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The aim of our research is to elucidate the mechanisms by which the normal regulatory pathways coordinating centrosome duplication with cell cycle events become uncoupled. Since negative (tumor suppressors) and positive (oncogenes) regulators of cell cycle progression can also regulate centrosome duplication, we propose that in breast cancer, alterations in growth factor signaling pathways, and/or inactivation of the p53 pathway act to deregulate G1/S and/or G2/M cell cycle checkpoints. Centrosome amplification may represent an early event in breast cancer development, while the degree of amplification may also increase during tumor progression accelerating the rate of chromosomal instability and aggressiveness. These studies will have an important impact in basic and clinical oncology for two reasons: first, they will help to define the molecular mechanisms leading to centrosome amplification in breast cancer; second, they will clarify if centrosome amplification represents a driving force in selecting hormone-independent and chemoresistant clones in breast cancer and its potential role as a new suitable marker of tumor aggressiveness.

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ANNUAL SUMMARY REPORT

CENTROSOME AMPLIFICATION: A POTENTIAL MARKER OF BREAST CANCER AGGRESSIVENESS

Antonino D'Assoro, M.D.

INTRODUCTION

The centrosome plays an essential role in the control of genomic stability through the establishment of the bipolar mitotic spindle and equal segregation of chromosomes during cell division (Lingle et al. 2002). The centrosome is duplicated once, and only once, during a normal cell cycle to give rise to two centrosomes that function as the spindles poles of the dividing cell (Kellog 1989). In order to maintain the integrity of the genome, centrosome duplication is strictly coordinated with DNA replication during cell cycle progression (D'Assoro et al. 2002, 2004, Sluder et al. 2000). Centrosome homeostasis results from the complex interaction of oncogenes and tumor suppressors operating at level of the G1/S and G2/M cell cycle checkpoints. In the development of cancer, deregulation of the centrosome duplication cycle leads to centrosome amplification and abnormal multipolar mitoses, and consequent chromosome mis-segregation. This, in turn, results in an increase in the rate of chromosomal instability and phenotypic diversity in the cancer cells, thereby promoting tumor progression, chemoresistance, and poor outcome.

The aim of our research is to elucidate the mechanisms by which the normal regulatory pathways coordinating centrosome duplication with cell cycle events become uncoupled. Since negative (tumor suppressors) and positive (oncogenes) regulators of cell cycle progression can also regulate centrosome duplication, we propose that in breast cancer, alterations in growth factor signaling pathways, and/or inactivation of the p53 pathway act to deregulate G1/S and/or G2/M cell cycle checkpoints. Centrosome amplification may represent an early event in breast cancer development, while the degree of amplification may also increase during tumor progression accelerating the rate of chromosomal instability and aggressiveness. These studies will have an important impact in basic and clinical oncology for two reasons: first, they will help to define the molecular mechanisms leading to centrosome amplification in breast cancer; second, they will clarify if centrosome amplification represents a driving force in selecting hormone-independent and chemoresistant clones in breast cancer and its potential role as a new suitable marker of tumor aggressiveness.

KEY RESEARCH ACCOMPLISHMENTS

Specific aim #1: Determine the role of 17-B estradiol and EGF growth factor receptor activation in the regulation of centrosome duplication.

Breast cancer is a hormone-dependent disease: estrogens play an essential role in the onset of breast cancer, however, the hormone-dependent phenotype is commonly lost during tumor progression. The EGF growth factor pathway also plays a key role not only in the development of breast cancer but also in the progression of the disease since advanced breast cancers often over-express the EGF receptor and show an hormone-independent and chemoresistant phenotype.

The goal of this first aim is to determine the role that 17-B estradiol and EGF growth factor pathways play in the regulation of centrosome duplication. We propose to develop and validate an in vitro model system for centrosome duplication that can be used to dissect the signaling events that are required for the development of centrosome amplification in breast cancer.

Our results to date demonstrate that in the MCF-7 breast cancer cell line, 17-B estradiol and EGF stimulate centrosome duplication and this process is well coordinated with the expression of key cell cycle regulators (cyclin D, E, A, B,) and with the phosphorylation status of the retinoblastoma tumor suppressor. Furthermore, in order to understand if abrogation of the p53 pathway may deregulate the centrosome duplication cycle in the MCF-7 cells stimulated with mitogens, we generated an MCF-7 cell line stable expressing a p53 dominant-negative construct (MCF-7Dnp53) to mask the function of the wild-type p53.

Interestingly, the MCF-7Dnp53 stimulated with estradiol and EGF exhibited a deregulation of the cell cycle regulators that was associated with an amplified centrosome phenotype, suggesting that development of centrosome amplification in breast cancer requires not only the acceleration of cell cycle progression through mitogens but also the inactivation of the p53 pathway with important consequences on the G1/S timing progression. These results suggest a model of multi-step neoplastic transformation where estrogens and growth factors initiate the development of breast cancer and the consequent abrogation of the p53 pathway during tumor progression promotes the development of centrosome amplification leading to genomic instability, chemoresistance and poor out-come.

A manuscript describing these experiments and results is currently in preparation.

Specific aim #2: Determine the relationship between cell cycle checkpoints and centrosome amplification.

The goal of this second aim is to determine the molecular mechanisms linking inactivation of the G1/S and G2/M cell cycle checkpoints to centrosome amplification in breast cancer. We have focused on the role of the G1/S checkpoint in the control of centrosome homeostasis. Our results demonstrate that induction of genotoxic stress induces centrosome amplification in breast cancer cell lines with an abrogated G1/S cell cycle checkpoint and have recently been published in *Oncogene* (D'Assoro et al, 2004).

