Estrogen-Induced Aurora Kinase-A (AURKA) Gene Expression is Activated by GATA-3 in Estrogen Receptor-Positive Breast Cancer Cells

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Abstract Aurora-A is a proto-oncogenic mitotic kinase that is frequently overexpressed in human epithelial malignancies including in breast and ovarian cancers. The mechanism of transcriptional upregulation of Aurora-A in human breast cancer is not yet elucidated. We report herein that Aurora-A transcription is positively regulated by GATA-3 in response to estrogen in estrogen receptor α (ER α)-positive cells. Transient expression of *aurora-A* promoter deletion mutants in luciferase constructs identified a GATA binding sequence motif as a functional regulatory element in ER α -positive breast cancer cells. Electrophoretic mobility shift assay identified the binding of regulatory proteins to the GATA element. Anti-GATA-3 antibody generated a supershifted complex. Recruitment of GATA-3 to the *aurora-A* promoter was verified by chromatin immunoprecipitation analysis with GATA-3 antibody. Ectopic expression of GATA-3 resulted in elevated expression of Aurora-A in both ER α -positive and negative cells while siRNA-mediated silencing

led to downregulation of endogenous Aurora-A in ER α -positive cells. Estrogen treatment of ER α -positive cells induced increased Aurora-A expression with enhanced recruitment of GATA-3 to the *aurora-A* promoter. Finally, in the ACI rat model of estrogen-induced breast cancer, known to be associated with elevated Aurora-A expression, we observed increased expression of GATA-3 in preinvasive and invasive mammary epithelial cells exposed to prolonged estrogen treatment and in developing breast tumors. These results demonstrate a direct positive role of estrogen in regulating Aurora-A expression through activation of the ER α -GATA-3 signaling cascade and suggest that this pathway may be critical in the origin of estrogen-stimulated sporadic breast cancer.

Keywords Aurora kinase-A · GATA-3 · Estrogen · Transcriptional control

Abbreviations

Aurora-A	Aurora kinase-A
ChIP	Chromatin immunoprecipitation
CIN	Chromosomal instability
DCIS	Ductal carcinoma in situ
ER α	Estrogen receptor α
EMSA	Electrophoretic gel mobility shift assay
E2	17 β -estradiol
MG	Mammary glands
MGT	Mammary gland tumors

Introduction

Aurora kinase-A is a critical regulator of mitotic cell proliferation playing essential roles in mitotic entry,

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centrosome maturation, mitotic spindle assembly, and chromosome segregation processes [1]. The Aurora kinase-A (AURKA) gene encoding the kinase, localized on the commonly amplified chromosome segment 20q13 in human cancers, was isolated by us as an amplified and overexpressed gene from human breast cancer cells [2] and referred to by the acronym of BTAK (breast tumor amplified kinase). Ectopic overexpression of the kinase induces chromosomal instability (CIN), centrosome anomalies, and tumorigenic transformation of human and rodent cells *in vitro* and *in vivo* [3, 4]. Aurora-A, thus, represents a unique proto-oncogenic mitotic kinase that is involved in the genetic pathway(s) underlying the origin of aneuploidy and centrosome aberrations, the two most commonly observed phenotypic alterations in human cancer cells. Elevated expression of Aurora-A has been found to occur frequently in various human epithelial malignancies including those of breast and ovary [4] with the incidence of overexpression, in some instances, reported to be predominantly associated with *in situ* carcinomas compared with invasive lesions of both breast and ovarian cancers [5, 6]. These findings together with the observation that overexpression of Aurora-A in cancer cells is more common than amplification of the encoding gene [7] indicate that altered transcriptional and/or posttranslational regulation rather than gene copy gain is the prevalent mechanisms responsible for elevated expression of the kinase in human tumors.

