a stochastic (random, probabilistic) model and a mutation model. According to the stochastic model, each cell in the population has equal probability of becoming polyploid during each cell cycle (24–26). Long term cell culture is the reflection of the period required for all of the individual cells to assume that state. The mutation model, on the other hand, postulates that there exists a small subpopulation of cells with additional mutation(s) that cooperate with Pim-1 to promote polyploidy.

We employed various strategies to distinguish between these possibilities. First, we took advantage of intermediate passage cells that have a mixed population of diploid and tetraploid cells (Fig. 3C and D). We sorted these cells based on DNA content into 2N-, 4N-, and >4N-derived cells by FACS, cultured them, and examined whether 2N-derived cells could progress to polyploidy. Fig. 4 shows the cell cycle profiles at passages 3–5 after cell sorting. 4N- and >4N-derived cells...
from Pim-1 and K67M expressing cells were polyploid, unlike those from Neo control cells, which were diploid. On the other hand, 2N-derived cells from all three cell lines were diploid. Fig. 4B shows expression of Pim-1 in the sorted cells. If the stochastic model is correct, one would expect 2N-derived Pim-1 cells to progress to polyploidy over time. As shown in Fig. 4C, the cell cycle profile examination of cells at passage 25 after sorting revealed that 2N-derived cells from RWPE-Pim-1 and RWPE-K67M have progressed to polyploidy, whereas 2N-derived cells from control RWPE-Neo remained diploid.

Next, we treated early passage cells with nocodazole to test whether Pim-1 overexpressing cells can be induced to become polyploid in line with the stochastic model. As shown in Fig. 4D, there was a significant increase of cells with 8N DNA content in both RWPE-1 and hTERT-HME1-Pim-1 cells after nocodazole treatment when compared with Neo controls. The 8N cell population was also increased in RWPE-K67M cells but not in hTERT-HME1-K67M cells. Pim-1 expression levels were high in both cell lines (Fig. 4E).

Finally, we tested single cell clones derived from pooled populations of Pim-1 overexpressing cells. The stochastic model predicted that all Pim-1 expressing clones will progress to polyploidy over time. Indeed, all the single clones (five out of five RWPE-Pim-1 clones) eventually developed polyploidy (data not shown). Taken together, these results from three lines of evidence supported the stochastic model.

Progressive Mitotic Checkpoint Defect in Pim-1 Expressing Cells—Late passage Pim-1 overexpressing cells have a defective mitotic spindle checkpoint (1). To determine whether mitotic checkpoint defect precedes the development of polyploidy, we examined different passages of Pim-1 expressing cells representing different degrees of polyploidy. Phospho-histone H3 analysis of Taxol-treated cells showed that a mitotic spindle checkpoint defect was evident in early passage Pim-1 cells when compared with control Neo expressing cells (Fig. 5A); this defect becomes progressively severe with increasing passage number (Fig. 5, B and C). K67M mutant cells also showed mitotic checkpoint defect, although the defect was always less pronounced than that in wild-type Pim-1 expressing cells (Fig. 5, B and C). Overall, there appeared to be a correlation between mitotic checkpoint defect and degree of polyploidy as the severity of checkpoint defect increases in cells with higher degrees of polyploidy. This relationship was confirmed when we assessed the mitotic checkpoint using sorted cells described above. We found that 4N- and >4N-derived cells from Pim-1 overexpressing cells had a more profound mitotic checkpoint defect when compared with 2N-derived Pim-1 expressing cells and 2N-derived Neo control cells (Fig. 5D). These data suggested that Pim-1 overexpression leads to mitotic checkpoint defect before polyploidy appears and that there is a correlation between mitotic checkpoint defect and degree of polyploidy.

Dysregulation of CDK1 Activity and Cyclin B1 in Pim-1 Overexpressing Cells—The cyclin B1/CDK1 (cdc2) complex is a key molecule for mitosis. Accumulation of mitotic cyclins and the activation of the Cdk/cyclin complexes are essential for mitotic entry and progression (27). On the other hand, the degradation of the cyclin B1 is required for inactivation of the cyclin B1/CDK1 and exit from mitosis (28). We
examined CDK1 kinase activity in Pim-1 overexpressing cells. Asynchronously growing and nocodazole-treated cells were immunoprecipitated with anti-CDK1 antibody, and kinase activity was measured using histone H1 as substrate. Interestingly, asynchronously growing RWPE-Pim-1 and RWPE-K67M cells showed 2.3–3.2-fold elevated CDK1 kinase activity when compared with control Neo cells (Fig. 6A). Furthermore, although nocodazole treatment increased CDK1 activity about 2-fold in Neo control cells, it had no significant effect in Pim-1 and K67M expressing cells. Together, these data demonstrated that CDK1 activity is higher and that proper regulation during mitosis is disrupted in Pim-1 overexpressing cells.

