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Upregulation of ATBF1 by progesterone-PR signaling and its functional implication in mammary epithelial cells

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ABSTRACT

Progesterone (Pg) is an essential steroid hormone during mammary gland development and tumorigenesis, including the maintenance of epithelial stem/progenitor cells. Pg functions through interaction with the progesterone receptors (PR) and Pg-PR signaling is thought to be mediated by key transcription factors, which are largely unidentified. In this study, we have identified the ATBF1 transcription factor as a transcriptional target of Pg-PR signaling in mammary epithelial cells. Pg treatment dramatically increased ATBF1 expression at both mRNA and protein levels in cultured cells and mammary tissues. As expected, the induction of *ATBF1* was PR-dependent, as it only occurred in PR-positive but not in PR-negative cells, and pretreatment with the Pg antagonist RU-486 or RNAi-mediated knockdown of PR abolished the upregulation of *ATBF1* by Pg. Promoter-reporter and ChIP assays further showed that Pg-activated PR directly binds to the *ATBF1* promoter to induce its transcription. Prevention of *ATBF1* induction inhibited the function of Pg in promoting progenitor cell transition, as indicated by colony formation in a Matrigel culture assay and expression of stem cell markers CD49f and CD44. These findings suggest that ATBF1 plays a crucial role in the Pg-PR signaling pathway in mammary epithelial cells.

1. Introduction

The progesterone (Pg) steroid hormone is a key player in the complex regulation of mammary gland development and mammary tumorigenesis. Pg functions through interaction with the two progesterone receptors, PR-A and PR-B [1]. PR is a prognostic marker in breast cancer, as its expression, along with that of the estrogen receptor (ER), occurs in more than 60% of ductal carcinomas of the breast, and is correlated with better prognosis and response to hormonal therapy [2]. Recent studies indicate that Pg also induces stem/progenitor cells through the upregulation of CK5 and CD49f, which is accompanied with an expansion of progenitor cells and enhanced tumorigenesis of breast cancer cells [2–6].

The AT-motif binding factor 1 (ATBF1, also named ZFHX3), originally identified as a transcriptional repressor of the human alpha-fetoprotein (AFP) [7], has been recognized as a differentiation inducer in different types of cells, because it is induced during neuronal differentiation [8–10], it regulates enterocyte maturation in the small intestine [11], and it activates the pituitary lineage determining factor 1 (Pit1) in pituitary gland [12]. Knockout of *ATBF1* leads to the dysregulation of a number of differentiation genes in the prostate (our unpublished data), and accelerates mammary gland development during puberty by enhancing estrogen-ER signaling [13].

In ER-positive breast cancer cells, ATBF1 was identified as an inhibitor of the function of ER in gene regulation and cell proliferation control [14]. In addition, there appears to be an autoregulatory negative feedback loop between ATBF1 and estrogen-ER signaling, as the transcription of *ATBF1* is increased by estrogen-ER while ATBF1 protein is degraded by the estrogen-responsive ubiquitin E3 ligase EFP [15,16]. PR is a well known target gene of estrogen-ER signaling [17], and ERα mediates progestin-induced tumor growth by interacting with PR [18]. We therefore examined whether ATBF1 is also involved in the function of Pg-PR signaling in mammary epithelial cells. We found that ATBF1 expression is upregulated by Pg via the binding of PR to the *ATBF1* promoter, and that ATBF1 is necessary for Pg to induce the expression of stem cell markers and the expansion of progenitor cells.

2. Materials and methods

2.1. Cell lines, mice and reagents

Human breast cancer cell lines (T-47D, BT-549 and MDA-MB-231), from the American Type Culture Collection (Manassas, VA),

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were cultured as previously described [19]. Mice were closely monitored and handled at Emory University according to the policies of the Institutional Animal Care and Use Committee. The following reagents were purchased from their respective vendors: Pg and RU-486, Sigma (St. Louis, MO); PR siRNA, Dharmacon (Lafayette, CO); anti-PR antibody, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-CD44 antibody, Novocastra Laboratories Ltd (Newcastle, UK); anti-CD49f antibody, Abcam (Cambridge, MA) and anti-β-actin antibody, Sigma. The ATBF1 siRNA and anti-ATBF1 antibody have been described in our previous studies [14].