We used breast cancer cell lines with different p53 backgrounds to investigate the relationship between DNA damage, G1/S cell cycle checkpoint integrity, and the development of centrosome amplification. Introduction of DNA damage in the MCF-7 cell line by treatment with hydroxyurea (HU) or daunorubicin (DR) resulted in the arrest of both G1/S cell cycle progression and centriole duplication. In these cells, which carry functional p53, HU treatment also led to nuclear accumulation of p53 and p21WAF1, retinoblastoma hypophosphorylation, and downregulation of cyclin A. MCF-7 cells carrying a recombinant dominant-negative p53 mutant (vMCF-7DNp53) exhibited a shortened G1 phase of the cell cycle and retained a normal centrosome phenotype. However, these cells developed amplified centrosomes following HU treatment. The MDA-MB 231 cell line, which carries mutant p53 at both alleles, showed amplified centrosomes at the outset, and developed a hyperamplified centrosome phenotype following HU treatment. In cells carrying defective p53, the development of centrosome amplification also occurred following treatment with another DNA damaging agent, DR. Taken together, these findings demonstrate that loss of p53 function alone is not sufficient to drive centrosome amplification, but plays a critical role in this process following DNA damage through abrogation of the G1/S cell cycle checkpoint. Interestingly, we were able to reactivate the G1/S checkpoint and revert the centrosome phenotype in the MDA-MB 231 cell line following genotoxic stress by using a potent cdk inhibitor, Roscovitine.

The novelty of these results demonstrate for the first time a clear relationship between DNA damage, abrogation of the G1/S checkpoint and the development of centrosome amplification. These observations have important clinical implications regarding the management of breast cancer patients: first, they suggest that breast cancers with compromised p53 function may develop centrosome amplification and consequent chromosomal instability following treatment with genotoxic anticancer drugs. Second, to overcome the complications associated with centrosome amplification for this subset of breast cancer patients, treatment with cdk inhibitors in combination with anticancer genotoxic drugs may provide a new attractive therapeutic approach.

CONCLUSIONS

Our studies suggest that the development and progression of breast cancer is a complex process involving the role of estrogens, growth factor signaling pathways and abrogation of the p53 protein leading to an inactivation of the G1/S cell cycle checkpoint. In turn, the abrogation of the G1/S checkpoint activity leads to the development of centrosome amplification that may represents the driven force to promote genomic instability and phenotypic heterogeneity in breast cancer. The development of these pathological processes may confer to the tumor cells high metastatic potential and chemoresistant properties with catastrophic consequences for breast cancer patients. We also suggest that the further characterization of the mechanisms leading to centrosome amplification in breast cancer proposed in this grant will help to define in the centrosome a suitable marker of tumor aggressiveness and a new target for breast cancer treatment,

D'Assoro, A. B., S. L. Barrett, et al. (2002). "Amplified centrosomes in breast cancer: a potential indicator of tumor aggressiveness." Breast Cancer Res Treat 75(1): 25-34.

D'Assoro, A. B., R. Busby, et al. (2004). "Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint." Oncogene.

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Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint

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Centrosome amplification plays a key role in the origin of chromosomal instability during cancer development and progression. In this study, breast cancer cell lines with different p53 backgrounds were used to investigate the relationship between genotoxic stress, G_1/S cell cycle checkpoint integrity, and the development of centrosome amplification. Introduction of DNA damage in the MCF-7 cell line by treatment with hydroxyurea (HU) or daunorubicin (DR) resulted in the arrest of both G_1/S cell cycle progression and centriole duplication. In these cells, which carry functional p53, HU treatment also led to nuclear accumulation of p53 and $p21^{WAF1}$, retinoblastoma hypophosphorylation, and downregulation of cyclin A. MCF-7 cells carrying a recombinant dominant-negative p53 mutant (vMCF-7^{DNp53}) exhibited a shortened G_1 phase of the cell cycle and retained a normal centrosome phenotype. However, these cells developed amplified centrosomes following HU treatment. The MDA-MB 231 cell line, which carries mutant p53 at both alleles, showed amplified centrosomes at the outset, and developed a hyperamplified centrosome phenotype following HU treatment. In cells carrying defective p53, the development of centrosome amplification also occurred following treatment with another DNA damaging agent, DR. Taken together, these findings demonstrate that loss of p53 function alone is not sufficient to drive centrosome amplification, but plays a critical role in this process following DNA damage through abrogation of the G_1/S cell cycle checkpoint. Furthermore, these studies have important clinical implications because they suggest that breast cancers with compromised p53 function may develop centrosome amplification and consequent chromosomal instability following treatment with genotoxic anticancer drugs.

