Progesterone Receptor A Stability Is Mediated by Glycogen Synthase Kinase-3β in the Brca1-deficient Mammary Gland*

Received for publication, April 10, 2013, and in revised form, July 17, 2013 Published, JBC Papers in Press, July 23, 2013, DOI 10.1074/jbc.M113.476556

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Background: Stabilization of progesterone receptors contributes to mammary tumorigenesis in Brca1-deficient mice.

Results: Deficiency in phosphorylation on serine 390 of progesterone receptor A by GSK–3β enhances the receptor stability.

Conclusion: Stabilization of progesterone receptor A is mediated by GSK-3β kinase in the Brca1-deficient mammary gland.

Significance: This finding provides a novel insight of how tumor suppression of Brca1 is mediated by PR-A.

Germ line mutations of the BRCA1 gene increase the risk of breast and ovarian cancer, but the basis of this tissue-specific tumor predisposition is not fully understood. Previously, we reported that the progesterone receptors are stabilized in Brca1-deficient mammary epithelial cells, and treating with anti-progesterone delays mammary tumorigenesis in Brca1/-p53 conditional knock-out mice, suggesting that the progesterone receptor has a critical role in breast carcinogenesis. To further explore how the stability of progesterone receptor is modulated, here, we have found that glycogen synthase kinase (GSK)-3β phosphorylation of progesterone receptor-A (PR-A) facilitates its ubiquitination. GSK-3β-mediated phosphorylation of serine 390 in PR-A regulates its subsequent ubiquitination and protein stability. Expression of PR-A S390A mutant in the human breast epithelial cells, MCF-10A, results in enhanced proliferation and formation of aberrant acini structure in the three-dimensional culture. Consistently, reduction of phosphorylation of serine 390 of PR-A and GSK-3β activity is observed in the Brca1-deficient mammary gland. Taken together, these results provide important aspects of tissue specificity of BRCA1-mediated suppression of breast carcinogenesis.

Progesterone receptors (PRs) play important roles in mammary gland development (reviewed in Ref. 1). PR activities have been linked to the expansion of mammary stem cells in a RANKL-dependent manner (2, 3). In hormone replacement randomized studies, postmenopausal women who received estrogen plus progestin have significantly increased breast cancer risk, by contrast, estrogen alone reduced the risk of breast cancer (4, 5). In mice, only the long isoform, PR-B but not PR-A, is required for mammary gland development; however, overexpression of PR-A leads to abnormal mammary gland development and ductal hyperplasia (6–8). These two isoforms differ in their amino terminus because of differential promoter usage.

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase that phosphorylates a broad range of substrates including glycogen synthase, β-catenin, Axin (13), Cdc25A (14), glucocorticoid receptor, and elf2B (15). Most GSK–3β substrates have been shown to contain the (pS/T)XXX(pS/T) motif; the GSK-3β-mediated S/T phosphorylation follows phosphorylation of the primary P+S position (13). Unlike most kinases, GSK-3β is active in resting cells, and stimulation of cells by mitogens or growth factors leads to its inactivation (15). Interestingly, it is noted that GSK-3 modulates the progesterone responsiveness during Xenopus oocyte maturation, because treatment with GSK-3 inhibitor 7-azaindolyl-pyrazinyl-maleimide increases progesterone response in Xenopus oocytes (16). This result suggests that PR may also be subjected to GSK-3 regulation.