Expression of Aurora-A in cells undergoing normal mitosis is regulated in a cell cycle stage-specific manner. The mRNA and protein levels progressively rise as the cells enter G2-M phases with subsequent degradation of the protein by ubiquitin proteasome pathway mediated by Cdh1-activated anaphase promoting complex/cyclosome as the cells exit mitosis [8]. The mechanisms of transcriptional regulation of Aurora-A through the cell cycle have been investigated in a limited number of published studies. These studies reported that Aurora-A is transcriptionally regulated by a member of the Ets family E4TF1 and the Ets-related transcription factor GABP [9, 10]. The transactivation function of GABP, in turn, is regulated through interaction with an evolutionarily conserved multi-subunit coactivator TRAP220/MED1 complex that is known to play a central role in serving as a functional interface between DNA-bound transactivators and the RNA polymerase II-associated basal transcription apparatus. In addition, a tandem repressor element CDE/CHR downstream of the E4TF1/GABP binding motif was found to be essential for G2/M-specific transcription of Aurora-A. More recently, a member of the E2F transcription factor family, E2F3, has been reported to directly bind the *aurora-A* promoter and activate expression during G2-M phases of the cell cycle [11]. Positive correlation of the E2F3 levels

with Aurora-A protein in human ovarian cancers was further suggested to indicate that E2F3 may be responsible for upregulation of Aurora-A in a subset of human ovarian cancer.

Besides the studies mentioned above, detailed mechanisms of tumor-associated transcriptional upregulation of Aurora-A in human cancers have not been well investigated, and a number of reports have just begun to address the subject in a systematic manner. In this regard, epidermal growth factor receptor (EGFR) signaling pathway, commonly upregulated in human cancers, has been reported to induce nuclear interaction between EGFR and the signal transducer and activator of transcription 5 to activate AURKA gene expression [12]. Additionally, it has been shown that the fusion gene product between the EWS gene and the Ets transcription factor family member Fli1 gene, found in Ewing sarcoma, directly regulates expression of the Aurora kinases by interacting with the Ets binding sites in the promoter sequences of the Aurora-A and -B genes [13].

In view of the well documented role of Aurora-A overexpression in inducing neoplastic transformation and CIN in mammalian cells and its high incidence (>75%) in the human ductal carcinoma in situ (DCIS) and invasive breast cancers [6], the common sporadic forms of which are known to be stimulated by estrogen (E_2) in majority of the cases, we began to investigate if E_2 directly activates AURKA gene expression in human breast cancer cells. This question gained credence in light of the recently published evidence of E_2 -mediating Aurora-A overexpression in a rat model of breast cancer [14].

It is generally accepted that growth of over two thirds of breast tumors is stimulated by E_2 through the activation of estrogen receptor α (ER α), a member of the nuclear receptor superfamily and a master regulator of these tumors [15, 16]. E_2 stimulation of ER α -positive breast cancer cell growth has been reported to be mediated, at least in some instances, by the transcription factor GATA-3, possibly playing a role in maintaining ER α expression and sensitivity to the growth stimulatory effect of E_2 in breast cancer by inducing pioneer factors such as FOXA1 keeping ER α -sensitive loci in a transcriptionally active conformation [17]. The role of this estrogen-ER α -GATA-3 signaling axis in modulating the cell cycle regulatory genes has not been studied. By analyzing the activity of a series of deletion constructs of the *aurora-A* promoter in ER α -positive and negative breast cancer cells, we show that E_2 mediates recruitment of GATA-3 to the *aurora-A* promoter activating its expression in ER α -positive breast cancer cells. These data establish a direct role of E_2 in regulating Aurora-A expression in breast cancer possibly by inducing increased proliferation and predisposition to CIN and centrosome anomalies in early and preinvasive lesions.