2.2. Gene expression analysis

RNA expression was measured by quantitative RT-PCR as described previously [14]. PCR primers used are shown in Supplementary Table S1. Protein expression was measured by Western blotting as previously described [14].

2.3. Dual luciferase assay

Promoter-luciferase reporter assay was performed as previously described [16,20]. T-47D cells were seeded in 24-well plates at 1×10^5 cells per well. On the next day, cells were transfected with the *ATBF1* promoter reporter plasmids constructed in our previous study [16] in regular medium. One day after transfection, cells were treated with 100 nM Pg for 12 h or 36 h, and luciferase activities were measured using the dual luciferase reporter assay system (Promega, Madison, WI).

2.4. Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed in T-47D cells with proper treatments following a protocol provided by Millipore (Billerica, MA), as previously described [16]. The primers for *ATBF1* and *β-actin* promoters are shown in Supplementary Table S1.

2.5. Immunofluorescence (IF) staining

Cells cultured on sterile glass cover slips were fixed with 4% paraformaldehyde and exposed first to antibody against CD44 (1:200 dilution) at 4 °C overnight, and then to FITC-conjugated secondary antibody at 1:1000 dilution in a dark humidified chamber for 1 h. Nuclei were stained with DAPI. Sections in anti-fade mounting solution were stored at −20 °C in the dark.

2.6. Matrigel culture, cell growth and cell cycle analysis

T-47D cells were seeded in 6-well plates at 2×10^5 cells per well. Sixteen hours later, the cells were transfected with 100 nM ATBF1 siRNA or negative control siRNA. After two days of incubation, cells were serum-starved for at least 24 h and treated with 100 nM Pg for 6 h. Matrigel culture was performed as previously described [4]. Eight-well chamber slides were loaded with 100 μl of growth-factor-reduced Matrigel (BD Biosciences, San Jose, CA) and set for 30 min. A total of 2500 treated cells were then overlaid onto the gel in RPMI-1640 medium supplemented with 5% FBS, and allowed to grow for 2 weeks. Identically-treated cells were also seeded in regular 24-well plates (plastic) at the same density, and cell growth was measured with the cell counting kit-8 (Dojindo Molecular Technologies, Rockville, MD). The remaining cells were then fixed in 70% cold ethanol for 24 h at −20 °C and stained with 0.025 mg/ml propidium iodide (PI) for 0.5 h at 37 °C in the dark as described previously [21].

2.7. Statistical analysis

Statistical analyses were performed using the SPSS® statistical software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to determine statistical differences. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Pg induces the expression of ATBF1 in mammary epithelial cells both in vitro and in vivo

To test whether Pg modulates ATBF1 expression, we used the PR-positive breast cancer cell line T-47D and Pg-treated C57BL/6 J mice. In T-47D cells, which express endogenous ATBF1 at quite a low level [22], Pg treatment significantly increased ATBF1 expression at both the mRNA (Fig. 1A and B) and protein (Fig. 1C and D) levels. Induction of ATBF1 by Pg was time- and dose-dependent, with doses as low as 1 nM Pg inducing significant *ATBF1* expression after 4 h of treatment (Fig. 1A). When 100 nM of Pg was applied, ATBF1 induction occurred as early as 2 h (Fig. 1B and D). In 8-week old female mice injected subcutaneously with 100 μl of Pg (10 mg/ml) or with solvent oil control, real-time PCR analysis showed that the *ATBF1* mRNA level more than tripled in Pg-treated mammary tissues after two days (Fig. 1E). Immunohistochemical staining confirmed that the increase of ATBF1 expression also occurred at the protein level (Fig. 1F). These results indicate that Pg induces the expression of ATBF1 both *in vitro* and *in vivo*.