It is noted that BRCA1 interacts with estrogen receptor α and two PR isoforms directly to modulate ligand-dependent and -independent transcription activities of estrogen receptor α and PRs. Both PR-B and PR-A become stabilized in Brca1-deficient mammary epithelial cells, correlating with the deregulated proliferation of mammary cells. Consistently, treatment with the progesterone receptor antagonist RU 486 delayed/prevented tumor development. These observations indicated that BRCA1 serves as a negative regulator for PRs (11). Similarly, increased PR expression and proliferation were reported in the normal breast epithelium of BRCA1 mutation carriers (17). BRCA1 contains E3 ubiquitin ligase at its amino-terminal region, which may directly dictate the stability of PR-A. However, direct evidence to support this observation remains obscure. It is likely that an additional factor such as phosphorylation by kinase may be needed.
In this study, we show that phosphorylation of PR-A at serine 390 by GSK-3β kinase is required for its ubiquitination and proteasome-mediated degradation. Expression of PR-A mutant with S390A substitution, but not wild-type PR-A, in nontumorigenic mammary epithelial cells, MCF-10A, resulted in increased proliferation and formation of abnormal acini structure. Consistently, in the Brca1/p53-mutated mammary gland, GSK-3β kinase activity was down-regulated, explaining the longer stability of PR-A. These results provide important aspects of tissue specificity of BRCA1-mediated suppression of breast carcinogenesis.

MATERIALS AND METHODS

Plasmids and siRNAs—PCR3.1 wild-type PR-A plasmid was a generous gift from Dr. Bert O’Malley. The PCR3.1 PR-A S390A construct was generated by site mutagenesis. To generate the His6-tagged PR-A, full-length PR-A cDNA was used as a template for PCR with a primer coding a His6 tag; the PCR fragment was subsequently subcloned into the pcDNA3.1 hygro vector. Full-length PR-A cDNA and PR-A S390A mutant were also subcloned into PQCXIP (Clontech). Wild-type GSK-3β, kinase dead GSK-3β, and HA-ubiquitin were as described in previous publication (18). GSK-3β siRNA was purchased from Santa Cruz Biotechnology, and control luciferase siRNA was customized at Dharmacon.

Antibodies—PR, cyclin D1, and GSK-3β antibodies were from Santa Cruz; GSK-3β serine 9 phosphospecific antibodies were from Abcam; HA antibody was from Sigma; and RANKL antibody was from R&D Systems. Vimentin, CK14, Bax, cytoplasmic GSK-3β, and GSK-β catalytic subunit were from Abcam; HA antibody was from Sigma; and Bcl-2 antibodies were from GeneTex. Phosphospecific antibodies recognizing phosphoserine 390 of PR-A was raised against a synthetic phospho-peptide column. Phosphospecific antibodies were obtained using a two-step phosphospecific column followed by binding and elution from the longer stability of PR-A. These results provide important aspects of tissue specificity of BRCA1-mediated suppression of breast carcinogenesis.

Pulse-Chase Studies for PR-A Stability—293T cells were grown in 100-mm culture dishes and transfected with the indicated plasmids or siRNAs for 48 h. Prior to harvest, cells were treated with 10 μM MG132 for 2 h, followed by 3 h of incubation with 20 nM R5020. The cells were lysed in a boiling solution containing SDS (1% in Tris-buffered saline) and then sonicated. Lysates were diluted 10-fold with Triton X-100 solution (1% in Tris-buffered saline). PR in the supernatants was immunoprecipitated using PR antibody, separated on SDS-PAGE, and visualized by immunoblotting using both HA and PR antibodies.

In Vivo Ubiquitination Assay—As previously reported, briefly, 1 μg of purified PR-A was preincubated with 2 units GSK3β (Sigma G1663) in the presence of the kinase buffer (50 mm Tris-HCl, pH 7.4, 5 mM NaF, 10 mM okadaic acid, 0.06 mM DTT, 2 mM Mg-ATP) at 30 °C for 30 min. The reaction products were added to the BRCA1-BARD1 complex that was immobilized to protein A beads with anti-FLAG antibody (15 μl in dried volume). The mixture was incubated at 4 °C for 60 min. The beads were then washed with buffer (100 mM NaCl, 0.5% Nonidet P-40, and phosphatase inhibitors) and used for the ubiquitination reactions. The ubiquitination reactions (30 μl) contained 1 μg of ubiquitin, 20 ng of E1, and 250 ng of E2, as well as 2 μM Mg-ATP in the ubiquitination buffer (50 mM Tris-HCl, pH 7.4, 2 mM NaF, 10 mM okadaic acid, 0.06 mM DTT) at 37 °C for 60 min. The reactions were terminated by boiling the samples in SDS buffer. The products were separated by SDS-PAGE and analyzed by Western blotting using anti-PR antibody.