Results

Identification of a Putative Positive Regulatory Region in the *aurora-A* Promoter in ER α -Positive Breast Cancer Cell Lines

To identify if the promoter activity of AURKA gene is differentially regulated in ER α -positive and negative breast cancer cells, we examined the protein expression levels of Aurora-A in several breast cancer cell lines and detected a trend of higher expression of Aurora-A in ER α ⁺ cells (Fig. 1a). It is important to note that although amplification of the AURKA gene in the breast cancer cell lines was seen irrespective of their ER α status [2, 4], Aurora-A protein expression appeared to be relatively higher in ER α ⁺ cells. For instance, while genomic copy numbers of the AURKA gene in ER α ⁺ BT474 cells and in ER α [−] MDA-MB-231 cells were similar, Aurora-A mRNA expression level is higher in ER α expressing BT474 cells than in MDA-MB-231 cells lacking ER α expression. These data implied that ER α may be regulating Aurora-A expression, which should be reflected in higher promoter activity in ER α ⁺ cells than ER α [−] cells. To test this hypothesis, we first performed a luciferase reporter assay to precisely measure the activity of *aurora-A* promoter in cells with or without ER α expression. We transiently transfected a luciferase construct of the full-length *aurora-A* promoter (pGL-1486) into ER α ⁺ BT474 cells and ER α [−] HMLE cells. As internal control, the luciferase activity was normalized with the activity of cotransfected renilla luciferase reporter plasmid (Fig. 1b and c). Consistent with the expression data of Aurora-A presented in Fig. 1a, the results showed higher luciferase activity in BT474 (ER α ⁺) than in HMLE (ER α [−]) cells, indicating that transcription factor(s) specifically expressed in an ER α ⁺ cell line positively regulated the activity of the *aurora-A* promoter.

To identify the cis-element(s) and respective trans-factor (s) responsible for activation of AURKA gene promoter in ER α ⁺ breast cancer cells, we examined the luciferase activities of promoter constructs with deletion to −415, −189, and −75 bp from the transcription initiation site. Deletion to −189 detected high promoter activity in BT474 cells compared to those of HMLE cells. However, deletion to −75 resulted in the dramatic loss of promoter activity which was similar to that observed in the HMLE cells, indicating that positive regulatory cis-element(s) is located between −189 and −75 bp (Fig. 1b and c). A search of the public database using Genomatix software identified several putative transcription factors binding to the elements in this region. Of particular interest in the list was the putative GATA element at −158 to −155 bp. Among six GATA transcription factors, the expression of GATA-3 is reported to have a strong association with the status of ER α

Fig. 1 Differential Aurora-A protein expression and promoter activities in ER α and GATA-3-positive and negative breast cancer cell lines. **a** Protein expression analyses of Aurora-A, GATA-3, and ER α in breast cancer cell lines. α -Tubulin was used as a loading control. Number represents relative amount of Aurora-A protein in the cancer cell lines compared to MCF-10A cells after normalization against amount of α -tubulin. **b** Schematic representation of the 5'-deleted *aurora-A* promoter-luciferase (*Luc*) construct. **c** BT474 (solid bars) and HMLE (open bars) cells were transiently transfected with the 5'-deletion *aurora-A* promoter-Luc constructs (pGL), and the relative luciferase activities were measured. Results are indicated as the ratio of luciferase activity of each construct relative to that of control vector. Data represent the means $\pm$ SD of three independent experiments.

expression [18]. Thus, we next investigated the possibility that GATA-3 was directly involved in the transcriptional regulation of the AURKA gene in ER α ⁺ breast cancer cells.

GATA-3 Binds to *aurora-A* Promoter

To determine whether GATA-3 binds to this putative cis-element, we first performed electrophoretic gel mobility shift assay (EMSA) of nuclear extracts prepared from GATA-3-positive cell lines MCF-7 and BT474 and GATA-3-negative cell line HMLE, with a nucleotide probe spanning the −166/−142 bp from the transcription initiation site of the AURKA gene. As shown in Fig. 2a, both GATA-