To examine whether dysregulation of CDK1 activity is due to the inappropriate regulation of cyclin B1 in Pim-1 overexpressing cells, we examined cyclin B1 expression before and after nocodazole treatment. Cyclin B1 protein expression was higher in Pim-1 overexpressing cells and was not increased further with nocodazole treatment, in contrast to Neo control cells, which showed the expected accumulation of cyclin B1 protein after nocodazole treatment (Fig. 6B). Similar results were obtained when the mitotic spindle checkpoint was activated by Taxol treatment (Fig. 6C). Expression levels of CDK1 protein were not changed in Pim-1 expressing cells (Fig. 6C).

Regulation of Cyclin B1 Expression by Pim-1—The results above prompted us to examine cyclin B1 expression in Pim-1 overexpressing cells in more detail. Cyclin B1 protein levels were determined in Pim-1 cells from different passages. As shown in Fig. 7A, Western blot analysis in RWPE-1 cells showed that cyclin B1 protein level is significantly increased in wild-type Pim-1 overexpressing cells. This increase was obvious in early passage cells and maintained in intermediate and late passage cells. In contrast, there was no significant change of the levels of cyclin B1 protein in Neo control cells regardless of cell passage number. Although there was no increase of cyclin B1 protein in early passage K67M mutant cells, its levels increased almost the same as those of wild-type Pim-1 overexpressing cells in intermediate passage cells. However, K67M "revertant" cells failed to maintain cyclin B1 protein in late passage cells. The K67M revertant cells that have lower levels of cyclin B1 also have lower expression of Pim-1 (Fig. 7A). At this stage, K67M cells have also largely lost polyploidy (Fig. 1A). Thus, cyclin B1 protein levels are closely correlated with Pim-1 expression levels and the degree of polyploidy. An increase in cyclin B1 protein level was also observed in early passage hTERT-HME1-Pim-1 cells (Fig. 7B). The close correlation between cyclin B1 and Pim-1 expression levels implied regulation of cyclin B1 by Pim-1. We tested this hypothesis directly by down-regulating Pim-1 using RNA interference. We stably knocked down Pim-1 expression using shRNA constructs in Pim-1 overexpressing cells. As shown in Fig. 7C, reduced Pim-1 expression also led to a reduction in cyclin B1 levels (Pim-1 shRNA clone 4), indicating that in these cells, cyclin B1 is under the regulation of Pim-1. To determine whether Pim-1 regulates cyclin B1 at the transcriptional level, we measured mRNA level of cyclin B1 using quantitative RT-PCR. There were no significant differences in cyclin B1 mRNA levels in either RWPE-1 or hTERT-HME1 cells due to expression of Pim-1 (Fig. 7D). Thus, Pim-1 overexpression led to increased cyclin B1 protein levels through a post-transcriptional mechanism.

Cyclin B1 Is Required for Cytokinesis Delay but Not Mitotic Checkpoint Defect in Pim-1 Overexpressing Cells—The two primary defects identified in Pim-1 overexpressing cells that likely underlie the development of polyploidy and chromosomal instability are a mitotic checkpoint defect and cytokinesis delay (1). Cyclin B has been implicated in both processes. Degradation of cyclin B1 is important for the ability to complete cytokinesis (29, 30), and recent reports indicate that overexpression of cyclin B can impair the mitotic spindle checkpoint (31). The high levels of cyclin B1 seen in Pim-1 overexpressing cells suggested that cyclin B1 elevation contributes to the development of polyploidy through impairment of either the mitotic checkpoint and/or cytokinesis.
delay. To distinguish between these possibilities, we sought to down-regulate cyclin B1 in Pim-1 expressing cells to levels that approximate those seen in Neo controls cells using siRNA. First, we show that cyclin B1 siRNA transfection reduced cyclin B1 levels without an effect on levels of Pim-1 expression (Fig. 8A). We then assayed for mitotic checkpoint defect and cytokinesis delay after cyclin B1 siRNA in our Pim-1 stable cell lines. As shown in Fig. 8B, Pim-1 expressing cells show lower expression of phospho-histone H3 after nocodazole treatment when compared with Neo cells, consistent with a mitotic checkpoint defect (Fig. 8B). However, reduction of cyclin B1 by siRNA did not discernibly alter this mitotic checkpoint defect. By contrast, analysis of cell division by tracking the decrease of a membrane-incorporated fluorescent dye PKH67 shows that the cytokinesis delay engendered by Pim-1 expression can be abrogated by reducing cyclin B1 levels using siRNA (Fig. 8C). Thus, the elevated cyclin B1 levels in Pim-1 expressing cells contributed to the development of polyploidy by delaying cytokinesis.