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Introduction

The progression of aggressive breast cancer is characterized by genomic instability leading to multiple genetic defects, phenotypic diversity, chemoresistance, and poor outcome (Lengauer *et al.*, 1998; Loeb, 2001). An imbalance between oncogene and tumor suppressor activities plays an important role in the onset of breast cancer through the inactivation of G_1/S and/or G_2/M checkpoints, which normally ensure the orderly progression of cell cycle events (Hartwell and Weinert, 1989). In normal cells, checkpoint activation in response to DNA damage is mediated by p53 through upregulation of its downstream targets, and consequent cell cycle arrest (Wang *et al.*, 1999; Jin and Levine, 2001; Taylor and Stark, 2001). Recently, the centrosome has been implicated in the pathogenesis of cancer through the development of multipolar mitotic spindles leading to chromosomal instability and clonal heterogeneity (Brinkley, 2001; D'Assoro *et al.*, 2002b). The centrosome is the major microtubule-organizing center of the cell and is duplicated once during a normal cell cycle to give rise to two centrosomes that function as the spindle poles during mitosis (Kirschner and Mitchison, 1986; Kellogg, 1989; Palazzo, 2003). Therefore, tight coordination between the centrosome duplication and DNA replication cycles is essential to ensure equal segregation of sister chromatids during cell division (Sluder and Hinchcliffe, 2000). However, centrosome duplication is not strictly dependent on DNA replication since the centrosome and DNA cycles can be uncoupled (Balczon *et al.*, 1995). In cancer, loss of coordination between the centrosome and DNA cycles may lead to centrosome amplification, increased frequency of multipolar mitoses, and chromosomal instability (Lingle *et al.*, 1998, 2002; Pihan *et al.*, 2001; D'Assoro *et al.*, 2002a). Tumor suppressors and cdk/cyclins play an important role in coordinating centrosome duplication with cell cycle events. In mouse model studies, loss of p53 by gene targeting and gain-of-function p53 mutations resulted in the development of centrosome amplification and aberrant mitoses (Fukasawa *et al.*, 1996; Fry *et al.*, 1999; Murphy and Rosen, 2000; Tarapore *et al.*, 2001). Furthermore, cyclins E and A have been demonstrated to be key regulators of the centrosome cycle (Hinchcliffe *et al.*, 1999; Meraldi *et al.*, 1999; Faivre *et al.*, 2002;

Okuda, 2002), and p53 mutations associated with cyclin E overexpression synergistically increase the frequency of centrosome defects (Mussman *et al*., 2000). Centrosome duplication is also coordinated with DNA synthesis through the phosphorylation status of retinoblastoma and consequent activation of the E2F transcription factor (Angus *et al*., 2002). A role for p53 and retinoblastoma (Rb) in this process is also suggested by studies where inactivation of these tumor suppressors after infection with high-risk human papillomaviruses carrying E6 and E7 viral oncogenes leads to centrosome amplification (Duensing and Munger, 2002; Riley *et al*., 2003). In human breast cancer, however, the relationship between cell cycle checkpoint defects, DNA damage and development of centrosome amplification has not been extensively investigated. To elucidate the molecular mechanisms linking G_1/S checkpoint activity to the centrosome duplication cycle, we studied the effect of drugs inducing genotoxic stress in breast tumor-derived cell lines with different p53 backgrounds. Our results demonstrate that p53 activity, through upregulation of $p21^{WAF1}$ and retinoblastoma hypophosphorylation, is essential for the maintenance of centrosome homeostasis following DNA damage. In the breast cancer models studied here, inactivation of the p53 pathway also leads to the upregulation of cyclin A, suggesting that control of centrosome duplication may depend on a balance between positive and negative regulators acting at the G_1/S transition of the cell cycle.

Results

p53 status and centrosome phenotype in breast cancer cell lines

In order to elucidate how p53 affects centrosome phenotype in breast cancer cell lines, we compared MCF-7 cells, which carry wild-type p53, a variant of this cell line carrying a dominant-negative p53 mutation (vMCF-7^{DNp53}), and MDA-MB 231 cells with mutant p53 at both alleles (Gartel *et al*., 2003). We analysed p53 expression, centrosome characteristics, and cell cycle profiles for each cell line. Western blot analysis for p53 showed that MCF-7 cells exhibited a low level of the wild-type protein, while vMCF-7^{DNp53} and MDA-MB 231 cells showed a high level of mutant p53 expression (Figure 1a). Immunofluorescence was performed to label centrioles and pericentriolar material using primary antibodies against the centrosome proteins centrin and pericentrin, respectively. MCF-7 cells showed a normal centrosome phenotype with two or four centrioles according to the phase of the cell cycle (Figure 1b, e). The vMCF-7^{DNp53} cells exhibited normal centrosomes; however, they showed a higher percentage of cells with duplicated centrosomes (total of four centrioles) compared to the parental cell line (Figure 1c, e). To determine if the difference in centrosome number was related to cell cycle progression, we performed FACS analysis and found that the vMCF-7^{DNp53} cell line showed a decrease in the proportion of cells in G_1 and a corresponding increase in the S and G_2/M phases of the cell cycle (Figure 1f). These results show that while partial inhibition of p53 function accelerated both entry into S phase and the centrosome duplication cycle, centrosome duplication nonetheless remained coordinated with DNA replication and cell cycle progression. To determine if the complete abrogation of p53 function may have different effects on centrosome characteristics, we further analysed centrosome phenotype in the breast cancer cell line MDA-MB 231, which carries only mutant p53. Immunofluorescence analysis (Figure 1d) showed amplified centrosomes, which were characterized by supernumerary centrioles and excess pericentriolar material in 56% of the cells. FACS analysis (Figure 1f) of these cells, however, showed a lower percentage of cells in the S and G2/M phases of the cell