Mammosphere Culture—Cells were derived from MCF-10A or HeLa cells and 293T cells (ATCC) were cultured in DMEM supplemented with 10% FBS. MCF-10A (ATCC) were cultured in DMEM/F-12 supplemented with 5% horse serum (HyClone), 10 μg/ml insulin (Sigma), 20 ng/ml EGF (Millipore), 100 ng/ml choleratoxin (Sigma), and 0.5 μg/ml hydrocortisone.

Pulse-Chase Studies for PR-A Stability—293T cells were cultured in 100-mm culture dishes and transfected with the indicated plasmids or siRNA. Twenty-four hours later, cells were incubated in a methionine- and cysteine-free medium, metabolically labeled with a [35S]methionine/[35S]cysteine mixture (PerkinElmer Life Sciences), and then chased with complete medium supplemented with 20 nM R5020 (PerkinElmer Life Sciences). Cells from one-third of each plate were harvested at each indicated time point and lysed in Nonidet P-40 buffer. PR was immunoprecipitated with PR antibody, separated on SDS-PAGE, and analyzed by autoradiography.

Immunochemistry—Mammary glands were removed from different strains of mice as indicated, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections were sliced at 5-μm thickness and subjected to immunochemical analysis. Immunostaining was performed following the instructions in the Vectastain Elite ABC kit (Vector Laboratories).

In Vivo Ubiquitination Assay—293T cells were transfected with the indicated plasmids or siRNAs for 48 h. Prior to harvest, cells were treated with 10 μM MG132 for 2 h, followed by 3 h of incubation with 20 nM R5020. The cells were lysed in a boiling solution containing SDS (1% in Tris-buffered saline) and then sonicated. Lysates were diluted 10-fold with Triton X-100 solution (1% in Tris-buffered saline). PR in the supernatants was immunoprecipitated using PR antibody, separated on SDS-PAGE, and visualized by immunoblotting using both HA and PR antibodies.
liquid chromatography pump (Agilent Technologies) with the Famos autosampler (LC Packings). The enzyme-digested protein samples were injected onto a self-packed precolumn (150-μm inner diameter × 20 mm, 5 μm, 200 Å). Chromatographic separation was performed on a self-packed reversed phase C18 nano-column (75-μm inner diameter × 300 mm, 5 μm, 100 Å) by using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 80% acetonitrile (mobile phase B). A linear gradient from 5 to 45% mobile phase B for 40 min at a flow rate of 300 nl/min was applied. Electrospray voltage was applied at 2 kV, and capillary temperature was set at 200 °C. A scan cycle was initiated with a full scan survey MS spectrum (m/z 300–2000) performed on the FT-ICR mass spectrometer with resolution of 100,000 at 400 Da. The ten most abundant ions detected in this scan were subjected to a MS/MS experiment; the normalized collision energy was set to 35%, activation time was 30 ms. Ions were fragmented by use of collision-induced dissociation; the normalized collision energy was set to 35%, activation Q was 0.3, and activation time was 30 ms. For data analysis, all MS/MS spectra were converted as DTA format from experiment RAW file by Bioworks (Thermo Fisher Scientific) and then merged into a single file for Mascot (version 2.2, Matrix Science) MS/MS ion search. The search parameters in Mascot including the error tolerance of precursor ions, the MS/MS fragment ions in spectra were 10 ppm and 0.8 Da, and the enzyme miss cleavage number was 5.

