RbAp46 Inhibits Estrogen-stimulated Progression of Neoplastigenic Breast Epithelial Cells

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Abstract. Background: Despite widespread agreement that estrogens are involved in the etiology of human breast cancer, there is uncertainty as to the molecular mechanisms of estrogen action in early development of breast cancer. Materials and Methods: MCF10AT3B cells, a cell line derived from a xenograft model of human proliferative breast disease, were used to study the estrogen-stimulated malignant progression of neoplastigenic mammary epithelial cells. A stable cell line was established from MCF10AT3B cells that ectopically expresses the retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46), a component of the histone modifying and remodeling complexes. Western blot and in vitro and in vivo growth assays were used to study the effects of constitutive RbAp46 expression on estrogen-stimulated cell proliferation. Results: Estrogen treatment downregulated RbAp46 expression in MCF10AT3B cells. Constitutive RbAp46 expression inhibited estrogen-stimulated cell growth in vitro. In nude mice, RbAp46 expression strongly suppressed estrogen-stimulated tumorigenesis of MCF10AT3B cells. Constitutive RbAp46 expression inhibited estrogen-stimulated cell growth in vitro. In nude mice, RbAp46 expression strongly suppressed estrogen-stimulated tumorigenesis of MCF10AT3B cells. In RbAp46-expressing tumors, β-catenin protein was highly phosphorylated and the steady state levels of β-catenin protein were significantly reduced. Conclusion: RbAp46 plays an important role in the regulation of mitogenic estrogen signaling and dysregulated RbAp46 expression may contribute to estrogen-stimulated breast cancer development.

Experimental evidence for the role of endogenous estrogens in the etiology of breast cancer comes from numerous studies of serum and urine hormone levels in populations at low and high risk for breast cancer, and from case control and cohort studies comparing serum hormone levels in breast cancer patients and healthy women (1). The effects of estrogens on the proliferation of breast epithelial cells are mediated through transactivation of specific genes such as c-Myc and cyclin D1, and activate cyclinE-Cdk2 complexes, all of which are rate limiting for the progression from the G1 to the S-phase of the cell cycle (2). Based on this evidence, it is believed that estrogen-induced cell growth is associated with the recruitment of non-cycling G0 cells into the cell cycle and an accelerated rate of progression through the G1-phase. However, it was reported that the early events induced by estrogen during the recruitment of quiescent cells into the cell cycle are insufficient to drive the cells to completion of the cell cycle (3). Other, still-unidentified, regulatory checkpoints must be crossed by the estrogen-stimulated cell in order for it to progress through the cell cycle.

The human cell line MCF10A originated from spontaneous immortalization of non-malignant breast epithelium. MCF10A cells transfected with T24 Ha-ras mutant (designated as MCF10AT) acquired the ability for xenograft growth in the dorsal flank of nude mice (4). The MCF10AT3B cell line was established from a hyperplastic lesion found in the third generation transplant of nude mice (5). It has been reported that expression of the estrogen receptor (ER) gene is activated in MCF10AT cells and cells of MCF10AT variants, all of which express a transfected mutated T24/ras gene (6). However, ER transcripts are undetectable in the parental MCF10A cells and in MCF10A cells transfected with normal c-Ha-ras and empty vector. Furthermore, estrogen treatment increases both the number and size of tumors formed by MCF10AT3B cells (7). Thus, MCF10AT3B cells provide an excellent model system to study estrogen-stimulated malignant progression of neoplastigenic mammary epithelial cells.

Retinoblastoma suppressor (Rb) associated protein 46 (RbAp46) was first identified as a protein interacting with Rb (8) and was later found to be a component of the mSin3 histone deacetylase (HDAC) complex (9), which is involved in the transcriptional repression mediated by transcription...
factors such as Rb (10). Previously, we isolated RbAp46 as a downstream target gene of the Wilms’ tumor suppressor gene product, WT1 (11). We have further found that constitutive expression of RbAp46 inhibits the transformed phenotype of adenovirus-transformed human embryonic kidney 293 cells (12) and suppresses tumorigenicity of neoplastigenic mammary epithelial cells (13). We and others have discovered that RbAp46 specifically interacts with the BRCT domain of the breast cancer and ovarian cancer susceptibility gene BRCA1 and modulates its transcriptional transactivation activity (14, 15), suggesting that RbAp46 may be involved in tumor suppressor activity of BRCA1 and the development of breast cancer. Recently, we found that RbAp46 expression is down-regulated in breast cancer cells and re-introduction of RbAp46 into breast cancer cells reverts transformed phenotypes of breast cancer cells (16). We studied the effect of estrogen treatment on RbAp46 expression in MCF10AT3B cells and the function of constitutive RbAp46 expression in estrogen-stimulated malignant progression of the neoplastigenic MCF10AT3B cells.