Fig. 2 GATA-3 binding to *aurora-A* promoter. **a** [γ - 32 P]-probe spanning -166/-142 bp of the *aurora-A* promoter was incubated with nuclear extracts from MCF-7 cells (lanes 2–4), BT474 (lanes 5–8), and HMLE (lane 9). Competition assays were performed with 200-fold molar excess amounts of unlabeled wild-type (WT) probe (lanes 3 and 6) or mutant (Mut) probe (lanes 4 and 7). For the supershift assay, the nuclear extract from BT474 cells was preincubated with antibody against GATA-3 (lane 8). Only labeled probe was also electrophoresed as negative control (lane 1). Arrows indicate specific DNA nuclear protein complex. **b** GATA-3 recruitment to putative GATA-3 site in the *aurora-A* promoter was determined by ChIP analysis of *aurora-A* promoter with antibody against GATA-3 or normal IgG as control in the indicated cell lines.

3-positive cell lines showed a specific band (lane 2 and 5) that was not seen in the nuclear extracts of HMLE cells. The formation of DNA–protein complexes was diminished by the addition of an excess amount of unlabeled probe (lanes 3 and 6). In comparison, competition with an excess amount of unlabeled mutant probe, with nucleotide substitutions in the putative GATA element (see “Materials and Methods”), did not affect the complex formation (lanes 4 and 7), indicating that the factor(s) forming the DNA–protein complex recognized the GATA element. We also performed supershift assay with a GATA-3-specific antibody to see if the binding factor was indeed GATA-3 and found that addition of the antibody generated a supershifted complex (Fig. 2a, lane 8).

We next determined whether GATA-3 was recruited to the *aurora-A* promoter in vivo. For this purpose, we performed chromatin immunoprecipitation (ChIP) assay with either a GATA-3 or a control IgG antibody followed by PCR analysis of the immunoprecipitated DNA with the specific primers which amplify the region surrounding the putative GATA element in the *aurora-A* promoter. The

result validated the specific recruitment of GATA-3 to *aurora-A* promoter (Fig. 2b).

GATA-3 Positively Regulates AURKA Gene Expression

In order to determine the effect of GATA-3 binding to the promoter activity of the AURKA gene in breast cancer cells, we first examined whether the ectopic expression of GATA-3 affected Aurora-A expression in GATA-3-negative

Fig. 3 GATA-3 positively regulates Aurora-A expression. **a** Either empty vector (–) or GATA-3 expressing vector (+) were transiently transfected into indicated cell lines for 24 h. The cell extracts were subjected to immunoblotting with indicated antibodies. α -Tubulin was used as a loading control. **b** Either control siRNA (–) or GATA-3 siRNA were transfected into indicated cell lines for 48 h. The cell extracts were subjected to immunoblotting with indicated antibodies. α -Tubulin was used as a loading control. **c** Expression level of Aurora-A mRNA in MCF7 and T47D cells transfected with either GATA-3 siRNA or control siRNA as described in **b** were measured by real-time RT-PCR. Data represent the means $\pm$ SD of three independent studies.

cells. We found that overexpression of GATA-3 significantly increases the protein levels of Aurora-A compared to an empty vector transfected into HMLE and MCF-10A cells (Fig. 3a). A similar effect was also observed in GATA-3-transfected BT474 cells (Fig. 3a). Next, we determined whether knock down of GATA-3 expression by small interference RNA (siRNA) reduced the promoter activity of the AURKA gene in GATA-3-positive cell lines. The results revealed a reduction of Aurora-A expression at both the mRNA and protein levels in cells transfected with GATA-3-specific siRNA compared to those transfected with control siRNA (Fig. 3b and c). These results demonstrate that GATA-3 is a transcription factor positively regulating AURKA gene expression in breast cancer cell lines.

