Downregulation of Aurora-A overrides estrogen-mediated growth and chemoresistance in breast cancer cells

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Abstract

Estrogen is known to play a causative role in the development of sporadic breast cancers and chemoresistance. However, studies on the mechanism and proteins involved in mediating the oncogenic effects of estrogen are very limited. Recently, Aurora-A, a centrosomal protein kinase, which induces centrosome amplification and genomic instability, has been shown to be upregulated during long-term treatment of rats with estrogen and was implicated in estrogen-induced oncogenesis. Herein, we present results of the studies carried out in short-term in vitro cultures to understand the regulation of Aurora-A by estrogen and the effect of downregulation of Aurora-A on estrogen-induced breast tumorigenesis and chemoresistance. Treatment of breast cancer cells with 10 nM 17β-estradiol (E2) resulted in the upregulation of Aurora-A levels in an estrogen receptor-dependent manner. However, the upregulation by E2 was not restricted to Aurora-A alone. Following release from the tamoxifen-induced arrest, the appearance of Aurora-A in the presence of estradiol in MCF7 cells coincided with the appearance of other mitotic markers suggesting that the spike in Aurora-A levels is an indirect consequence of estrogen-mediated cell proliferation. Thus, at least in short-term in vitro studies, Aurora-A is not a specific direct target of estrogen. However, downregulation of Aurora-A by RNA interference led to a significant decrease in estrogen-induced, anchorage-dependent, and independent growth of MCF7 cells. Moreover, knockdown of Aurora-A could overcome estrogen-induced decrease in docetaxel sensitivity of MCF7 cells. Cumulatively, we propose that the upregulation of Aurora-A by estrogen in short-term in vitro cultures is an indirect consequence of estrogen-mediated cell proliferation. Nevertheless, Aurora-A inhibitors could be exploited to override the effects of estrogen on breast tumorigenesis and chemoresistance.

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Introduction

Estrogen plays a major role in promoting the proliferation of both normal and neoplastic breast epithelium (Bernstein & Ross 1993, Pike et al. 1993). Recent epidemiological studies show that the prolonged exposure to estrogen can be a risk factor in the development of sporadic breast cancer (Clemens & Goss 2001). The receptor-mediated hormonal response is one of the few mechanisms considered responsible for the carcinogenicity of estrogen. It has been shown that estrogen stimulates cell proliferation in a receptor-dependent mechanism resulting in accumulation of genetic damages that lead to carcinogenesis (Ikeda & Inoue 2004). The levels of estrogen receptor (ER) are higher in two-thirds of breast tumor tissues than normal breast (Early Breast Cancer Trialists’ Collaborative Group 1998). Moreover, correlation exists between ER expression and the breast cancer risk in postmenopausal woman (Sharma et al. 2006). It has been observed that the highly proliferative ER-positive type 1 lobules are highly susceptible to transformation in vitro and the site of origin of ductal carcinomas (Russo & Russo 2003). Nevertheless, the studies on the identification of key molecules involved in the oncogenic effects of estrogen are still minimal. Currently, hormone-related cancers are clinically managed by endocrine therapy with anti-estrogen
drugs (Howel 2000, Ali & Combos 2002). However, a considerable portion of the breast cancer patients eventually develops resistance against these anti-estrogen drugs. Alternatively, paclitaxel, a microtubule-interfering agent, has been widely used for both breast and ovarian cancer treatment. In the presence of estrogen, the extent of apoptosis induced by paclitaxel, however, was effectively reduced in breast and ovarian cell lines in vitro (Razandi et al. 2000) suggesting a role for estrogen as a survival factor also (Lippman & Dickson 1989).