3.2. Induction of ATBF1 by Pg is PR-dependent

To investigate whether the induction of ATBF1 transcription by Pg is mediated by PR, we first knocked down the expression of PR in T-47D cells using two different small interfering RNAs (siRNAs) against *PR* and analyzed gene expression. Efficient knockdown of PR was confirmed for both siRNAs (Fig. 2A, panel at left). Knockdown of PR significantly blocked the induction of ATBF1 by Pg (Fig. 2A). We also used the Pg antagonist RU-486 to block PR function and observed that in T-47D cells treated with RU-486, the induction of ATBF1 by Pg was completely blocked (Fig. 2B). In PR-negative breast cancer cell lines MDA-MB-231 and BT-549, Pg did not have a significant effect on the expression of ATBF1 (Fig. 2C). These results indicate that Pg functions via its receptor to induce *ATBF1* expression.

3.3. Pg activates ATBF1 transcription by mediating the binding of PR to ATBF1 promoter

Pg-PR signaling regulates gene expression through the binding of PR to gene promoters. To test whether this is also the case for Pg-induced *ATBF1* transcription, we transfected three *ATBF1* promoter-luciferase reporter plasmids constructed in our previous study [16], as illustrated in Fig. 3A, into T-47D cells, and measured their promoter activities in response to Pg treatment. We observed a significant increase in the activity of all three *ATBF1* promoters after Pg treatment. pATBF1-Luc1 showed the strongest and earliest response to Pg (Fig. 3B), suggesting that the segment of the *ATBF1* promoter present in pATBF1-Luc1 but not in pATBF1-Luc2 is more crucial for Pg-induced *ATBF1* transcription.

To more directly evaluate whether PR binds to the *ATBF1* promoter, we performed ChIP experiments in T-47D cells that were serum-starved and treated with Pg for 1 and 4 h or with Pg and RU-486 for 4 h. We designed four pairs of PCR primers to amplify different segments of the *ATBF1* promoter (Fig. 3A).

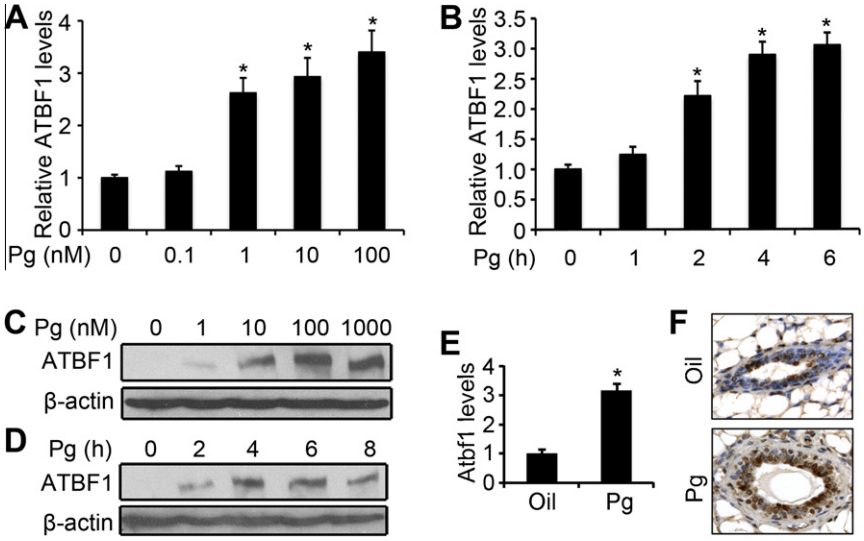

Fig. 1. Progesterone (Pg) increases ATBF1 expression in the PR-positive breast cancer cell line T-47D (A–D) and in normal mouse mammary tissues (E, F). T-47D cells treated with Pg at different concentrations for 4 (A) and 6 h (C) or at 100 nM for the indicated times (B, D) were subjected to real-time PCR for mRNA expression (A, B) or Western blotting for protein expression (C, D). Mammary tissues from mice injected subcutaneously with 100 l Pg (10 mg/ml in sesame oil) or the oil alone were harvested after 48 h and subjected to real-time PCR (E) or immunohistochemical (IHC) staining (F, original magnification 200). *GAPDH* was used as an internal control for human cDNA analysis, while *β-actin* was used for mouse cDNA. $^{*}P < 0.01$. Statistical significance was analyzed by one-way ANOVA (in all figures).