Estrogen-Induced AURKA Gene Expression is Mediated by GATA-3

GATA-3 is known to be a target gene of the ER α and to mediate gene expression of a number of its target genes in response to E₂ treatment [17]. A previous study has demonstrated upregulation of Aurora-A after E₂ treatment in cells grown *in vitro* [19]; however, the precise mechanism of the transcriptional control of AURKA gene in response to E₂ remains unclear. Based on the above findings, we postulated that E₂ treatment enhances the recruitment of GATA-3 to the GATA element in the *aurora-A* promoter resulting in an increased expression of the AURKA gene. We first confirmed that E₂ treatment enhances AURKA gene expression in our system. Consistent with published results, upregulation of Aurora-A protein expression was observed after E₂ treatment in

MCF-7 cells in a time-dependent manner (Fig. 4a). When ChIP assay was performed under similar conditions using an antibody against GATA-3, we detected an increase in the immunoprecipitated DNA fragment of the *aurora-A* promoter after E₂ treatment compared with that of untreated control cells (Fig. 4b). These results confirmed that GATA-3 is a critical transcription factor mediating E₂-stimulated AURKA gene expression.

Association Between Aurora-A and GATA-3 Expressions in the Female ACI Rat Model of Breast Cancer

We have previously demonstrated that prolonged administration of estrogen to female ACI rats leads to induction of Aurora-A expression at both the mRNA and protein levels in 4.0-month E₂-treated mammary glands, as well as in the mammary gland tumors (MGT) after 5.0–6.0 months of E₂ treatment [14]. To determine whether this E₂-induced Aurora-A expression was accompanied by the induction of GATA-3 expression, we examined the temporal expression levels of GATA-3 and Aurora-A in mammary glands from control and ACI rats treated for 4.0 to 5.0 months with E₂ (4ME2, 5ME2), as well as in primary mammary gland tumors isolated from rats on E₂ for at least 5 months (Fig. 5). GATA-3 expression was detected uniformly in luminal cells of intact normal cycling untreated female ACI rat mammary glands (Fig. 5a) and randomly in hyperplastic mammary cells after 3.0 to 4.0 months of E₂ treatment (Fig. 5b). GATA-3 expression was markedly elevated in both incipient and large focal dysplasias, present after 3.5 to 4.0 months of E₂ treatment (Fig. 5c, d), while in DCISs and in tumors, GATA-3 was abundantly detected between 4.5 and 5.0 months of E₂ treatment (Fig. 5e, f). Western blot analyses showed that Aurora-A expression was ~10.0-fold above control untreated levels after 4.0 to 5.0 months of E₂ treatment, a period when the occurrence of DCIS was maximal (Fig. 5g). The expression of Aurora-A in DCIS was frequently greater than that seen in primary invasive ACI rat breast tumors (6.6–6.8-fold) when compared to untreated cycling age-matched mammary glands. Moreover, GATA-3 expression rose significantly after 4.0 to 5.0 months of E₂ treatment (2.7–3.4-fold) as in primary breast tumors (7.0–7.8-fold). In view of the *in vitro* experimental findings described above, these *in vivo* results are consistent with the notion that an early induction of GATA-3 mediates E₂-stimulated activation of Aurora-A expression in mammary gland epithelial cells and tumors.

Fig. 4 Estrogen enhances GATA-3 recruitment to *aurora-A* promoter. **a** MCF-7 cells were treated with 17 β -estradiol (E₂) for various periods of time, and the cell extracts were subjected to immunoblotting with indicated antibodies. **b** MCF7 cells were treated with either E₂ (+) or ethanol (–) for 1 h and harvested for ChIP analysis of *aurora-A* promoter with anti-GATA-3 antibody or control IgG.

Discussion

Epidemiological and experimental studies have provided convincing evidence that exposure to both systemic and

Fig. 5 Immunohistochemical and immunoblotting analyses of Aurora-A and GATA-3 protein expression in estrogen-treated ACI rat mammary glands and mammary tumors. **a–f** GATA-3 Immunohistochemical staining in **a** cholesterol-treated control mammary glands and (**b–f**) after E_2 treatment. **b** Hyperplasia (arrows) after 3.0 months. **c** Incipient focal dysplasia after 3.0 months. **d** Dysplasia after 4.0 months. **e** DCIS after 4.5 months. **f** Mammary tumor after 5.0 months. Notice that the GATA-3-stained cells in hyperplasia are

larger and mainly confined to cells within dysplastic lesions, DCIS, and tumors. Magnification 20×. **g** Immunoblotting analysis of cholesterol-treated control mammary glands (cont.), mammary glands E_2 treated for 4.0 and 5.0 months (4ME2, 5ME2), and E_2 -induced MGT. β -Actin was used as a loading control. Numbers at the bottom indicate relative fold expression levels of Aurora-A and GATA-3 in the treated samples compared with untreated control normalized against β -actin loading control.