Aurora-A kinase is an important member of the subfamily of aurora kinases that play essential roles in mitotic events (Giet & Prigent 1999). Overexpression of Aurora-A results in defective spindle assembly checkpoint, which monitors abnormal chromosome separation leading to aneuploidy (Bischoff & Plowman 1999). Aurora-A is overexpressed in many cancer types and mapped to human chromosome 20q13 region that is frequently amplified in many human cancers (Tanaka et al. 1999, Gritsko et al. 2003, Li et al. 2003). Higher levels of Aurora-A in human and rodent cells induce centrosome amplification, aneuploidy, transformed phenotype, and tumorigenesis in nude mice (Bischoff et al. 1998, Zhou et al. 1998). Overexpression of Aurora-A significantly correlates with induction of aneuploidy, centrosome anomaly, poor prognosis, and invasiveness of the primary human tumors and of experimental tumors in animal model systems (Sakakura et al. 2001, Buschhorn et al. 2005).

Breast cancers and solid tumors generally exhibit centrosome amplification characterized by abnormalities in centrosome number, organization, and behavior (Lingle et al. 2002). Similar centrosome amplification has been observed in a well-characterized rat mammary tumor model where animals were exposed to methylnitrosourea (Sivaraman et al. 1998). Female August/Copenhagen/Irish (ACI) rats treated with physiological levels of 17β-estradiol (E2) developed mammary gland tumors with characteristics similar to those in ductal carcinoma in situ (DCIS) and invasive sporadic ductal breast cancer (Li et al. 2002). Prolonged treatment of estrogen in these rats for 4 months caused centrosomal abnormalities, aneuploidy generated by random and non-random chromosome changes and upregulation of key molecules such as myc and Aurora-A (Li et al. 2004). Similarities in the phenotypes led them to propose that upregulation of myc and Aurora-A could be responsible for the phenotypic changes associated with the estrogen-induced mammary gland tumorigenesis. In this communication, attempts were made to understand whether Aurora-A could be exploited as a therapeutic target to abrogate estrogen-induced transformation and chemoresistance of breast cancer cell line in short-term in vitro cultures. We observed that downregulation of Aurora-A led to a significant decrease in estrogen-induced, anchorage-dependent, and independent growth of MCF7 cells. Knockdown of Aurora-A could restore estrogen-induced decrease in docetaxel (Doc) sensitivity of MCF7 cells suggesting that Aurora-A inhibitors could be exploited to override the effects of estrogen on breast tumorigenesis as well as chemoresistance.

Materials and methods

Cells culture and hormone treatments
MCF7 cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin–streptomycin at 37°C with humid atmosphere at 5% CO2. For experiments with E2 (Sigma), cells were cultured in medium without phenol red (Sigma) supplemented with 5% charcoal–dextran-treated FBS (stripped serum; Hyclone, Logan, UT, USA) and 100 U/ml penicillin–streptomycin. Typically, cells were cultured in stripped serum-containing medium for 24 h before treatment with 10 nM E2. To block MCF7 cells in G1 phase, cells were treated with 1 μM of tamoxifen for 48 h. Mitogen-activated protein kinase kinase 1 and 2 (MEK1 and MEK2) inhibitor U0126 was used at 10 μM. MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or DMEM without phenol red (Sigma) supplemented with various components as above.

Small interfering RNA (siRNA) transfection
MCF7 cells grown in regular medium or in medium with stripped serum were plated at 0.75 × 105 per well in six-well plates. Transfection of double-stranded siRNA was done with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction. The targeting sequence, siAurora–A (5′-UGG CAA AUG CCC UGU CUU ACU GUC-3′) and control sequence, luciferase gene siGL2 (5′-ACA UCA CGU CGG AAU ACU UCG A-3′) were synthesized by Invitrogen.

Western blotting
Cells were lysed in 1× Laemmli buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS) with pulsed sonication and an equal amount of cell lysates were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose and the blots were probed with respective antibodies. The antibodies used were mouse anti-
Aurora-A (IAK monoclonal; BD Biosciences Pharmin-
gen, San Diego, CA, USA), rabbit anti-cyclin B1 (Santa Cruz), rabbit anti-cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti- β-tubulin (E7, Developmental Studies Hyridoma Bank, University of Iowa). Signals were detected with horseradish per-
oxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and either SuperSignal West Pico or Super-
Signal West Dura enhanced chemiluminescene solution (Pierce, Rockford, IL, USA).