RESULTS

GSK-3β Kinase Enhances PR-A Ubiquitination and Degradation—Upon ligand binding, progesterone receptors become phosphorylated at multiple sites; the receptors are subsequently polyubiquitinated and degraded by the proteasome. To test whether GSK-3β has any effect on PR-A polyubiquitination and degradation, we performed an in vitro ubiquitination assay in the presence of E1, E2, and BRCA1/BARD1 complexes as previously described (11). As shown in Fig. 1A, PR-A was polyubiquitinated upon phosphorylation by GSK-3β (Fig. 1A, lane 4). To confirm this observation, we performed an in vivo polyubiquitination assay by expressing either wild-type, kinase dead GSK-3β (GSK-3β KD), or depleting GSK-3β with siRNA or control siRNA, and PR-A expression was examined as described in C. Lanes 1–3, GSK-3β siRNA, 0, 3, and 6 h, respectively; lanes 4–6, control siRNA, 0, 3, and 6 h, respectively. Ubiquitination of PR-A was examined by Western blot analysis using HA and PR antibodies; the HA immunoblot identified ubiquitinated PR.
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Consistently, expressing GSK-3β KD inhibited PR-A degradation (Fig. 1C, lanes 1–3) compared with that of expressing wild-type GSK-3β (Fig. 1C, lanes 4–6). On average, the half-life of PR-A was approximately 3 and 6 h in wild-type GSK-3β- and GSK-3β KD-expressing cells, respectively. Similarly, the half-life of PR-A was ~4.5 h in GSK-3β-depleted cells (Fig. 1D, lanes 1–3) but was ~3 h in control cells (Fig. 1D, lanes 4–6). Taken together, these results suggest that GSK-3β promotes ligand-induced ubiquitination and degradation of PR-A.

GSK-3β Binds to and Phosphorylate PR-A—To test whether PR-A is a direct substrate of GSK-3β, we performed co-immunoprecipitation using cells expressing FLAG-tagged PR-A and HA-tagged GSK-3β. HA-GSK-3β was presented in the anti-FLAG immunoprecipitates only when HA-GSK-3β was co-expressed (Fig. 2A), indicating that GSK-3β and PR-A interact with each other.

Next, we performed in vitro kinase assay using purified PR-A and GSK-3β followed with mass spectrometry analyses. Purified His₆-tagged PR-A from SF-9 cells in the presence of R5020 had phosphorylation sites at serines 26, 63, 112, 236, and 394. Upon incubation with GSK-3β in vitro, two additional phosphorylated sites, serines 164 and 390, were identified (Fig. 2, C and D). Mass spectrometric analyses revealed that serine 390 but not serine 164 was phosphorylated when His₆-tagged PR-A was purified from 293T cells treated with R5020 (Fig. 2, C and D). In addition to serine 390 phosphorylation, we also confirmed the previously reported phosphorylation sites, including serines 26, 63, 130, 236, and 512 in the latter setting (Fig. 2, C and E).

Characterization of Antibodies Specifically Recognize Serine 390 of PR-A—To confirm whether phosphorylation of serine 390 is GSK-3β-dependent, we generated phosphospecific antibodies against a synthetic phospho-peptide, SEASQ(pS)PQYS-FESL, which corresponds to PR-A amino acids 385–398. These antibodies detected PR-A in R5020-treated human cervical cancer HeLa cells expressing wild-type PR-A, but not at the zero time point of treatment (Fig. 3A, lanes 1–3). No signals were detected in cells expressing PR-A mutant with serine 390 changed to alanine PR-A mutant (PR-A⁵³⁹₀A; Fig. 3A, lanes 4), suggesting that the antibodies are specific in recognizing PR-A with phosphorylated serine 390. These phospho-peptide antibodies also gave an immunostaining signal that co-localized with PR in cells treated with R5020 (data not shown).