Regular and real-time PCR analysis of DNA bound by the anti-PR antibody showed that *ATBF1* promoter DNA was present in the precipitated DNA spanning primer pairs F1/R1, F2/R2 and F3/R3, but not F4/R4 (Fig. 3C and D). These findings indicate that PR directly binds to the *ATBF1* promoter at binding sites in or near the region between primer sites F1 and R3, which contains three previously-identified potential PR binding sites (Fig. 3A) [23].

3.4. Expression of ATBF1 is necessary for Pg to regulate gene expression and progenitor cell expansion

Recent studies indicate that Pg can also reprogram a subset of differentiated cells into basal-like progenitor cells [24]. To determine whether Pg-induced ATBF1 expression is necessary for Pg to function in this process, we knocked down ATBF1 expression by RNAi in T-47D cells, treated cells with Pg, and cultured them in Matrigel for 2 weeks. Consistent with previous studies indicating the induction of stem/progenitor cell expansion by Pg [4], Pg-treated T-47D cells formed large colonies in Matrigel (Fig. 4A). When ATBF1 expression was significantly reduced by *ATBF1* siRNA transfection (Fig. 4B and C), Pg-induced colony expansion was significantly reduced, while cells without Pg treatment did not show visible changes (Fig. 4A). This result indicates that Pg-induced ATBF1 expression is necessary for Pg to regulate progenitor cell expansion in T-47D cells.

We also evaluated whether upregulation of ATBF1 by Pg is necessary for Pg to regulate gene expression. CD44 and CD49f, two classical progenitor cell markers that are upregulated by Pg [6,25], were indeed induced by Pg in T-47D cells as demonstrated by real-time PCR (Fig. 2B, Fig. 4B), and blocking PR suppressed their expression (Fig. 2B). Their induction was also detectable at the protein level, as demonstrated by Western blotting for CD49f (Fig. 4C) and by IF staining for CD44 (Fig. 4D). When ATBF1 was knocked down however, Pg failed to induce CD49f (Fig. 4B and C) and the induction of CD44 by Pg was also compromised (Fig. 4B and D).

When T-47D cells were cultured on a plastic surface (2-D culture), Pg did not promote cell growth (Fig. 4E). In fact, cell numbers