Flow cytometry
Cells were harvested and fixed in 70% ethanol overnight at 4 °C. The fixed cells were pelleted and resuspended in PBS (Cells were pelleted, resuspended in propidium iodide staining solution (50 µg/ml propidium iodide, 100 µg/ml RNAse A and 0.1% Triton X-100) and incubated in the dark for at least 1 h before analysis using FACS Calibur system (Beckton Dickinson). The data were analyzed using Modfit (Beckton Dickinson) software.

Cell proliferation assay
MCF7 cells (0.75 × 10^5) were plated in six-well plate. Next day, cells were transfected with or without siRNA as described earlier. Twenty-four hours post-transfection, the medium was changed with experimental medium with or without E2 and was replaced every 24 h. After 72 h hormonal treatment, cells were harvested by trypsinization and the viable cells were counted by trypan blue dye exclusion. The number of cells in the control sample without siRNA and E2 treatment was referred as 100%.

BrdU assay
Cells were grown on glass cover slips in 35 mm tissue culture dish. Twenty-one hours after treatment with or without E2, cells were treated with 10 µM BrdU (Sigma) for 3 h to allow incorporation of BrdU. Cells were fixed with methanol/acetic acid (3:1) for 20 min at −20 °C. Cells were washed three times with PBS and incubated with 2 M HCl/0.5% Triton X-100 for 30 min at room temperature and again washed three times with PBS and incubated with 100 mM Tris–HCl (pH 7.8) for 15 min at room temperature. Cells were then washed three times with PBS, blocked with 1% BSA/0.5% Triton X-100/PBS for 30 min at room temperature and incubated with FITC-conjugated anti-BrdU (eBiosciences, San Diego, CA, USA) at 1:10 dilution together with 0.1 µg/ml propidium iodide in 1% BSA/0.5% Triton X-100/PBS for 1 h in dark at room temperature. Again, cells were washed three times with PBS and once with distilled water and mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA, USA). The slides were observed under fluorescence microscope (Carl Zeiss, Jena, Germany) for positive staining of cells. Two hundred nuclei were counted and the percentage of BrdU-positive cells were calculated and presented.

Clonogenic assay
Approximately, 10^4 cells were plated in 60 mm dishes. The medium were replaced by medium with or without E2 and cultured for 4 weeks. Medium with the supplements was changed every 2 days. The cells were stained with 0.05% crystal violet/0.02% acetic acid (v/v) before photography. Experiments were performed in triplicates and two independent experiments were carried out.

Soft agar assay
Sixty mm dish agar plates were prepared by using 0.7% Bacto-agar (Oxoid, Basingstoke, Hampshire, UK) in RPMI without phenol red supplemented with 5% charcoal–dextran-treated FBS and 100 U/ml penicillin–streptomycin with or without E2 as bottom layer. Six thousand cells were mixed with 0.35% Bacto-agar in experimental medium with or without E2 and were poured on top of the bottom agar. Cultures were grown at 37°C with humid atmosphere at 5% CO2. Experimental medium with or without E2 was replenished every 2 days and a number of colonies with diameter more than 100 µm in a total of 500 colonies were counted after 4 weeks. Experiments were performed in triplicates and at least two independent experiments were done.

Doc sensitivity assay
MCF7 cells (0.75 × 10^5) were transfected with or without siRNA as described earlier. Eight hours post-transfection, cells were pretreated with or without 10 nM E2 for 24 h. Subsequently, cells were treated with either 100 nM Doc (Sigma) alone or in combination with 10 nM E2 for 72 h. At the end, cells were harvested and viable cells were counted using trypan blue dye exclusion. The cell lysates prepared under identical conditions were analyzed for poly (ADP ribose) polymerase (PARP) cleavage by western blot analysis.