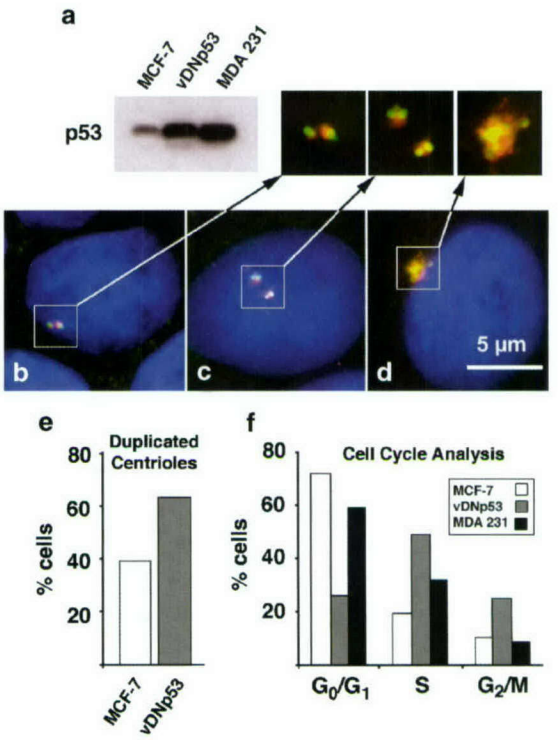

Figure 1 Analysis of p53 expression, centrosome phenotype, and cell cycle characteristics in breast cancer cell lines. (**a**) Western blot analysis of p53 expression in MCF-7, vMCF-7^{DNp53}, and MDA-MB 231 cell lines (20 μg protein whole-cell lysate was loaded in each lane). Centrosome phenotype for MCF-7 (**b**), vMCF-7^{DNp53} (**c**), and MDA-MB 231 (**d**) was determined by immunofluorescence: centrosomes were stained with antibodies against centrin (green) and pericentrin (red) to label the centrioles and pericentriolar material, respectively. Arrows and insets illustrate the centrosomes from (**b–d**) at higher magnification. Nuclei were stained blue with DAPI. (**e**) Graph showing percentage of cells with duplicated centrioles for MCF-7 and vMCF-7^{DNp53}. Cells carrying the dominant-negative p53 mutation show normal centrosomes, but a higher percentage of duplicated centrioles compared to the parental cell line. (**f**) Cell cycle analysis based on flow cytometry for MCF-7, vMCF-7^{DNp53}, and MDA-MB 231 cells. The vMCF-7^{DNp53} cell line shows a higher proportion of proliferating cells, with a decrease in G_0/G_1 and increase in S and G_2/M phase of the cell cycle, compared to the parental cell line MCF-7 and to MDA-MB 231 cells

cycle compared to the vMCF-7^{DNp53} cell line. Taken together, these observations led us to investigate further the role of p53 abrogation in uncoupling the centrosome and DNA cycles and the generation of amplified centrosomes.

Effect of genotoxic stress on the centrosome duplication cycle in breast cancer cells

In normal cells, p53 functions to monitor genomic integrity, and following DNA damage this tumor suppressor mediates molecular responses leading to cell cycle arrest, DNA repair, or programmed cell death. We used hydroxyurea (HU), an inhibitor of ribonucleotide reductase activity, to induce replication stress DNA damage (Sakano *et al*., 2001), activate the G_1/S checkpoint, and to determine how the centrosome duplication cycle was affected. In these experiments, MCF-7, vMCF-7^{DNp53}, and MDA-MB 231 cells were treated with 2 mM HU for 48 h and analysed for DNA synthesis arrest by FACS analysis and centrosome phenotype by immunofluorescence and electron microscopy. FACS analysis showed that HU treatment blocked DNA replication in all three cell lines regardless of their p53 status (Figure 2). High magnification immunofluorescence images, of cells before and after HU treatment, labeled for pericentriolar material and centrioles, using pericentrin and centrin antibodies, respectively, are illustrated in Figure 3a. Quantitative analysis of centrosome number and size for each cell type showed a different centrosome phenotype following HU treatment (Figure 3b and c and Table 1). In MCF-7 cells, the centrosome duplication cycle was arrested following HU treatment since most of the cells showed normal centrosomes with two or four centrioles and did not show an increase in centrosome size (Figure 3a upper row, b and c and Table 1). Therefore, the DNA and centrosome cycles in MCF-7 cells remained tightly coordinated, even following experimentally introduced

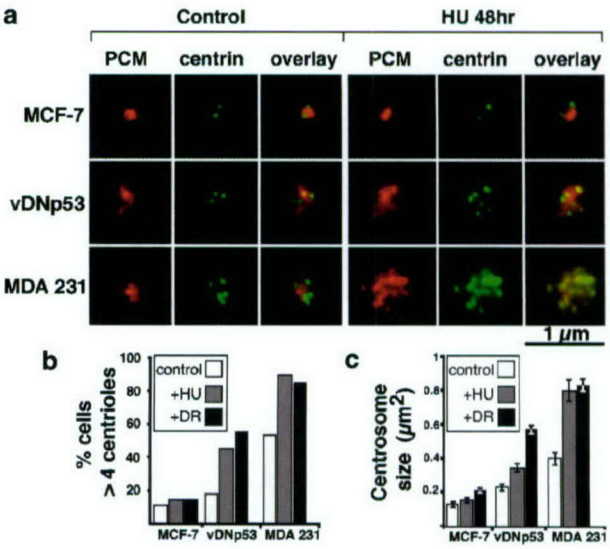

Figure 3 Analysis of centrosome characteristics in the three breast cancer cell lines. (**a**) Immunofluorescence of centrosomes in MCF-7 (upper row), vMCF-7^{DNp53} (middle row), and MDA-MB 231 (lower row) cells before (Control) and after HU treatment (48 h). These are high magnification images showing the centrosome as a maximum projection and each panel represents approximately 1 μm^3 of the cytoplasm. The bar at the lower right is 1 μm. Pericentriolar material (PCM) and centrioles were labeled for pericentrin (red) and centrin (green), respectively. Each row illustrates the individual fluorescence channels and an overlay image of the two. MCF-7 cells showed two or four centrioles surrounded by a similar amount of PCM, regardless of treatment. In contrast, vMCF-7^{DNp53} cells showed normal centrosomes in untreated cells and centrosome amplification following HU treatment. MDA-MB 231 cells showed amplified centrosomes at the outset and centrosome hyperamplification following HU treatment. (**b**) Graph showing the percentage of cells with more than four centrioles before and after HU or DR treatment (48 h). The centriole cycle is arrested in MCF-7, while vMCF-7^{DNp53} and MDA-MB 231cells showed centriole overduplication in response to DNA damage. (**c**) Quantitative digital image analysis of centrosome size in breast cancer cells before and following HU or DR treatment ($\pm$s.d.). Centrosome size did not significantly change in MCF-7 cells, while vMCF-7^{DNp53} and MDA-MB 231 cells showed an increase in centrosome size following HU or DR treatment