exogenously ingested E_2 plays a role in the initiation and progression of sporadic breast cancer [16, 20]. It has been reported that E_2 stimulates cell proliferation in an $ER\alpha$ -dependent manner with accumulation of genetic damage leading to carcinogenesis [21]. Furthermore, $ER\alpha$ expression levels have been correlated with breast cancer risk in postmenopausal women [22]. Despite these interesting correlative findings on the oncogenic effects of estrogen, the genetic factors and their signaling pathways involved in the process have not yet been elucidated. The most notable evidence of an oncogenic event induced by E_2 came from studies performed in female ACI rats exposed to relatively modest physiological serum E_2 levels over an extended period of time, which revealed elevated expression of Aurora-A in parallel with developing mammary gland tumors displaying histologic, molecular, and ploidy changes similar to that seen in human DCIS and invasive

sporadic ductal breast cancers [14]. The role of the E_2 signaling cascade in the elevated Aurora-A expression remains unexplored. However, previous reports of similar phenotypic alterations of aneuploidy and resistance to Paclitaxel shared between E_2 -induced mammary tumors, mammary epithelial cells [23, 24], and those induced by overexpression of Aurora-A [7, 25] suggest that an overlapping molecular mechanism may be underlying these events. E_2 treatment has been found to increase the steady state level of Aurora-A in an $ER\alpha$ -dependent manner, while down regulation of Aurora-A has been found to override E_2 -mediated growth and chemoresistance in breast cancer cells in vitro [19]. However, the possibility that E_2 may induce tumor-associated phenotypic changes in mammary epithelial cells by activating Aurora-A expression directly has been disputed in the past since other cell cycle proteins such as cyclins A and B1 also increase in response to

estrogen treatment. Instead, it has been proposed that higher levels of Aurora-A expression following E_2 treatment reflects an indirect effect of cell proliferation. While this may be partially true, our present finding of GATA-3 positively regulating Aurora-A expression in $ER\alpha^+$ cells following recruitment to the promoter in response to estrogen treatment indicates that E_2 also directly activates Aurora-A expression in an $ER\alpha$ -dependent manner. Since Aurora-A inactivates p53 and BRCA1 tumor suppressor proteins [26, 27], affecting the DNA and spindle damage checkpoint response pathways, it is logical to suggest that prolonged E_2 exposure may cause deregulated activation of Aurora-A with consequential induction of malignant transformation in $ER\alpha^+$ mammary epithelial cells.