Phosphorylation of Serine 390 in PR-A Is GSK-3β-dependent—Next, we assessed whether phosphorylation of serine 390 in PR-A is GSK-3β-dependent using these antibodies. Phosphorylation of serine 390 in PR-A became prominent upon R5020 treatment in HeLa cells expressing wild-type PR-A (Fig. 3B, lanes 2 and 3). In contrast, significantly reduced serine 390 phosphorylation was found in HeLa cells with GSK-3β depletion, whereas total PR-A levels were compatible in
GSK-3β phosphorylation of PR-A is mediated by GSK-3β. Knockdown or control cells (Fig. 3B, lanes 4–6). Serine 390 phosphorylation was significantly reduced in HeLa cells expressing GSK-3β/KD mutant compared with cells expressing GSK-3β WT upon ligand treatment (Fig. 3C, lanes 2 and 4). Similarly, phosphorylation of serine 390 was also reduced when cells were treated with GSK-3 inhibitor, BIO, on phosphorylation of serine 390 in PR-A (Fig. 3D, lane 4 versus lane 2). Taken together, these results suggest that ligand-induced phosphorylation of serine 390 in PR-A is mediated by GSK-3β.

PR-AΔ390A Has Less Ubiquitination and Longer Half-life upon Ligand Treatment—If GSK-3β-mediated phosphorylation of PR-A serves as a signal for subsequent ubiquitination and proteasomal degradation, the PR-AΔ390A mutant would have a longer half-life than the wild-type PR-A. To explore this possibility, we introduced either wild-type PR-A or PR-AΔ390A and HA-ubiquitin into 293T cells that were treated with R5020 and MG132 for 3 h. Cell lysates were immunoprecipitated using PR antibodies followed by immunoblotting with HA antibody to detect ubiquitinated PR-A. As shown in Fig. 4A, PR-AΔ390A was less polyubiquitinated compared with the wild type (Fig. 4A, lane 1 versus lane 3). Furthermore, the stability of wild-type PR-A and PR-AΔ390A was compared in HeLa cells and immortalized human mammary MCF-10A cells treated with R5020 and cycloheximide. Based on the amount of PR-A at various time points, the half-life of PR-A was ∼3.8 h in HeLa.

FIGURE 3. Serine 390 of PR-A is a GSK-3β phosphorylation site. A, the specificity of antibodies against phosphoserine 390 in the PR-A. Immunoblotting was performed using lysates from HeLa cells transfected with either wild-type PR-A (lanes 1 and 2) or PR-AΔ390A mutant (lanes 3 and 4) and treated with R5020 for 2 h (lanes 2 and 4) or left untreated (lanes 1 and 3). Total PR and phosphorylated PR levels are indicated. B, phosphorylation of serine 390 in PR-A in cells with GSK-3β knockdown. Immunoblotting was performed using lysates from HeLa cell transfected with PR-A and control siRNA (lanes 1–3) or GSK-3β siRNA (lanes 4–6) treated with R5020. C, phosphorylation of serine 390 in PR-A in cells overexpressing either wild-type or GSK-3β KD mutant. Immunoblotting was performed using lysates from HeLa cell transfected with PR-A and GSK-3β WT plasmid (lanes 1 and 2) or GSK-3β KD (lanes 3 and 4) treated with R5020 for 2 h (lanes 2 and 4) or untreated (lanes 1 and 3). D, the effects of GSK-3 inhibitor, BIO, on phosphorylation of serine 390 in PR-A. Phosphorylation was examined in HeLa cells transfected with PR-A, followed by either control dimethyl sulfoxide (lanes 1 and 2) or BIO (lanes 3 and 4) treatment. The cells were treated with R5020 for 2 h (lanes 2 and 4). p-PR, phosphorylated PR; siLuc, luciferase siRNA; siGSK-3, GSK-3β siRNA; DMSO, dimethyl sulfoxide.