Annexin V staining
Floating and adherent cells were collected, washed twice in PBS, resuspended in the binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl and
25 mM CaCl₂) at a concentration of 1 × 10⁶ and stained with Annexin V-FITC (BD Biosciences Pharmingen) and propidium iodide, according to the manufacturer’s instruction. Cells were analyzed immediately after staining by using FACSCalibur flow cytometry and CellQuest software (BD Biosciences).

Results

Higher levels of Aurora-A was observed in E₂-treated cells in an ER-dependent manner

Long-term treatment of estrogen to female rats resulted in centrosome amplification, mammary tumorigenesis, and upregulation of c-myc and Aurora-A (Li et al. 2004). It has been postulated that Aurora-A might be responsible for the phenotypes associated with estrogen-induced tumorigenesis in vivo. To understand the functional link between estrogen and Aurora-A, we initiated studies in short-term cultures of breast tumor cell line MCF7 in vitro. Treatment of MCF7 cells with E₂ resulted in higher Aurora-A protein levels compared with the control untreated cells (Fig. 1A). This increase in Aurora-A protein levels was followed up to 72 h with replenishment of E₂ every 24 h. Analysis of the Aurora-A transcript levels by real-time PCR showed that Aurora-A transcripts levels also increased twofold as early as 3 h and a fourfold induction was observed at around 24 h after E₂ treatment (Supplementary Figure 1, which may be viewed online at http://erc.endocrinology-journals.org/supplemental/). Intriguingly, levels of another mitotic marker cyclin B1 were also upregulated suggesting that the effect of E₂, however, is not specific to Aurora-A. However, similar effect of E₂ on Aurora-A was absent in another breast cancer cell lines, MDA-MB-231, lacking the ER, suggesting that E₂ effect on Aurora-A is receptor dependent. (Fig. 1B).

It is noteworthy, that higher levels of Aurora-A is normally observed in cycling cells (Giet & Prigent 1999) and estrogen has a pro-proliferative role on receptor-positive breast cancer cell lines (Pike et al. 1993). Hence, we speculated that in the absence of any specificity of E₂ effects on Aurora-A, higher levels of Aurora-A observed in the presence of E₂ could be an indirect consequence of the pro-proliferative effect E₂ on MCF7 cells. To address this, we analyzed the cell cycle profiles of MCF7 cells under identical conditions described in Fig. 1A. The results presented in Fig. 1C, show that E₂ treatment decreased the percentage of cells in G1 phase, in comparison with the cells grown in the absence of E₂ in stripped serum, with a concomitant increase in S and G2/M phase population. Cell proliferation assessed by BrdU incorporation also showed that in the presence of E₂ nearly 61% of the cells showed BrdU positivity in comparison with 32% in the control (Supplementary Figure 2, which may be viewed online at http://erc.endocrinology-journals.org/supplemental/). These data supported our interpretation that E₂ effect on Aurora-A could be due to increased proliferation of MCF7 cells.

Higher levels of Aurora-A observed in the presence of E₂ is cell cycle dependent

To further demonstrate the cell cycle-dependent nature of E₂ effect on Aurora-A, we arrested MCF7 cells at G1
phase of the cell cycle by tamoxifen treatment for 48 h followed by release into medium containing E₂ with or without the MEK1 and MEK2 inhibitor U0126 for up to 24 h. Cell cycle profiling of these cells at different time intervals (Fig. 2A) showed that at 8 h after release, all the samples were similar to the G₁-arrested, 0-h control. However, at 16 and 24 h after release, one could observe an increase of S phase population in E₂-treated cells, while the profiles of E₂ and U0126-treated samples were similar to that of the untreated cells. This suggested that U0126 was able to retard E₂-mediated cell cycle entry. Immunoblot analysis of the lysates from cells under conditions described in Fig. 2A for Aurora-A, cyclin A, and cyclin B₁ revealed (Fig. 2B) that the levels of Aurora-A were upregulated in tandem with other cell cycle proteins such as cyclin A and cyclinB₁ in the presence of E₂. Moreover, blocking the cell cycle progression with U0126 aborted the E₂-mediated increase in Aurora-A as well as cyclin B₁ and cyclin A levels confirming our speculation that the higher Aurora-A levels observed in the presence of E₂ is due to cell proliferation.