GATA-3 and $ER\alpha$ expression patterns are highly correlated in breast cancer [28, 29]. A positive cross-regulatory loop between these two genes reciprocally activating their expression has been proposed to account for the robust coexpression of GATA-3 and $ER\alpha$ in breast cancer cells [17]. On the other hand, GATA-3 is necessary for the determination and maintenance of luminal cell fate in the adult mammary gland [30, 31]. While there is a strong correlation of high GATA-3 expression with the luminal A subtype of breast cancers, in addition to being an excellent predictor of $ER\alpha$ status, low GATA-3 expression is a predictor of poor clinical outcome, high tumor grade, and positive lymph node status [32]. Since in an earlier study, expression of Aurora-A was found elevated only in DCIS but low in the invasive lesions of the same tumors [6], it is worth investigating if high GATA-3-driven Aurora-A-elevated expression is restricted to early and preinvasive stages of breast cancer. Intriguingly, since luminal differentiation genes are expressed in the absence of $ER\alpha$ [33], which typically induces cell proliferation, and GATA transcription factors have been implicated in cellular quiescence, it appears that GATA-3 and $ER\alpha$ pathways may have nonoverlapping functions in mammary ductal luminal cells. However, a functional interaction between GATA-3 and $ER\alpha$ has also been demonstrated through other factors such as FOXA1, a critical GATA-3-driven pioneer factor that maintains $ER\alpha$ -induced genes open for transcriptional activation through $ER\alpha$ [34]. At the same time, the transcriptional activity of GATA-3 is known to be regulated by the Friend of GATA (FOG) transcriptional effectors with GATA-3/FOG-2 interaction considered necessary for lobulo-alveolar development [35], which also requires Stat5 α , a recently reported activator of Aurora-A expression [12]. Therefore, it is likely that aberrant expression of the GATA-3 gene regulatory network in $ER\alpha^+$ mammary epithelial cells leads to deregulated expression of Aurora-A, predisposing the normal epithelium to acquire centrosome anomalies and CIN associated with development of

naturally occurring breast tumors in humans [36] and also in the experimental models of mammary carcinogenesis in rats [14, 37]. Prolonged E_2 exposure may be one physiological trigger capable of inducing such changes, as reported in the estrogen-induced model of mammary oncogenesis in female ACI rats [14]. Aurora-A overexpression accompanying centrosome anomaly and CIN are predominantly present in preinvasive DCIS in humans [6], and centrosome amplification together with Aurora-A overexpression was also shown to be an early event in rat mammary carcinogenesis [37]. Therefore, it is likely that CIN and centrosome aberrations are induced early following aberrant expression of $ER\alpha$ -GATA-3 signaling cascade in response to prolonged estrogen exposure leading to elevated Aurora-A level in mammary epithelial cells. Detailed analyses of Aurora-A expression profiles in reference to those of $ER\alpha$ and GATA-3 together with the incidence of CIN and centrosome aberrations in early and preinvasive lesions will help address these issues. In addition, characterization of the gene regulatory networks of Aurora-A in relation to $ER\alpha$ and GATA-3 may be informative prognostic markers of clinical outcome.

Materials and Methods

Cell Culture

The cells were cultured in RPMI 1640 medium (BT474), DMEM medium (MDA-MB-231, HBL-100), and DMEM/F-12 (T47D and MCF-7), supplemented with 10% FBS and penicillin/streptomycin. Both immortalized human mammary epithelial cell (HMLE) and MCF-10A cells were cultured in MEGM medium. HMLE cell line, established in the laboratory of Dr. Robert A. Weinberg at the Whitehead Institute for Biomedical Research, Cambridge, MA, was kindly provided by Dr. Sendurai Mani in the Department of Molecular Pathology at UTMD Anderson Cancer Center. All other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). For estradiol (E_2) treatment, cells were cultured in phenol red-free DMEM with 5% charcoal/dextran-treated FBS 24 h prior to treatment. Cells were cultured in fresh medium with 10 nM E_2 and were harvested as indicated and used in subsequent analysis. For the control treatment, ethanol (solvent of E_2) was used.

Luciferase Reporter Assays

The dual-reporter luciferase assay was performed according to manufacturer's instructions (Promega). Briefly, 1 μ g of full-length or deletion constructs of an *Aurora-A* promoter

(−1486~−75) in pGL3 vector (kindly provided by Dr. Ishigatsubo) were cotransfected with 100 ng of *renilla* luciferase reporter plasmid, a transfection efficiency control. Twenty-four hours after transfection, cells were lysed, and both reporter and control luciferase activities were measured and normalized.

Immunoblot Analysis

Whole cell extracts were prepared in RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Roche)). Antibodies used were Aurora-A (610939, BD Transduction Laboratory), ER α (F-10, Santa Cruz Biotechnology), and GATA-3 (HG3-31; Santa Cruz Biotechnology).