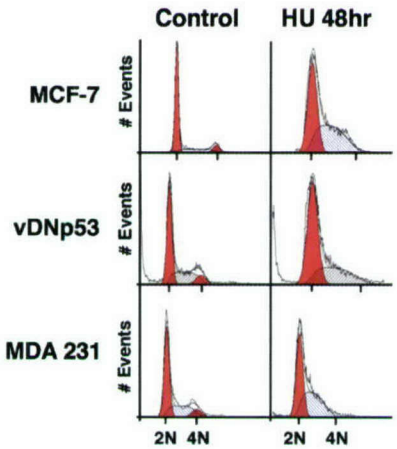

Figure 2 FACS analysis of cell cycle progression in the breast cancer cell lines before and after HU treatment. Each cell line showed a block in the G_1/S phase of the cell cycle due to HU-induced DNA replication blockade

DNA replication blockade. In contrast, vMCF-7^{DNp53} cells generated amplified centrosomes with 45% of the cells showing greater than four centrioles after treatment with HU (Figure 3a middle row, b). Interestingly, in these cells, centriole overduplication was not accompanied by a dramatic increase of the centrosome size (Figure 3c and Table 1). However, a striking change in centrosome structure was observed in MDA-MB 231 cells following HU treatment (Figure 3a lower row). These cells, which showed amplified centrosomes at the outset, developed *hyperamplified* centrosomes characterized by dramatic centriole overduplication and a further increase in centrosome size (Figure 3a lower row, b and c and Table 1). Ultrastructural analyses of centrosomes confirmed the immunofluorescence findings. Figure 4 shows representative images of MCF-7 centrosomes, where more than four centriole profiles were never seen in the same section. In MDA-MB 231, however, cell sections with more than four centriole profiles were

Table 1 Centrosome size based on centrin and pericentrin labeling in human breast cancer cell lines

<0.3 μm² *Normal*	*0.3–0.6 μm²* *Amplified*	*>0.6 μm²* *Hyperamplified*
MCF-7	vMCF7^{Dnp53} + HU	MDA-MB 231 + HU
MCF-7 + HU	vMCF7^{Dnp53} + DR	MDA-MB 231 + DR
MCF-7 + DR	MDA-MB 231	
vMCF7^{Dnp53}	MDA-MB 231 + HU + Ros	

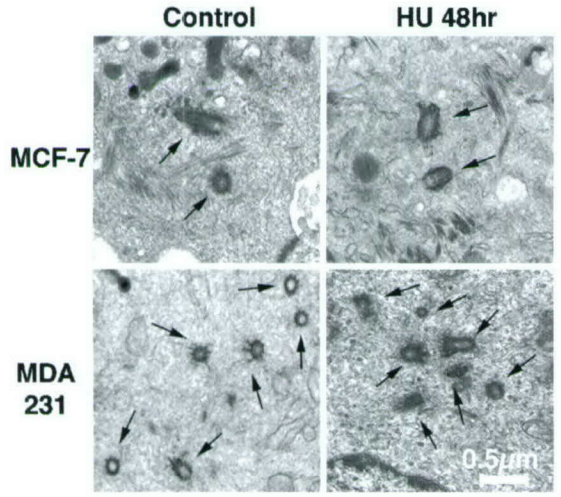

Figure 4 Ultrastructural analysis of centrosome morphology in MCF-7 and MDA-MB 231 cell lines before and after HU treatment (48 h) confirmed the immunofluorescence observations. Bar = 0.5 μm

frequently observed, particularly following HU treatment. To determine if other genotoxic agents result in the development of centrosome amplification, we investigated the effect of the daunorubicin (DR) on centrosome duplication in the three breast cancer cell lines. The anthracycline DR induces damage to DNA through intercalation-induced distortion of the double helix, or stabilization of the cleavable complex formed between DNA and topoisomerase II (Rubin and William, 2003). Following treatment with DR, MCF-7 cells maintained a normal centrosome phenotype similar to that seen following HU treatment (Figure 3b, c and Table 1). While vMCF-7^{Dnp53} and MDA-MB 231 cells developed centrosome amplification and hyperamplification, respectively, following DR treatment (Figure 3b, c and Table 1). These findings suggest that, in cells with intact p53 function, genotoxic stress may trigger cell cycle checkpoints that normally ensure coordination of the centrosome duplication and DNA cycles.