**Aurora-A knockdown retards estrogen-induced cell proliferation**

The results presented above indicate that Aurora-A could not be a direct target of estrogen, at least in short-term *in vitro* cell cultures. However, Aurora-A is one of the established anticancer target as well as a chemosensitizer (Anand *et al.* 2003, Yang *et al.* 2006) and inhibitors of Aurora family of kinases are currently on clinical trials (Shi *et al.* 2007). To explore the possibility whether downregulation of Aurora-A could counteract estrogen-mediated tumorigenesis and chemoresistance, we downregulated Aurora-A levels in MCF7 cells using RNAi (RNA interference) technology. The results presented in Fig. 3A indicate that efficient knockdown of Aurora-A was observed in MCF7 cells at concentrations as low as 1 nM of siRNA. Similar efficacy on the knockdown of Aurora-A with 1–10 nM siRNA could be demonstrated even in the presence of E₂ (Fig. 3B). To investigate the effect of Aurora-A downregulation on cell proliferation, cells were treated with different concentrations of Aurora-A siRNA in the presence and absence of E₂ and cell cycle profiles were then acquired. The data in Fig. 3C show that increasing concentrations of Aurora-A siRNA lowered the percentage of S phase population in both control and E₂-treated MCF7 cells. The effect was more obvious at higher concentrations (5 and 10 nM) of siRNA suggesting that knockdown of Aurora-A could impede E₂-dependent proliferation. This effect was more evident in cells synchronized with tamoxifen for 48 h and released in the presence of estrogen as cells treated with Aurora-A siRNA showed lower percentage of cells in S phase (Fig. 3D) than the control and mock-treated cells.

**Aurora-A knockdown reduces estrogen-induced, anchorage-dependent, and independent growth potential of MCF7 cells**

To extrapolate our findings that downregulation of Aurora-A levels reduced E₂-induced proliferation, we...
studied the effect of Aurora-A knockdown on the growth of MCF7 cells in the presence and absence of E2. We observed a nearly 100% increase in growth of mock and control luciferase siRNA transfected cells after 72 h in the presence of E2. However, this E2-induced increase in growth was progressively reduced in cells treated with increasing concentrations of Aurora-A siRNA (Supplementary Figure 3, which may be viewed online at http://erc.endocrinology-journals.org/supplemental/), suggesting that depletion of Aurora-A might have lead to cell death as observed by others (Du & Hannon 2004). To study the effects of knockdown of Aurora-A on cell growth in the absence of its apoptotic phenotype, the lowest concentrations of siRNA that showed the modest knockdown of Aurora-A with minimal apoptosis were determined. At 2.5 nM siRNA concentrations, the knockdown of Aurora-A was the modest and had minimal effect on apoptosis as judged by the Annexin V staining (Supplementary Figure 4, which may be viewed online at http://erc.endocrinology-journals.org/supplemental/). Since Aurora-A siRNA at 2.5 nM was effective both in the downregulation of Aurora-A and in reducing E2-induced growth and showed minimal cell death, anchorage-dependent growth of MCF7 cells in the presence of increasing concentrations of E2 was assessed by clonogenic assay and cell proliferation assay in the presence of either 2.5 nM control luciferase siRNA or Aurora-A siRNA. The data presented in Fig. 4A indicate that increasing concentrations of E2 promotes colony-forming potential of MCF7 cells in both mock and luciferase siRNA-transfected cells, while 2.5 nM Aurora-A siRNA tends to suppress the E2-induced growth. Similar results were mirrored in the growth assay where cell growth was monitored by cell counting. The results presented in Fig. 4B show that knockdown of Aurora-A had a minimal effect (20%) on the growth of MCF7 cells compared with the control or mock-transfected cells. However, it retarded growth of estrogen-treated cells more than 40% indicating that Aurora-A knockdown could counteract E2-induced cell growth. To address the relevance of Aurora-A knockdown in E2-induced tumorigenesis, the anchorage-independent growth of MCF7 cells on agar was also assessed. As shown in Fig. 5A and B, E2-treatment resulted in the growth of bigger and more number of colonies on agar. However, pre-treatment with Aurora-A siRNA reduced
Figure 4 Downregulation of Aurora-A impedes estrogen-induced growth. (A) MCF7 cells were transfected with either 2.5 nM control siRNA or 2.5 nM Aurora-A-specific siRNA. Eight hours post-transfection, 10,000 cells were plated in 60 mm dish and treated with different concentration of E2 for 4 weeks. Medium was changed every 2–3 days and the cells were stained with Crystal violet before photography. (B) MCF7 cells were transfected with either 2.5 nM control siRNA or 2.5 nM Aurora-A-specific siRNA. Twenty-four hours post-transfection, 10 nM E2 was added and cells were allowed to grow for further 72 h. Medium with E2 was replaced every 24 h. After 72 h hormonal treatment, viable cells were counted by trypan blue dye exclusion. Mock-treated sample without siRNA and E2 treatment was taken as 100%. Mock-treated control (□), Luciferase siRNA (siGL2) (○), and Aurora-A siRNA (△).