FIGURE 4. Serine 390 to alanine mutation in PR-A decreased PR-A ubiquitination and led to its stabilization. A, ubiquitination of wild-type PR-A and PR-AΔ390A. Immunoprecipitation followed by immunoblotting analyses of anti-PR immunoprecipitates from 293 cells co-transfected with HA-ubiquitin, and wild-type PR-A, PR-AΔ390A, or control vector, treated with R5020 and MG132 for 3 h. Cell lysates were immunoprecipitated using PR antibodies followed by immunoblotting with HA antibody to detect ubiquitinated PR-A. As shown in Fig. 4A, PR-AΔ390A was less polyubiquitinated compared with the wild type (Fig. 4A, lane 1 versus lane 3). Furthermore, the stability of wild-type PR-A and PR-AΔ390A was compared in HeLa cells and immortalized human mammary MCF-10A cells treated with R5020 and cycloheximide. Based on the amount of PR-A at various time points, the half-life of PR-A was ∼3.8 h in HeLa.
whereas PR-AS390A was greater than 8.0 h (Fig. 4B). In MCF-10A cells, the half-life for the wild-type PR-A was 4.4 h, and that for PR-AS390A was 7.0 h (Fig. 4C). These results suggest that phosphorylation of serine 390 of PR-A by GSK-3 determines its stability.

Expression of PR-AS390A Promotes MCF-10A Cell Mammosphere Formation and Alters Its Growth Behavior in Three-dimensional Culture—To access the biological consequences of phosphorylation of serine 390 of PR-A by GSK-3β, we expressed wild-type or mutant PR-A in MCF-10A cells via retrovirus-mediated gene transfer. As shown in Fig. 5A, the expression of wild-type and mutant PR-A was comparable.

Using these cells, we compared their ability to form mammospheres because progesterone has been shown to promote mammary stem cell self-renewal (2, 3). Under a modified culture condition that contained R5020, instead of EGF, PR-AS390A-expressing cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.

Next, we cultured these cells in Matrigel in low EGF-containing medium for 16 days to assess the effects of wild-type PR-A expression on mammosphere formation. As shown in Fig. 5, D and E, PR-AS390A cells formed ~18 spheres, wild-type PR-A formed ~9 spheres, but the vector only did not form any mammosphere (Fig. 5, B and C). The mammospheres formed under these conditions possessed multiple cell types as evidenced by the expression of vimentin, CK14, CD10, and MUC1 (data not shown). Interestingly, wild-type PR-A cells treated with the GSK-3β inhibitor BIO formed more mammospheres than mutant PR-A cells (Fig. 5C). These results suggest that the failure of phosphorylation of serine 390 of PR-A promotes stem cell renewal.
and PR-A<sup>S390A</sup> on their growth behavior. All three groups formed mammary acini (Fig. 5D), which consist of CK-14-positive and -negative cells (data not shown); interestingly, PR-A<sup>S390A</sup>-expressing cells had the largest acini. Using National Institutes of Health ImageJ software and statistical analyses of more than 100 acini in each group, we found that acini formed by PR-A<sup>S390A</sup>-expressing cells were 2.20-fold larger than the vector control group, whereas acini formed by the wild-type PR-A-expressing cells were 1.27-fold larger than the control group (Fig. 5E). Immunostaining of Ki-67 at day 16 revealed strong patched Ki-67 staining in acini formed by PR-A<sup>S390A</sup>-expressing cells, whereas control and wild-type PR-A-expressing cells had weak and even staining (Fig. 5F). These data suggest that PR-A<sup>S390A</sup> promotes proliferation. Although the PR-A<sup>S390A</sup> cells formed larger acini, there were fewer acini compared with the wild-type and control groups. Judging from stronger immunostaining of anti-cleaved caspase-3 antibodies in PR-A<sup>S390A</sup>-expressing cells compared with wild-type PR-A and control cells, an elevated apoptosis in PR-A<sup>S390A</sup>-expressing cells was observed (Fig. 6A and B). In addition, we used antibodies recognizing PR target genes to compare their expressions among these three groups. RANKL expression was increased in PR-A<sup>S390A</sup>-expressing cells (Fig. 6C), whereas no changes in cyclin D1 expression were detected (Fig. 6D). Interestingly, Bcl-2 expression was decreased in cells expressing the mutant PR-A, which correlated with the increased cell death seen in these cells (Fig. 6E). Taken together, these results indicate that failure of the phosphorylation of serine 390 of PR-A promotes proliferation and apoptosis of MCF-10A cells in the three-dimensional culture.