Loss of p53 activity uncouples the centrosome cycle from DNA synthesis through deregulation of the G_1/S cell cycle checkpoint

To determine the mechanism by which p53 integrates the centrosome duplication and DNA replication cycles, we investigated the expression and localization of p53 and its downstream target p21^{WAF1}, following DNA damage. Immunofluorescence labeling of MCF-7 cells showed a quantitative increase in both p53 and p21^{WAF1} nuclear localization in HU-treated cells (Figure 5a upper row, b). In contrast, vMCF-7^{DNp53} cells, regardless of a higher nuclear localization of p53 following HU treatment, showed a reduced p21^{WAF1} nuclear accumulation compared to the parental cell line (Figure 5a middle row, b). MDA-MB 231 cells showed the mutated form of p53 highly expressed and localized in the nucleus before and following DNA damage. However, in these cells, HU treatment did not result in p21^{WAF1} induction, indicating the complete abrogation of the p53 pathway (Figure 5a lower row, b). We then performed a time course Western blot analysis in order to establish the relationship between integrity of the p53 pathway and activation of the G_1/S cell cycle checkpoint in the three cell lines. Activation of the G_1/S checkpoint was present only in the MCF-7 cell line where HU treatment resulted in a rapid increase of p53 protein that was followed by induction of p21^{WAF1} and subsequent hypophosphorylation of retinoblastoma (Figure 6). In contrast, both vMCF-7^{DNp53} and MDA-MB 231 cells showed a high level of mutant p53 expression, low p21^{WAF1} expression, and a high level of retinoblastoma phosphorylation (Figure 6). These results indicate a defect in G_1/S checkpoint activation after DNA damage in the cell lines with abrogated p53 function. Interestingly, while

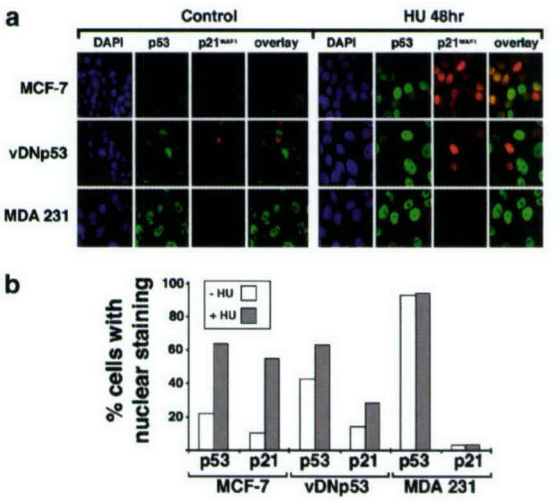

Figure 5 Fluorescence analysis of G_1/S checkpoint activation following HU treatment (48 h). (**a**) Analysis of p53 and p21^{WAF1} nuclear localization in MCF-7 (upper row), vMCF-7^{DNp53} (middle row), and MDA-MB 231 (lower row) cells before (Control) and after HU treatment (48 h). Nuclei were stained in blue with DAPI, while p53 and p21^{WAF1} were labeled green and red, respectively. Each row illustrates the individual fluorescence channels and an overlay image of p53 and p21^{WAF1}. Following HU treatment, MCF-7 cells showed an increase of p53 and p21^{WAF1} nuclear accumulation, while vMCF-7^{DNp53} cells also showed a higher level of nuclear p53, but did not show a corresponding increase of nuclear p21^{WAF1}. MDA-MB 231 cells showed nuclear localization of p53 in most cells, but there was no induction of p21^{WAF1}, following HU treatment. (**b**) Graph showing quantitative analysis of nuclear accumulation of p53 and p21^{WAF1} before and after HU treatment (48 h)

Figure 6 Western blot analysis of the breast cancer cell lines following HU treatment. The abundance of key cell cycle regulators (p53, $p21^{WAF1}$, phospho-Rb, cyclin E, and cyclin A) was analysed before and in a time course series following HU treatment. Whole-cell lysate (20 μg) was loaded in each lane. β-Actin was used as loading control

following HU treatment the level of cyclin E in all three cell lines remained high, expression of cyclin A was downregulated in MCF-7 cells following DNA damage, while vMCF-7^{DNp53} and MDA-MB 231 cells showed a high expression and overexpression of cyclin A, respectively (Figure 6). Therefore, loss of p53 function and increased cyclin A expression together may uncouple the centrosome and DNA cycles following genotoxic stress.

Cdk activity is required for centrosome hyperamplification following HU treatment in the MDA-MB 231 cell line

In order to further investigate the role of cyclin/cdk activity in the development of centrosome amplification following DNA damage, we determined the effect of roscovitine, a potent inhibitor of the cdk activity (Mgbonyebi *et al*., 1999), on the level of expression of key G_1/S cell cycle checkpoint regulators and on HU-induced centrosome hyperamplification in the MDA-MB 231 cell line. Roscovitine treatment resulted in a decrease in both Rb phosphorylation and cyclin A expression in HU-treated cells, indicating an effective arrest in the progression of the G1/S cell cycle machinery (Figure 7a). Immunofluorescence analysis also demonstrated that roscovitine treatment effectively reduced centrosome hyperamplification induced by HU (Figure 7b–f, Table 1). These experiments demonstrated that in cancer cells lacking p21 expression, it is possible to experimentally block centrosome hyperamplification through the inhibition of cyclin/cdk activity. These results further substantiate an interplay between the p53-mediated G1/S cell cycle checkpoint, retinoblastoma function, and cyclin A expression in the maintenance of centrosome homeostasis following genotoxic stress in breast cancer cells.