Figure 5 Aurora-A knockdown reduces estrogen-induced anchorage-independent growth of MCF7 cells. (A) MCF7 cells were transfected with either 10 nM control siRNA or 1–10 nM Aurora-A-specific siRNA. Eight hours post-transfection, 6000 cells were seeded in triplicates on soft agar and allowed to grow for 4 weeks in the presence and absence of E2 before staining with crystal violet. The experiment was repeated twice and a representative image is presented. (B) The soft agar assays were performed as in (A) and the number of colonies with diameter more than 100 μm in a total of 500 colonies observed were counted and presented as the percentage of colony formed. The results represent the mean of two independent experiments carried out in triplicates.
this colony-forming potential on agar surface. At the optimal 2.5 nM Aurora-A siRNA concentration, one could observe a 30% drop in the E2-effect compared with the mock or control luciferase siRNA. These data support the anti-tumor role of Aurora-A knockdown as well as its capacity to override E2-mediated transformation of MCF7 cells.

**Aurora-A knockdown overrides estrogen-mediated decrease in Doc sensitivity**

It is known that Aurora-A levels could modulate the sensitivity of cancer cells to drugs such as paclitaxel, cisplatin, etoposide, etc. (Anand et al. 2003, Yang et al. 2006). Also known is that estrogen protects breast and ovarian cancer cells from taxol-induced apoptosis (Mabuchi et al. 2004, Sui et al. 2007). Based on our data that Aurora-A downregulation can override E2-induced growth and transformation, we speculated that downregulation of Aurora-A might counteract the estrogen-mediated decrease in Doc sensitivity as well. To this end, we performed a viability assay by counting the number of viable cells grown under conditions described in Fig. 6A. Doc treatment decreased the viability of MCF7 cells by 60%, while presence of E2 protected the cells by decreasing the viability only by 45%. Luciferase siRNA-treated cells behaved similar to mock-treated control cohort. In contrast, cells pretreated with Aurora-A siRNA increased the sensitivity to Doc and decreased the viability of MCF7 cells by 65 and 72% at 2.5 and 10 nM respectively. Moreover, the E2-dependent protection was totally counteracted by the pretreatment with Aurora-A siRNA. Intriguingly, one could observe the highest percentage (80%) drop in the viability with pretreatment of Aurora-A siRNA at 10 nM concentrations in the presence of E2. Subsequently, we verified the effect of Aurora-A knockdown on the E2-mediated Doc resistance in MCF7 cells by Annexin V staining and flow cytometry. The data presented in Fig. 6B show that knockdown of Aurora-A restored the estrogen-mediated drop in the percentage of Annexin V-positive Doc-treated cells. To provide additional evidence, cleavage of PARP, an indicator of ongoing apoptosis, was analyzed in the lysates prepared from cells under similar conditions described in Fig. 6A. Analysis of the data indicated that E2 rendered a protective effect