Materials and Methods

Cell culture and stable cell lines. MCF10AT3B cells were obtained from Karmanos Cancer Institute (Detroit, MI, USA). Cells were maintained at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 5% horse serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), EGF (2 ng/ml) and cholera toxin (0.1 ng/ml). MCF10AT3B cells stably transfected with RbAp46 expression vector (46-3B) and control cells transfected with empty expression vector (control) were described previously (13). For cell growth assays, cells were maintained in phenol-red free DMEM/F12 medium plus 5% charcoal-dextran stripped fetal calf serum for three days and then were treated with 1 nM 17β-estradiol, or vehicle as controls. The cells were seeded at 1x10⁶ cells per well in a 24-well plate and counted every other day using a hemacytometer.

Western blot analysis and antibodies. Tumors dissected from nude mice were homogenized in [T-PER tissue] protein extract reagent (Pierce, Rockford, IL, USA). The protein concentrations of the lysates were measured and proteins were denaturated by boiling in a gel-loading buffer and separated by SDS/PAGE in 10% gels. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with different first antibodies, appropriate HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The same membranes were stripped and reprobed with an antibody against β-actin as loading controls.

Antibodies against α- and β-catenin were purchased from BD Biosciences Transduction Laboratories (Lexington, KY, USA). Antibodies specific for phosphorylated β-Catenin and HA-tag were purchased from Upstate Biotechnology (Charlottesville, VA, USA). Antibodies against β- and γ-catenin were purchased from BD Biosciences Transduction Laboratories (Lexington, KY, USA). Antibodies specific for phosphorylated β-Catenin and HA-tag were purchased from Upstate Biotechnology (Charlottesville, VA, USA).

RNA extraction and Northern blot analysis. Total cellular RNA was isolated using Trizol from Invitrogen (Frederick, MD, USA), according to the manufacturer’s instruction. Ten μg of total RNA were separated by electrophoresis on a 1.2% formaldehyde gel and blotted onto a nylon membrane, Hybond-N from GE Healthcare (Buckinghamshire, UK). The blots were prehybridized for 1 hour and hybridized for 2 hours in Quick-Hybridization solution (GE Healthcare, Buckinghamshire, UK) at 65°C. The probes included: a cDNA fragment of pS2 purchased from Incyte Corporation (Wilmington, DE, USA) and an RbAp46 cDNA probe. The blots were washed twice with 2x SSC and 0.1% SDS for 15 minutes at room temperature and twice with 0.1x SSC and 0.1% SDS for 15 minutes at 55°C. Blots were then autoradiographed using intensifying screens at –70°C overnight.

Tumor formation in nude mice. Forty-eight female athymic nude (nu/nu) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were ovarioctomized and a 60-day release estradiol pellet containing 1.7 mg of 17β-estradiol (E2; Innovative Research of American, Sarasota, FL, USA) was placed under the skin of twenty-four mice. As controls, placebo pellets were implanted into the remaining twenty-four mice. One week before the injection, the culture medium of cells was switched to phenol red-free Improved Minimum Essential Medium (IMEM) plus 5% charcoal-dextran stripped fetal calf serum. Five days after implantation of estrogen or placebo pellets, 1x10⁷ cells of the RbAp46 transfected (46-3B) or empty vector transfected control (control) MCF10AT3B cells suspended in 200 μl of sterile Matrigel (BD Biosciences, San Jose, CA, USA) were subcutaneously injected into the mammary fat pad of ovarioctomized mice. Four groups of mice, control-E2, control+E2, 46-3B-E2 and 46-3B+E2, were used. Animals were monitored weekly, beginning at two weeks after the injection, to check the rate of tumor growth. After 10 weeks, all mice were sacrificed, and tumors were dissected and weighed. Representative tumors were then used to examine protein expression with Western blot analysis.

Results

RbAp46 expression is down-regulated in estrogen-treated MCF10AT3B cells. It was reported that estrogen signaling strongly stimulated malignant progression of MCF10AT3B cells, a mammary epithelial cell line derived from a xenografts model of human proliferative breast disease that highly express estrogen receptor (7). We decided to examine whether estrogen signaling regulates expression of RbAp46, a potent growth inhibitor. MCF10AT3B cells were maintained in medium containing charcoal-dextran treated serum that is stripped of steroid hormones for three days, and 1 nM E2 was added for different periods of time. Northern blot analysis was then performed to examine RbAp46 expression using a fragment of RbAp46 cDNA as a probe. We found that levels of RbAp46 transcripts decreased after estrogen treatment while the expression level of pS2, an estrogen-inducible gene, was greatly increased (Figure 1). This data demonstrated that RbAp46 expression is down-regulated by estrogen signaling and
suggested that down-regulation of RbAp46 may be a prerequisite for estrogen to stimulate cell growth.