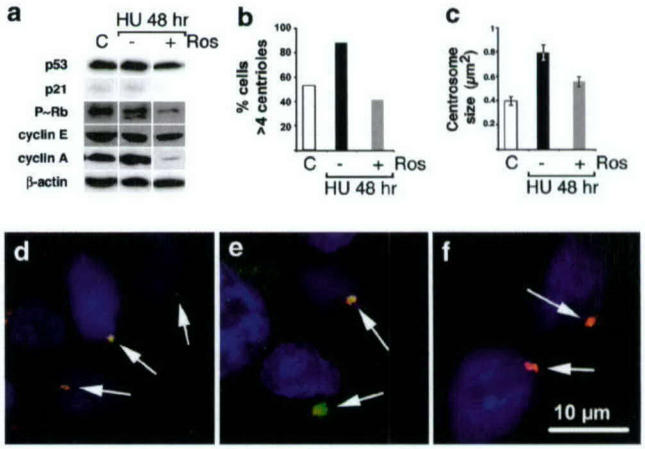

Figure 7 Effect of the cdk inhibitor roscovitine on expression of key cell cycle regulators and centrosome hyperamplification in HU-treated MDA-MB 231 cells. **(a)** Western analysis of the abundance of key cell cycle regulators (p53, $p21^{WAF1}$, phospho-Rb, cyclin E, and cyclin A) before and following treatment with HU alone or together with roscovitine. Whole-cell lysate (20 μg) was loaded in each lane. β-Actin was used as loading control. Graph showing the percentage of cells with more than four centrioles **(b)** and quantitative digital image analysis of centrosome size **(c)** before and following treatment with HU alone or together with roscovitine (48 h). Representative immunofluorescence of centrosomes (pericentrin stained red and centrin stained green) before **(d)** and following treatment with HU alone **(e)** or together with roscovitine **(f)** for 48 h. These results show that inhibition of cdk/cyclin by roscovitine reduces the level of phospho-Rb and cyclin A and consequent inhibition of centrosome hyperamplification following genotoxic stress

Discussion

Deregulation of cell cycle checkpoints, development of genomic instability, and aneuploidy are hallmarks of breast cancer (Anbazhagan *et al*., 1991; Wenger *et al*., 1993; Gunther *et al*., 1997). Genomic instability represents a major problem in the management of breast cancer patients due to the generation of diverse tumor cell phenotypes, which in turn may facilitate the development of chemoresistance and tumor progression (Khong and Restifo, 2002; Pinto *et al*., 2003). However, molecular mechanisms associated with the development of genomic instability and aneuploidy are poorly understood in breast cancer. Several studies suggest that deregulation of the centrosome cycle may lead to the development of chromosomal instability through the formation of multipolar mitotic spindles and unequal chromosome segregation (Lingle and Salisbury, 1999; Goepfert *et al*., 2002; Kramer *et al*., 2002; Lingle *et al*., 2002; Duensing and Munger, 2003; Pihan *et al*., 2003). The tumor suppressor gene p53, mutated in more than 50% of human cancers (Lane and Benchimol, 1990; Levine *et al*., 1991), plays an important role in the maintenance of centrosome homeostasis since loss of p53 function can lead to centrosome defects (Fukasawa *et al*., 1996; Borel *et al*., 2002; Tarapore and Fukasawa, 2002). However, since p53-null mice develop normally and have normal centrosomes in most of their cells, the development of centrosome amplification in this model may result only after failure of p53 function following genotoxic stress. Furthermore, the cdk/cyclins have also been implicated in the control of centrosome duplication (Hinchcliffe *et al*., 1999; Lacey *et al*., 1999; Meraldi *et al*., 1999) and changes in their expression correlate with centrosome amplification (Kronenwett *et al*., 2003), suggesting an interplay between p53 and cdk/cyclin

regulatory pathways in coordinating centrosome duplication with other cell cycle events.

In order to investigate the role of the G_1/S cell cycle checkpoint in the development of centrosome amplification in cancer, centrosome characteristics were monitored in human breast cancer cell lines with intact or abrogated G_1/S checkpoint function. We compared the behavior of the breast tumor-derived cell lines MCF-7, vMCF-7^{DNp53}, and MDA-MB 231 and determined that the progressive loss of p53 function leads to the acquisition of increasingly severe centrosome defects following genotoxic stress. MCF-7 cells, which have wild-type p53, arrested the centrosome duplication cycle when DNA damage was introduced by treatment with either HU or DR. In this cell line, activation of the G_1/S cell cycle checkpoint was indicated by an increase of p53 and p21^{WAF1} expression and their nuclear localization, retinoblastoma hypophosphorylation, and reduction of cyclin A levels. Therefore, in these cells, activation of the G1/S checkpoint in response to genotoxic stress led to inhibition of centrosome overduplication and maintenance of a normal centrosome phenotype. In contrast, DNA damage led to centrosome amplification in vMCF-7^{DNp53} cells. In these cells, the introduced dominant-negative p53 led to weak induction of p21^{WAF1} expression, high level of phosphorylated retinoblastoma, and deregulation of cyclin A expression, indicating a failure of G_1/S cell cycle checkpoint activation. Interestingly, in vMCF-7^{DNp53} cells, centrosome amplification was seen only after induction of DNA damage, since in untreated cells the majority of centrosomes showed a normal phenotype. MDA-MB 231 cells, which have mutant p53 and displayed amplified centrosomes at the outset, developed a higher grade of centrosome defect following DNA damage. In these cells, the development of *centrosome hyperamplification* was also associated with failure of the G1/S cell cycle checkpoint through lack of p21^{WAF1} expression, retinoblastoma hyperphosphorylation, and cyclin A overexpression. Importantly, centrosome hyperamplification in these cells could be blocked by roscovitine, a potent inhibitor of cyclin/cdk activity that is currently under clinical trial (Senderowicz, 2003). Roscovitine treatment mimicked a role for p21 in the p53-defective cell line MDA-MB 231 by effectively rescuing G1/S cell cycle checkpoint function, which was characterized by Rb hypophosphorylation and low levels of cyclin A expression.