DISCUSSION

Chromosomal instability (CIN) leading to an abnormal chromosomal number (aneuploidy) is a prominent feature of many cancers; however, the causes of aneuploidy are not fully understood. Defects in the mitotic spindle checkpoint can contribute to the chromosomal mis-segregation and instability. In fact, mutations in some mitotic checkpoint genes have been reported in a subset of human cancers and cell lines, which have CIN (32–36). However, the significance of alterations in mitotic checkpoint genes in cancer development is unclear due to the low frequency of such mutations (37). In this regard, the finding of inherited mutations in the BUB1B gene in families with a cancer predisposition disorder called mosaic variegated aneuploidy is intriguing (38). This finding provides direct evidence that CIN due to a defective mitotic checkpoint predisposes to cancer development. Aneuploidy can also arise from a wide variety of defects in different phases of the cell cycle.
cycle including cytokinesis. A cell that has attempted but failed to complete cytokinesis will end up in a G1-like state with an unsegregated genome. Survival of such cells results in polyploidy (39). In this report, we showed that Pim-1-induced polyploidy occurs in a passage-dependent manner consistent with a stochastic mode of progression. The stochastic model predicted that the individual cell in the population has a small but equal probability of becoming polyploid during each cell cycle. Thus, the time required for cells to become polyploid reflected the period required for all individual cells in the population to assume that state. Three pieces of evidence presented in this study supported the stochastic progression model: 2N-derived Pim-1 overexpressing cells progressed to polyploidy, early passage Pim-1 overexpressing cells progressed to polyploidy when exposed to mitotic spindle poisons, and all the single clones derived from Pim-1 overexpressing cells progressed to polyploidy. A similar gradual development of polyploidy with prolonged in vitro culture has also been reported for cells in which c-Myc overexpression is combined with either p53 loss or cyclin B1 overexpression (25, 40).

Pim-1 kinase activity is not essential for induction of polyploidy as RWPE-1 cells expressing the K67M kinase-dead Pim-1 mutant consistently developed polyploidy, albeit at a lower rate when compared with cells expressing wild-type Pim-1. The two cell lines (RWPE-1 and hTERT-HME1) differ concerning their p53 status; RWPE-1 cells are currently investigating the precise mechanisms by which Pim-1 overexpression alters cyclin B1 protein levels. Finally, it is of interest to note, however, that cyclin B1 has been reported to be a transcriptional target of c-Myc (40). Therefore, these cooperating oncogenes (c-Myc and Pim-1) may use distinct mechanisms (transcriptional and post-transcriptional), respectively, to up-regulate cyclin B1 expression.

Pim-1 overexpression leads to dysregulation of cyclin B1, which results in the observed cytokinesis delay but not mitotic checkpoint defect. High levels of cyclin B1 and CDK1 kinase activity have been reported to precede the onset of aneuploidy in tumor cells (43–45). Our results indicated that early passage Pim-1 overexpressing cells and sorted 2N-derived Pim-1 cells had defects in the mitotic checkpoint before polyploidy emerged. This was associated with an increase in the levels of cyclin B1 protein and its associated CDK1 kinase activity. The mitotic spindle checkpoint inhibits sister chromatid separation until all chromosomes have aligned on the metaphase plate (46). This control mechanism is well conserved in mammalian cells, and defects in this pathway can lead to the chromosomal abnormality including polyploidy. Some reports indicate that overexpression of B-type cyclins can impair the mitotic checkpoint and lead to chromosomal missegregation and polyploidy (31, 40). However, we found that knocking down cyclin B1 by siRNA to levels that approximate those in empty vector transfected cells did not affect the mitotic checkpoint defect in Pim-1 cells. Rather, elevated levels of cyclin B1 contribute to the cytokinesis delay in Pim-1 overexpressing cells since the cytokinesis delay engendered by Pim-1 expression can be abrogated by inhibition of cyclin B1 expression with cyclin B1-specific siRNA. Cyclin B1 is degraded to allow mitotic exit and cytokinesis (29, 30).

Our data indicated that Pim-1 regulates cyclin B1 levels through a post-transcriptional mechanism, as cyclin B1 mRNA levels were not affected by Pim-1 expression, and that this function of Pim-1 is inde-

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