Constitutive expression of RbAp46 suppresses estrogen-stimulated cell growth and tumor progression in MCF10AT3B cells. To test whether constitutive expression of RbAp46 influences estrogen-stimulated cell growth in MCF10AT3B cells, we used MCF10AT3B cells that constitutively express high levels of recombinant RbAp46, 46-3B, and the control MCF10AT3B cells that were transfected with the empty expression vector (13). Control and 46-3B cells were treated with 1 nM of E2 for eight days, and cell number was counted every other day. We found that estrogen treatment strongly stimulated growth of the control MCF10AT3B cells, consistent with the previous reports that MCF10AT cell variants express estrogen receptor and strongly respond to mitogenic estrogen signaling (6, 7). However, constitutive expression of recombinant RbAp46 inhibited estrogen-stimulated cell proliferation in 46-3B cells (Figure 2).

We then tested whether constitutive RbAp46 expression inhibits estrogen-stimulated progression of the preneoplastic MCF10AT3B cells in nude mice. Ovariectomized female nude mice supplemented with or without estrogen pellets were injected with control and 46-3B cells in the mammary fatty pad. In the absence of estrogen pellets, control cells formed small tumors after 10 weeks which could be totally inhibited by RbAp46 in 46-3B cells (Figure 3). In the presence of estrogen pellets, control MCF10AT3B cells formed bigger tumors (Figure 3), consistent with the previous reports that estrogen treatment stimulates tumor progression of the MCF10AT3B cells (6, 7). However, RbAp46 expression strongly suppressed estrogen-stimulated tumor formation of the preneoplastic MCF10AT3B cells (Figure 3).

Constitutive expression of RbAp46 suppresses estrogen-stimulated β-catenin accumulation in tumors formed by MCF10AT3B cells. Recently, we reported that in RbAp46-expressing mammary epithelial cells, β-catenin protein was highly phosphorylated and the steady state levels of β-catenin protein were significantly decreased (17). We then examined β-catenin phosphorylation and expression levels in tumors formed in nude mice using the phospho-specific or non-specific antibodies against β-catenin. Western blot analysis revealed that β-catenin protein was significantly accumulated in tumors formed by the control MCF10AT3B...
cells treated with estrogen, and β-catenin phosphorylation at Ser31/35/Thr41 residues and the Thr41/Ser45 residues was dramatically down-regulated (Figure 4). However, the steady state level of β-catenin protein was dramatically decreased in tumors formed by 46-3B cells associated with increased β-catenin phosphorylation in the presence of estrogen (Figure 4). These results indicated that estrogen treatment strongly induces stabilization and accumulation of β-catenin protein, which is abrogated by constitutive RbAp46 expression.

Discussion

In this study, we revealed that RbAp46 is a downstream target gene of the estrogen-signaling pathway that is subjected to down-regulation by mitogenic estrogen signaling. It was reported that the early events, such as induction of c-Myc and cyclin D1 by estrogen signaling, during the recruitment of quiescent cells into cell cycle are insufficient to drive the cells to completion of the cell cycle (3). It was suggested that some unidentified regulatory checkpoints must be crossed by the estrogen-stimulated cell in order for it to progress through the cell cycle (3). Based on our results that RbAp46 expression is down-regulated by estrogen signaling and constitutive RbAp46 expression greatly inhibits estrogen-stimulated cell proliferation and tumorigenesis, we speculate that RbAp46 may be one of the checkpoints that need to be crossed for estrogen to stimulate cell proliferation, and down-regulation of RbAp46 is a prerequisite for mitogenic estrogen signaling.

In this study, we also found that estrogen treatment dramatically reduced phosphorylation levels of β-catenin and increased the steady state levels of β-catenin protein in tumors formed by control MCF10AT3B cells. β-Catenin is an important component of the Wnt/Wingless signaling pathway and can act as a co-activator for the LEF/TCF family transcription factors to activate growth promoting genes such as c-Myc and Cyclin D1 and to stimulate cell proliferation (18). It has been reported that estrogen treatment up-regulated β-catenin expression in an osteoblast cell line (19). It has also been reported that the Wnt/β-catenin signaling pathway converges with the estrogen-signaling pathway in vivo, presumably through a direct interaction between estrogen receptors and β-catenin (20). Our data, together with these reports, strongly suggest estrogen signaling activates the β-catenin signaling pathway through stabilization and accumulation of β-catenin protein in tumors formed. Thus, the Wnt/β-catenin signaling pathway plays an important role in estrogen-stimulated mammary tumorigenesis.

In this study, we further demonstrated that β-catenin was highly phosphorylated in RbAp46 expressing 46-3B cells independent of the presence of estrogen. As a result, the steady state levels of β-catenin protein were significantly reduced, which may attenuate the β-catenin/TCF/LEF signaling activity, consistent with our finding that RbAp46-transfected 46-3B cells exhibit a reduced cell growth. Taken together, our data demonstrated that constitutive expression of RbAp46 suppresses estrogen-stimulated mammary tumorigenesis presumably though an enhancement of proteasome mediated β-catenin degradation and down-regulation of the β-catenin/TCF/LEF signaling pathway.

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References


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