In conclusion, the findings reported here suggest that p53 and cyclin/cdk pathways regulate centrosome homeostasis by ensuring the integrity of the G1/S cell cycle checkpoint. Furthermore, they also demonstrate that loss of p53 function alone is not sufficient to cause the development of centrosome amplification, but rather failure of the G_1/S checkpoint activation following genotoxic stress is required to induce centriole overduplication. This mechanism may play an important role in the development of centrosome amplification during breast cancer development. However, these findings also have important clinical implications for the treatment of breast cancer because they suggest that tumor cells with defective G_1/S cell cycle checkpoint function may develop or increase centrosome amplification following treatment with genotoxic anticancer drugs. This process may facilitate the development of higher clonal heterogeneity leading to chemoresistance and poor outcome. To overcome the complications associated with centrosome amplification for this subset of breast cancer patients, treatment with cdk inhibitors in combination with anticancer genotoxic drugs may provide an attractive therapeutic approach.

Materials and methods

Cell lines

The human breast cancer cell lines (MCF-7 and MDA-MB 231) were obtained from ATCC (Manassas, VA, USA) and maintained in EMEM medium containing 5 mM glutamine, 1% penicillin/streptomycin, and 10% FBS at 37°C in 5% CO_2 atmosphere. MCF-7 cells were supplemented with 20 μg insulin/ml, and MCF-7 cells carrying a dominant-negative p53 mutant (vMCF-7^{DNp53}) were grown in selection medium containing 500 μg/ml G148.

Generation of dominant-negative p53 clones

The pCMV-p53mt135 expression vector (Clontech, Palo Alto, CA, USA) was used to generate MCF-7 clones carrying a dominant-negative p53 mutant that had a G to A conversion at nucleotide 1017. This mutant, which is characterized by a Val 135 substitution, forms a mixed tetramer with the p53 wild-type protein that is unable to activate its downstream targets, without exerting gain of function activity (Harvey *et al*., 1995). MCF-7 cells were plated at a density of 3×10^5. After 24 h, cells were transfected using Fugene 6 reagent (Roche, Indianapolis, IN, USA) following the manufacture's instructions. After 48 h, cells were washed in PBS, and were selected over 3 weeks in medium containing 500 μg/ml G418. Colonies derived from single cells were isolated using cloning cylinders, followed by limiting dilution. Individual subclones were grown to 80% confluence and frozen. The subcellular localization and expression of the dominant-negative p53 in MCF-7 clones were confirmed by immunofluorescence and Western blotting analysis. Clone 8 was used for the experiments described in this study.

Induction of genotoxic stress

To investigate the relationship between centrosome duplication and G_1/S checkpoint activation, cell lines were plated at a density of 3×10^5. After 48 h, cells were treated with 2 mM HU or 1 μM DR to induce DNA damage and analysed at different time points up to 48 h. Roscovitine (10 μg/ml) was also used to inhibit cdks activity in combination with HU in the MDA-MB 231 cell line for 48 h.

Cell cycle profile

For fluorescence-activated cell sorting (FACS) analysis, cell lines were washed with cold PBS, fixed in 95% ethanol, stained with propidium iodide for 30 min and analysed by flow cytometry using Facscan by Becton Dickinson (Franklin Lakes, NJ, USA). The resulting cell cycle profiles, based on 20 000 events, were analysed using the ModFit program by Verity Software House (Topsham, ME, USA).

Immunofluorescence

For indirect immunofluorescence analysis, cells were grown on coverslips, fixed in methanol at −20°C for 10 min, blocked in 5% normal goat serum, 1% glycerol, 0.1% BSA, 0.1% fish skin gelatin, 0.04% sodium azide and incubated with primary antibodies directed against the following proteins: p53 (Santa Cruz, Santa Cruz, CA, USA) and $p21^{WAF1}$ (Oncogene, Darmstadt, Germany). For the staining of centrioles and pericentriolar material, we used antibodies against the proteins centrin and pericentrin, respectively. Coverslips were washed three times in PBS followed by incubation with secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR, USA), washed three times in PBS and finally incubated in DAPI to stain DNA. Immunofluorescence images were digitally recorded using a Nikon FXA fluorescence microscope equipped with computer-controlled focus, CCD digital camera, and Metamorph TM (Universal Imaging Corp, Downingtown, PA, USA) software. Fields were recorded at multiple focal planes and analysed and printed as maximum projections to assure that all centrioles and centrosomal structures were imaged.

Quantitative analysis of centrosome size and number

The NIH image J program was used for the quantitative analysis of centrosome size. For each cell line and treatment, centrosome area (μm^2) was determined by measuring the fluorescence signal for overlaid images of centrin and pericentrin defined by a contiguous region of fluorescence after adjusting threshold levels to eliminate background staining. The percentage of cells with duplicated centrioles and those showing more than two centrosomes was also determined. The values reported represent the average of 100 cells in each of two independent experiments.

Western blotting

For Western blot analysis, 20 μg protein of whole-cell lysate were run in 12% SDS–PAGE, transferred to PVDF membrane, fixed in 0.25% glutaraldehyde, blocked in 5% nonfat dry milk and incubated with primary antibodies against the following proteins: p53 (D07 DAKO A/S), $p21^{WAF1}$, phosphoretinoblastoma (Sigma, St Louis, MO, USA), cyclin E and cyclin A (Santa Cruz), and β-actin (Sigma) as loading control. After washing in Tween-20 buffer, PVDF membranes were incubated with HRP secondary antibodies (Amersham, Piscataway, NJ, USA), and signal was detected using the ECL-plus reagent (Amersham) following the manufacture's instruction.

Electron microscopy

Cells were fixed in 2% glutaraldehyde in sodium phosphate buffer, pH 7.2, for 2 h, postfixed in osmium tetroxide, dehydrated through an ethanol series, and embedded in Spurr's resin. Silver-gold sections were stained in 2% aqueous uranyl acetate and Reynold's lead citrate, observed using a JEOL 1200 EX electron microscope and recorded digitally.

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