**FIGURE 6.** PR-A<sup>S390A</sup> expression altered growth behavior of MCF-10A cells in three-dimensional culture and expression of PR target genes. PR-A WT, PR-A S390A, or control cells were cultured in a Matrigel-coated chamber slide with medium containing 2% Matrigel. At the indicated time, acini were fixed and stained by antibody against cleaved caspase-3. A and B, acini after 6 (A) and 16 (B) days of culture. C, RANKL expression in MCF-10A cells stably expressing control vector, wild-type PR-A, or S390A mutant PR-A. The cells were fixed after 8 days culture in the three-dimensional Matrigel. D, cyclin D1 expression. See C for details. E, Bcl-2 expression. See C for details.
test this possibility, mammary glands were harvested from mice at the estrous or diestrous stage, and PR-A and phospho-PR-A levels were analyzed by Western blot. As shown in Fig. 7A, phosphorylation of serine 390 was significantly reduced in the Brca1f/f p53f/fWapCrec mice both at estrous and diestrous compared with that of the wild-type mammary gland (Fig. 7A, lanes 3 and 4 versus lanes 1 and 2). Higher levels of total PR-A were found in Brca1f/f p53f/fWapCrec mice as previously reported (11). These results further suggest that phosphorylation of serine 390 of PR-A by GSK-3β plays an important role in BRCA1-mediated breast carcinogenesis.

It was reported that BRCA1 negatively regulated AKT activity, which phosphorylates serine 9 of the GSK-3β and inhibits GSK-3β kinase activity (21). Thus, it is likely that GSK-3β activity is down-regulated in Brca1-deficient mammary epithelial cells. To test this possibility, we performed and compared immunostaining using mammary tissue sections from wild-type, Brca1f/f p53f/fWapCrec mice and WT mice. As shown in Fig. 7B, the levels of GSK-3β and phosphorylation on serine 9 were similar in Brca1f/f p53f/fWapCrec mice and WT mice (Fig. 7B, upper panels), whereas the total GSK-3β levels were compatible in these two groups (Fig. 7B, lower panels), indicating that GSK-3β activity is not affected in the mammary epithelial cells of the Brca1f/f p53f/fWapCrec mice.

**DISCUSSION**

In this study, we found that progesterone receptor-A is a physiological substrate of GSK-3β kinase. Phosphorylation of PR-A on serine 390 by GSK-3β enhances its ubiquitination and degradation. Expression of PR-AΔ390Δ mutant in the human breast epithelial cells, MCF-10A, results in increased proliferation and formation of abnormal acini structure. Consistently, in the Brca1f/f p53f/f-deficient mammary gland, phosphorylation on serine 390 of PR-A is down-regulated. These results suggest the importance of phosphorylation of PR-A by GSK-3β in BRCA1-mediated suppression of breast carcinogenesis.

Progesterone receptors, especially the dominant short form PR-A, accumulate in the mammary gland of Brca1f/f knock-out mice because of stabilization of the receptor (11). The PR activity is tightly modulated by phosphorylation. Both the long and short forms of PR become phosphorylated upon ligand binding, and several phosphorylation sites have been identified. For example, PR serine 400 (e.g., serine 236 in PR-A) is phosphorylated to mediate the ligand-independent activity of PR (12). Phosphorylation of PR serine 345 (e.g., serine 181 in PR-A) by p38 MAPK is required for tethering PR to specificity protein 1 to promote cell proliferation (19). Phosphorylation of PR serine 294 (e.g., serine 130 in PR-A) by p42/44 MAPK coupled with sumoylation of PR-B is essential for derepression of PR action (20). Consistently, phosphorylation of PR-A on serine 390 by GSK-3β, as described here, is essential for modulating PR-A stability.