![Figure 6](image-url)

**Figure 6** Aurora-A knockdown overrides estrogen-mediated decrease in docetaxel sensitivity. MCF7 cells were transfected with or without siRNA. Eight hours post-transfection, cells were pretreated with or without E2 for 24 h. Subsequently, cells were treated with either 100 nM docetaxel (Doc) alone or in combination with 10 nM E2 for 72 h. Cells were harvested and counted by trypan blue dye exclusion (A), or analyzed for apoptosis by Annexin V staining (B), or analyzed for cleaved PARP by immunoblot analysis (C).
on Doc-induced apoptosis as observed by a decrease in PARP cleavage (Fig. 6C). Higher levels of cleaved PARP in cells pretreated with Aurora-A siRNA was observed in the presence of E2. Quantitation of band intensities of cleaved PARP in the immunoblot showed that the pretreatment with Aurora-A siRNA increased PARP cleavage marginally compared to cells treated with Doc alone. Intriguingly, Aurora-A knockdown-dependent cleavage of PARP showed a further increase in the presence of E2. Collectively, these data support our previous observation made in the viability assay that Aurora-A knockdown could override the E2-induced decrease in Doc sensitivity.

Discussion

Estrogen plays a positive role in the development of sporadic breast cancer. However, the carcinogenic nature of estrogen has not been understood well. The three proposed mechanisms responsible for the carcinogenicity of estrogens involve i) the receptor-mediated hormonal response; ii) cytochrome P450-mediated metabolic activation that leads to genotoxic effects, and iii) induction of aneuploidy by estrogen (Russo et al. 2003). Recently, it has been shown that long-term treatment of estrogen in rats resulted in mammary gland tumorigenesis with characteristics of DCIS and was accompanied by the upregulation of oncogenes such as myc and Aurora-A (Li et al. 2004). Similarly, in an estrogen-induced Syrian hamster kidney tumor model, centrosome amplification and overexpression of Aurora-A and Aurora-B was observed in a receptor-dependent fashion (Hontze et al. 2007). These information lend hand to the proposition that receptor-mediated alteration in the key cell cycle regulators such as Aurora-A might play an important role in estrogen-induced tumorigenesis. Alternatively, in addition to being pro-proliferative, estrogen has been shown to be a survival factor also. Estrogen has been shown to abrogate the apoptotic response of ovarian cancer cells to paclitaxel (Mabuchi et al. 2004) and ER-positive breast cancer cells tend to be more resistant to anti-neoplastic drugs such as paclitaxel and Doc (Su et al. 2007). On the other hand, Aurora-A has been emerging as an anti-cancer target for reasons related to its role in cell cycle regulation, checkpoint evasion, centrosome amplification, and aneuploidy. In this study, we tried to understand the link between estrogen and Aurora-A in short-term in vitro cell cultures with regard to estrogen-induced tumorigenesis and chemoresistance.

We have observed that E2 treatment increased the steady state levels of Aurora-A in an ER-dependent manner. However, the increase in protein levels is not restricted to Aurora-A alone. Concomitant increase of other cell cycle proteins such as cyclin A and cyclin B1 and absence of any increase when cell cycle entry is prevented by MAP kinase kinase inhibitor, U0126, indicate that higher levels of Aurora-A seen following E2-treatment is mainly due to cell proliferation. Based on these in vitro studies, it can be conceived that the consistent E2-induced upregulation of Aurora-A levels observed during long-term E2 treatment of rats in vivo and in Syrian hamster kidney tumor model could have also resulted from increased proliferation of target cells. This is supported by the observation that in rats the higher levels of Aurora-A mRNA were present during early pregnancy when proliferation levels are the highest and also during hormone-induced cell proliferation in virgin rats treated with hormones (Goepfert et al. 2002). However, we cannot exclude the possibility that an alternative mode of regulation of Aurora-A levels operates under in vivo conditions.