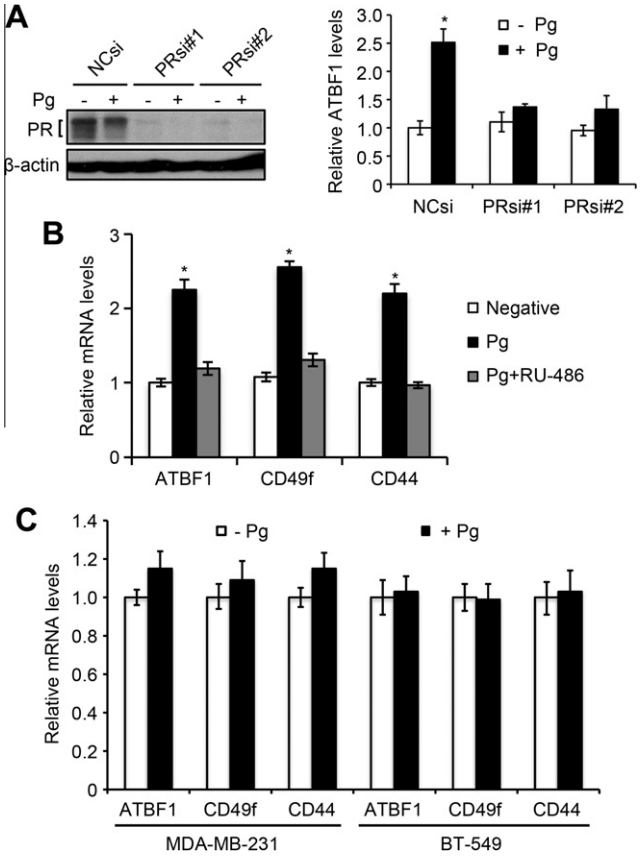

Fig. 2. Pg-induced *ATBF1* expression is dependent on PR. Pg-induced *ATBF1* expression is dependent on PR. (A) Knockdown of PR by two different siRNA against *PR* (PRsi#1 and PRsi#2), as confirmed at the protein level by Western blotting (blots at left), prevents Pg from inducing *ATBF1* expression when compared to negative control siRNA (NCsi), as detected by real-time PCR assay. (B) Pg antagonist RU-486 abolishes the effect of Pg on *ATBF1* mRNA expression in T-47D cells, as detected by real-time PCR. Known target genes of Pg, *CD49f* and *CD44*, were used as controls. (C) Pg has little effect on the expression of *ATBF1*, along with control genes *CD49f* and *CD44*, in PR-negative breast cancer cell lines MDA-MB-231 and BT-549, as detected by real-time PCR. $^{*}P < 0.01$.

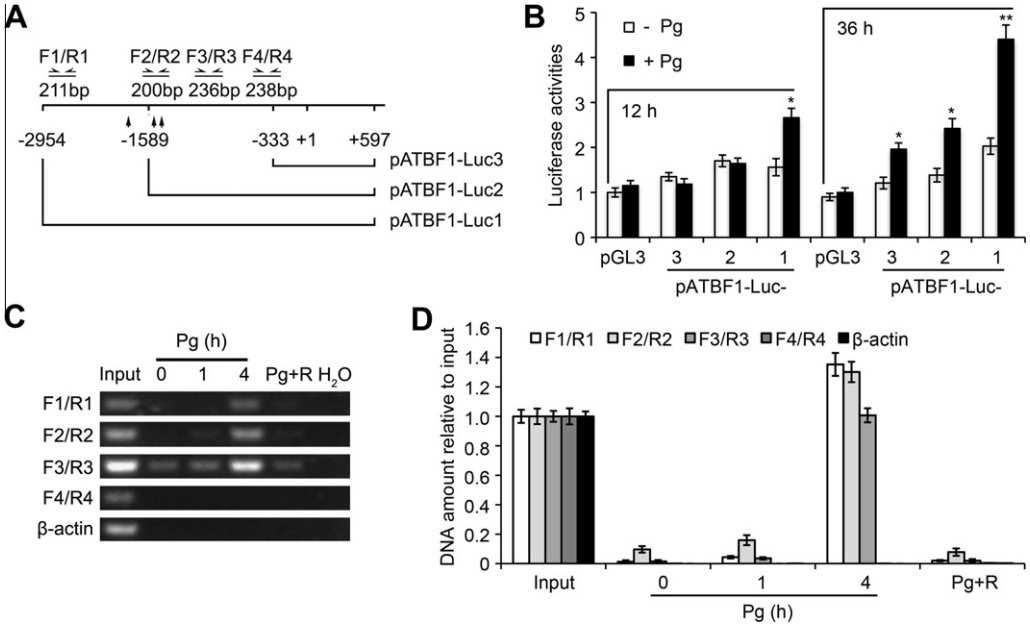

Fig. 3. Pg induces *ATBF1* promoter activities and the binding of PR to the *ATBF1* promoter. (A) Schematic of *ATBF1* promoter region from +597 to −2954 relative to its transcriptional initiation site, showing the fragments used in the promoter-reporter assay and the location and product sizes of four pairs of PCR primers used in the ChIP assay. Arrows indicate predicated PR response elements. (B) Relative luciferase activities of different *ATBF1* promoter-reporter constructs (pATBF1-Luc1/2/3, described previously [16]) in T-47D cells treated with 100 nM Pg for 12 or 36 h. (C, D) Detection of PR-bound *ATBF1* promoter DNA by ChIP assay in combination with regular (C) and real-time (D) PCR in T-47D cells treated with 100 nM Pg for 1 or 4 h or 4 h with RU-486 pretreatment. *β-actin* served as the control for input and water as the negative control for PCR. $^{*}P < 0.05$; $^{**}P < 0.01$.