GSK-3β is a key regulator of sex steroid receptors (reviewed in Ref. 21) and phosphorylates different steroid receptors including estrogen receptor α (22), glucocorticoid receptor (23), and androgen receptor (24). GSK-3β regulates the progesterone response in Xenopus oocyte meiotic entry, and treating with GSK-3β inhibitor enhances the progesterone sensitivity of Xenopus oocytes (16). Consistently, GSK-3β is the key kinase to modulate PR-A stability as described here.

Interestingly, many of GSK-3β substrates including glucocorticoid synthase (13), CDC25A (14), and SRC-3 (steroid receptor coactivator 3) contain a (S/T)XXX(pS/T) motif for phosphorylation. However, phosphorylation of these substrates by GSK-3β requires a prior phosphorylation on priming site (the n + 4 S/T) by other kinases. On the other hand, many GSK-3β substrates such as Axin and Tau (15), steroid receptors (21, 22, 24), do not need priming phosphorylation. It was noted that phosphorylation of glucocorticoid receptor serine 404 by GSK-3β is also essential for modulating its stability (23). Interestingly, the surrounding amino acid sequences of serine 390 of PR-A share high homology with that of the glucocorticoid receptor serine 404, suggesting a potential recognition motif for GSK-3β for this class of proteins. This possibility warrants further exploration.

It has been shown that progesterone triggers two waves of proliferation in the mammary epithelial cells: the first short wave is dependent on cyclin D1 and acts in an autocrine fashion; the second long wave is dependent on RANKL and acts in a paracrine fashion (25). Progesterone induces mammary stem
cell expansion (3). Interestingly, Hilton et al. (26) showed that the presence of PR- and basal markers-positive bi-potent progenitor cells in normal human breast. In contrast to the luminal epithelial cells, these PR-positive bi-potent progenitor cells are refractory to estrogen. We show that Brca1/p53-deficient mammary cells are uniquely sensitive to progesterone, in part because of stabilization of PR. Because PR degradation by the proteasome is the major pathway for termination of progesterone signaling, the degradation needs to be tightly regulated because prolonged progesterone response can trigger abnormal proliferation in the mammary gland (3). Consistently, the expression of PR-A S390A mutant triggered abnormal proliferation of MCF-10A cells in three-dimensional culture as indicated by larger acini and extensive Ki-67 staining (Fig. 6). In addition, increasing apoptosis was seen in the mutant PR-A group, which correlated with decreased levels of Bcl-2 expression. Bcl-2 is a known PR target (27). Although an extended PR-A half-life could lead to more Bcl-2 expression, the reduced Bcl-2 expression in these cells suggests that phosphorylation of PR-A S390A may have an opposite role in Bcl-2 transcriptional regulation. Further investigation is needed to clarify this issue.

Interestingly, Akt activity is up-regulated in the mammary gland of Brca1 knock-out mice (28); Brca1-deficient cells accumulate phosphorylated nuclear Akt, which increases phosphorylation of the Akt substrates including GSK-3β (29). Phosphorylation of GSK-3β by Akt inhibits GSK-3β kinase activity (30). Connecting these observations together, the longer half-life of PR-A in the BRCA1-deficient cells may be resulted from higher Akt activity, which inhibits GSK-3β kinase activity and reduces phosphorylation of serine 390 of PR-A. Thus, these results provide a plausible explanation for the tissue specificity of BRCA1-mediated suppression of breast carcinogenesis through progesterone receptors.

Acknowledgments—We thank Dr. Bert O’Malley for the His-PR-A overexpressed in the insect cells, Dr. Carol A Lange for PR reporter plasmid, Dr. Win-Hwa Lee for critical reading and Connie Tat and Serena Abbondante for comments.

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