The anti-neoplastic role of Aurora-A in other cancer cell lines and its emerging roles beyond cell cycle led us to propose that Aurora-A, despite not being a direct target of E2, could still be able to override the E2-induced tumorigenesis and resistance to Doc in receptor-positive breast cancer cells. Indeed, we found that knockdown of Aurora-A decreased the proliferation rate of MCF7 cells in the presence of E2, which resulted in the decrease of anchorage-dependent growth and this could be achieved under condition where there is minimal Aurora-A-driven apoptosis. Further, we showed that downregulation of Aurora-A suppressed E2-driven anchorage-independent growth of MCF7 cells also confirming the anti-tumor role of Aurora-A in this context also. Mechanistically, knockdown of Aurora-A decreased the percentage of cells in the S phase (Fig. 3C) indicating the Aurora-A knockdown could influence cell proliferation. Similar effect of Aurora-A knockdown on the reduction in S phase population and cell proliferation was observed with another ER-positive cell line IBEP-2 in the regular growth medium ruling out that the effect observed was neither cell line specific nor growth condition dependent (Supplementary Figure 5, which may be viewed online at http://erc.endocrinology-journals.org/supplemental/). We did not observe any visible G2 arrest as observed by others under our Aurora-A knockdown conditions probably due to low concentrations of siRNA employed. However, it should be mentioned that we were able to observe higher percentage of G2/M cells if we synchronize
and release the cells after Aurora-A knockdown (data not shown).

Aurora-A also influenced the protective effect of E2 in Doc-induced apoptosis. As observed with other anti-neoplastic agents (Hata et al. 2005, Tanaka et al. 2007), Aurora-A knockdown increased Doc sensitivity. Furthermore, it counteracted the E2-dependent decrease in apoptosis. Indeed, Aurora-A knockdown was able to cause more apoptosis in the presence of E2 compared with untreated sample consistently (Fig. 6). The reason for this increased apoptosis is currently unclear. However, we observed a higher percentage of G2/M-arrested cells following Aurora-A knockdown with 100 nM siRNA in IBEP-2 cells in the presence of E2 (data not shown), suggesting that E2 could potentiate Aurora-A effect. The exact mechanism by which Doc exerts its cytotoxic effect on cancer cells is not clearly understood. It has been shown that paclitaxel, another member of the taxane family, has been shown to exert its apoptotic effect in ovarian cancer cells through phosphorylation of Akt and E2 significantly reduced the phosphorylation of Akt (Mabuchi et al. 2004), providing a mechanistic explanation for the anti-apoptotic role of E2. Recently, it has also been observed that Aurora-A activates Akt and induces chemoresistance in a p53-dependent manner in ovarian cancer cells (Yang et al. 2006). Based on these observations, it is tempting to speculate that similar pathways involving Akt might be responsible for the observed phenotypes following Aurora-A knockdown in the breast cancer cell line MCF7 (p53-proficient) also. However, the NF-kB signaling pathway, which is also targeted by paclitaxel (Huang et al. 2000), E2 (Kalaitzidis & Gilmore 2005), and Aurora-A (Sun et al. 2007), also seems to be another promising candidate to be investigated. It has been observed that while Aurora-A overexpression could influence the extent of chromosomal instability and Doc sensitivity in ER-negative tumors, it is ineffective in ER-positive tumors (Noguchi 2006) suggesting that there exists a crosstalk between estrogen and Aurora-A signaling pathways. The overriding effect of Aurora-A knockdown on E2-induced growth and chemoresistance could mean that the pathways/components influenced by Aurora-A knockdown might be dominant over the E2-mediated signaling. Conceivably, the findings presented in this work, has the potential to expand the clinical applicability of the Aurora-A inhibitors. The exciting new chapter in this study will be to explore the molecular links in these observed effects of Aurora-A knockdown on estrogen effects and if Aurora-A knockdown will have similar effects in vivo.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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