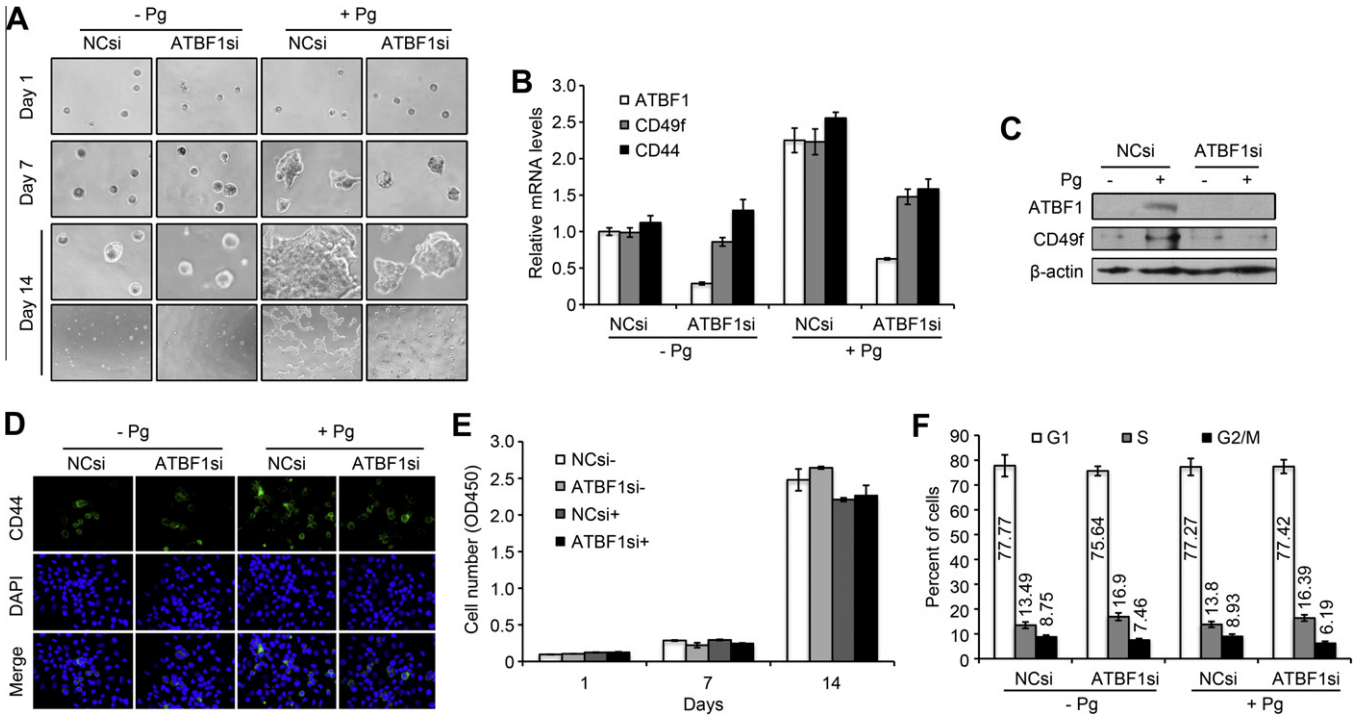

Fig. 4. Knockdown of ATBF1 attenuates the function of Pg in gene regulation and cell dedifferentiation in T-47D cells. (A) Colony formation induced by Pg in Matrigel was attenuated by the knockdown of ATBF1. Phase contrast images are from cells grown in Matrigel for 1, 7 and 14 days, with lower-magnification fields also shown for day 14 at the bottom. (B–D) Pg-treated cells were subjected to real-time PCR for mRNA expression of *ATBF1*, *CD49f* and *CD44* (B), Western blotting for protein expression of ATBF1 and CD49f (C), and IF staining for protein expression and cellular localization of CD44 (D, green, original magnification 200). DAPI was used to show nuclei (blue). (E) The proliferation of T-47D cells on 2-D plastic plates was measured by the cell counting kit-8. (F) The cell cycle distribution in 2-D culture was measured by flow cytometry assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were somewhat decreased by day 14 of Pg treatment, regardless of ATBF1 status (Fig. 4E). In addition, neither Pg treatment nor the knockdown of ATBF1 changed the cell cycle distribution (Fig. 4F).