EMSA

Nuclear extracts were prepared from cells in 70–90% confluence by using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). The following sequences of the wild-type and mutant GATA binding probes were used: (1) wild-type probe: 5'-GCTTCACCGATAAATGGCCGACCGC-3' (forward) and 5'-GCGGTCGGCCATTATCGGTGAAGC-3' (reverse) and (2) mutant probe: 5'-GCTTCACCA $\underline{\underline{AA}}$ $\underline{\underline{CAA}}$ ATGGCCGACCGC-3' (forward) and 5'-GCGGTCGGCCA $\underline{\underline{TTT}}$ $\underline{\underline{GTT}}$ GGTGAAGC-3' (reverse). Mutated sites are underlined. The procedures and conditions were carried out as previously described [38]. For the supershift assay, anti-GATA-3 antibody was mixed with nuclear extracts on ice for 1 h before the radioactive probe was added.

GATA-3 Expression Plasmid, siRNA, and Transfection

GATA-3 cDNA was amplified by PCR from pMIG-GATA-3 retrovirus vector (kindly provided from Dr. Werb) with the primers, 5'-GAGATCTATGGAGGTGACGGCGG-3' and 5'-GGAATTCCTAACCCATGGCGGTGAC-3'. The PCR product was digested with BglII and EcoRI, and later subcloned into the BamHI and EcoRI site of pcDNA3.1 vector (Invitrogen). Both GATA-3 siRNA and control siRNA (Dharmacon) protocols were previously described [17]. The transfection of the plasmids or siRNAs (40 nM) was carried out for 48 h using Lipofectamine or Oligofectamine (Invitrogen), respectively, according to the manufacturer's instructions.

RNA Preparation and Real-Time PCR

Total RNA from transfected cells was prepared using Trizol reagent (Invitrogen). Real-time PCR for the quantification of Aurora-A mRNA and 18S rRNA was performed using ABI 7900 and ABI SYBR green for Aurora-A with the

primers previously described [39] and Taqman Kit for 18S rRNA (ABI). The target cDNA quantitation was performed according to the manufacturer's instruction, normalizing the threshold cycle number of Aurora-A relative to that of 18S rRNA. Later, Aurora-A and 18S rRNA were quantified from two independent cDNA preparations from each sample, and the final relative quantification was represented as the average of two measurements.

ChIP

The ChIP was performed as previously described [40]. Ten percent of the chromatin DNA before each immunoprecipitation was utilized to isolate DNA by reverse cross-linking and extraction, which was subsequently used as a source for input DNA in each PCR. The PCR primers used were as follows: 5'-GGCTGTTGCTTCACCGATA-3' (forward) and 5'-ACTTGCTCCCTAAGAACCCG-3' (reverse). PCR conditions were 95°C for 2 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s.

Animals and Treatment

Immunoblotting and immunohistochemical analysis were conducted in intact 6–8-week female ACI rats (Harlan Sprague Dawley, Inc.). They were housed individually in an AAALAC-accredited facility with an approved animal protocol. The rats were acclimated for 1 week prior to treatment, randomly distributed into control and treatment groups, and treated with a single pellet containing either 20 mg cholesterol alone or 3 mg of E₂ plus 17 mg of cholesterol (Hormone Pellet Press, Leawood, KS) implanted in the shoulder region for 3.0–6.0 months as previously described [14]. The serum E₂ levels were as previously reported (123.5 $\pm$ 4.8 and 121.8 $\pm$ 3.0 pg/ml at 4.0 and 6.0-month E₂ treatment) [23].

Immunohistochemistry

Five- to six-micrometer formalin-fixed paraffin-embedded mammary glands were analyzed by immunohistochemistry as previously described [23]. Anti-GATA-3 (HG3-31, Santa Cruz Biotechnology) was used. Negative controls were performed in the absence of primary antibody. Sections were then incubated with a biotinylated antirabbit secondary antibody, and signal amplification was accomplished using Vector Laboratories Elite ABC reagent (Burlingame).

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