4. Discussion

Steroid hormones, including estrogen, Pg and prolactin, and their receptors play essential roles in the proliferation and differentiation

of mammary epithelial cells [26]. Although estrogen-ER signaling can activate Pg-PR signaling by inducing PR expression, the latter clearly has distinct functions in ductal branching and lobular-alveolar development of the mammary gland during the estrous cycle and pregnancy as well as in breast carcinogenesis [24,26]. The functions of hormonal signaling have been demonstrated to be mediated by key transcription factors [26,27]. In this study, we found that Pg activated the transcription of *ATBF1*, that the transactivation by Pg occurred in both ER- and PR-positive human breast cancer cells in culture and in mouse mammary glands *in vivo*, and that the transactivation was mediated by Pg-induced direct binding of PR to the promoter of *ATBF1* (Fig. 1–3). Furthermore, induction of ATBF1 plays a necessary role in the function of Pg-PR signaling in gene regulation and cell differentiation control (Fig. 4). These findings suggest that ATBF1 is a key transcription factor in the Pg-PR signaling pathway in the mammary gland.

Pg, together with pituitary-derived prolactin, provides key signals to orchestrate mammary epithelial differentiation during pregnancy [28–30]. Implication of ATBF1 in the function of Pg-PR signaling in this study suggests that ATBF1 could be an important functional mediator of Pg-PR signaling in ductal side-branching and lobular-alveolar development during pregnancy. Although this prediction remains to be tested, a function of ATBF1 in cell differentiation of different tissues has been documented [8,10,11]. For example, ATBF1 expression is induced during the differentiation of mouse mammary gland at puberty and lactation stages [13], differentiation of the human mammary breast epithelial cell line MCF10A in Matrigel [13], and during neuronal differentiation [8–10].

In addition to a pro-differentiation role in the mammary gland, Pg can induce CK5 expression and increase the number of $CD49f^{hi}$ mammary stem/progenitor cells in both mice [31] and humans [32], including stem-like cells in human breast cancer [4,24]. Pg-mediated expansion of stem/progenitor cells involves both paracrine and autocrine signals, and RANKL has been identified as a functional mediator of this process [6]. Using the same cell system used to study the role of Pg in stem-like cells in breast cancer [4], i.e. T-47D cells grown in Matrigel, we found that ATBF1 was induced during the expansion of stem-like cells and that inhibition of ATBF1 expression attenuated the expansion of stem-like cells (Fig. 4). These results suggest that ATBF1 could also mediate Pg-induced stem cell expansion in normal mammary glands, a hypothesis that warrants further exploration.

In our previous studies, we found that ATBF1 and another steroid hormone, estrogen, are involved in an autoregulatory feedback loop that regulates gene expression and cell proliferation by estrogen-ER signaling [14–16]. On one hand, ATBF1 inhibits the function of estrogen-ER signaling in gene regulation and cell proliferation control [14]. On the other hand, whereas estrogen upregulates *ATBF1* transcription by inducing the binding of ER to the *ATBF1* promoter, it causes the degradation of ATBF1 protein via the induction of EFP, an estrogen-responsive E3 ubiquitin ligase [15,16]. Although our current study indicates that Pg-PR signaling also induces *ATBF1* transcription, which suggests that ER-upregulated *ATBF1* could also be mediated by ER-upregulated PR [33], it is unknown whether ATBF1 binds to PR and regulates PR activities. In addition, Pg-PR signaling did not cause the degradation of ATBF1 protein, at least in T-47D cells (Fig. 1). Therefore, Pg-PR signaling might not involve an autoregulatory relationship with ATBF1.

In summary, we have demonstrated that Pg-PR signaling upregulates *ATBF1* transcription by inducing the binding of PR to the *ATBF1* promoter in mammary epithelial cells, and that this induction of ATBF1 is required for the functions of Pg-PR. These findings suggest a role of ATBF1 in multiple biological processes regulated by Pg-PR, including cell proliferation, cell differentiation, expansion of stem/progenitor cells, and tumorigenesis in the mammary gland. Whether and how ATBF1 modulates these processes remains to be determined.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.009.

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