Cell Cycle Defects Contribute to a Block in Hormone-induced Mammary Gland Proliferation in CCAAT/Enhancer-binding Protein (C/EBPβ)-null Mice*

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In contrast to hormone-dependent breast cancer, steroid hormone-induced proliferation in the normal mammary gland does not occur in the steroid-receptor positive cells but rather in adjacent cells via paracrine signaling involving several local growth factors. To help elucidate the mechanisms involved in the block in proliferation in hormone-receptor positive cells, we have utilized a CCAAT/enhancer binding protein (C/EBPβ)-null mouse model. Loss of this transcription factor results in increased steroid and prolactin receptor expression concomitant with a 10-fold decrease in proliferation in response to pregnancy hormones. To determine the basis for this decrease, several markers of cell cycle progression were analyzed in wild type and C/EBPβ-null mammary epithelial cells (MECs). These studies indicated that cell cycle progression in C/EBPβ-null MECs is blocked at the G₁/S transition. C/EBPβ-null mammary glands display substantially increased levels of the activated form of transforming growth factor β, a potent inhibitor of epithelial cell proliferation, as well as increased downstream Smad2 expression and signaling. While cyclin D1 levels were equivalent, cyclin E expression was markedly reduced in C/EBPβ-null as compared with wildtype MECs. In addition, increased p27 stability and retention in the nucleus and decreased levels of the cc25a phosphatase contributed to a significant loss of cdk2 kinase activity. Collectively, these changes prevent C/EBPβ-null mammary epithelial cells from responding to hormone-induced proliferative signals.

The ovarian hormones, estrogen (E)² and progesterone (P), and the pituitary hormone, prolactin (PrL), are key mediators of mammary gland proliferation during lobuloalveolar development. Estrogen receptor (ERα), progesterone receptor (PR), and prolactin receptor (PrLR) are co-expressed in mammary epithelial cells (MECs), and their expression is non-uniform along ducts and does not co-localize with proliferating cells (1–3). Growth factors expressed in steroid receptor- and PrLR-positive cells act on neighboring cells to induce proliferation in a paracrine fashion (3).

Transforming growth factor β (TGF-β) is known to play an anti-proliferative role in mammary gland development (reviewed in Ref. 4), affecting the expression of a number of key regulators of the G₁/S phase transition. While the most well studied TGF-β response in normal epithelial cells is growth inhibition, chronic exposure to TGF-β may also affect differentiation and alter polarity of epithelial cells (5). Recently, it was shown that activated TGF-β1 co-localizes with ERα expression and acts in an autocrine fashion to block the proliferation of steroid receptor-positive mammary epithelial cells (6). Although Tgfβ1⁻/⁻ mice die at 3 weeks of age, loss of one allele down-regulates TGF-β1 expression by 90% and results in accelerated mammary gland development and increased proliferation after hormone treatment (7). Conversely, overexpression of TGF-β1 leads to reduced mammary gland development (8). Many cell cycle components have been shown to play a role in mammary gland development and have functional consequences when deleted in mice. A number of mice with germ line deletion of key cell cycle regulators do not undergo lobuloalveolar development in response to pregnancy, including those lacking cyclin D1, progesterone receptor, Id2, TGF-β1, and CCAAT/enhancer binding protein (C/EBPβ) (7, 9–13).

C/EBPs are a family of bZIP transcription factors involved in proliferation and differentiation in many cell and tissue types, including the mammary gland (reviewed in Refs. 14 and 15). Cebpb encodes an intronless gene that generates a single mRNA that is alternatively translated into three isoforms. W,e, and others (12, 13), have previously characterized the mammary gland phenotype in mice with a germ line deletion of C/EBPβ. Ductal elongation appears normal, and ducts fill the fat pad, but hormone-induced proliferation is impaired (3). Coincident with a 10-fold decrease in 5-bromo-2′-deoxyuridine (BrdUrd) incorporation, a marker of S-phase, is a nearly 3-fold increase in the percent of ERα/PR/PrLR-positive mammary epithelial cells in C/EBPβ-null mice (3, 15, 16). Despite decreased proliferation and altered steroid receptor and PrLR patterning, these two cell populations remained distinct.

A key question in the mammary gland and breast cancer fields is what keeps steroid receptor-positive cells from dividing, and why is this proliferative block lost during breast cancer progression. The opposite effect is observed in C/EBPβ-null mammary glands, where increased numbers of ERα/PR-positive cells are accompanied by decreased proliferation. The precise role of C/EBPβ is not clear because of its many protein-protein interactions and post-translational modifications (reviewed in Refs. 17 and 18). To better understand how loss of C/EBPβ affects hormone-induced mammary gland proliferation, we used wild type (WT) and C/EBPβ-null mammary tissue to analyze the expression and activity of G₁/S cell cycle molecules after acute (2 days with estrogen and progesterone (E + P)) hormone treatment. These types of studies cannot be duplicated in cell culture, since almost all mammary cell lines lose expression of steroid receptors and cell-cell interactions in tissues.
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and paracrine effects of growth factors cannot be accurately recapitu-
lated in culture. We found that loss of C/EBPβ and the altered steroid
receptor patterning results in up-regulation of active TGF-β1, decreased
cyclin E expression, increased p27, decreased cdc25A expres-
sion, and loss of cdk2 kinase activity, all of which contribute to an insur-
mountable block in cell cycle progression in response to hormone stim-
ulation. Thus, the C/EBPβ-null mouse model has provided valuable
new insights into the mechanisms that prevent steroid receptor-positive
cells from proliferating in response to pregnancy hormones and may
help us understand how these mechanisms are abrogated in breast can-
cer progression.

EXPERIMENTAL PROCEDURES

Animals and Tissue Isolation—Mice with targeted germ line deletion
of C/EBPβ have been previously described (19). Animal care and pro-
cedures were approved by the Institutional Animal Care and Use Com-
mitee of Baylor College of Medicine and were in accordance with the
procedures detailed in the Guide for Care and Use of Laboratory Ani-
mals (National Institutes of Health publication 85-23). Twelve-week-
old, ovary-intact, nulliparous mice were treated for 48 h with a single
interscapular subcutaneous injection of 17β-estradiol benzoate (1 µg)
and progesterone (1 mg) in 100 µl of sesame oil (all from Sigma). Two
hours before sacrifice, animals were injected intraperitoneally with
BrdUrd (0.03 mg/g of body weight; Sigma). Mammary glands were
removed and fixed in 4% paraformaldehyde for 2 h at 4 °C. After embed-
ing in paraffin, tissues were sectioned (5–7 µm) onto Probe-On Plus
and the altered steroid

immunostaining. At least

experimental sample was then normalized to the levels of load-
ing control.

Image Capturing, Counting, and Statistical Analyses—An Olympus
BX40 light microscope connected to a MagnaFire digital camera was
used to capture images from immunohistochemical staining. At least
8–12 individual 40× fields per sample were captured for counting. The
number of positively stained MEC nuclei was expressed as a percentage
of the total number of luminal epithelial cells. Statistical significance
was determined by Student’s t test (two-sample assuming unequal variance).
Fluorescent images of phospho-p27 immunostaining were digitally cap-
tured using an Olympus BX50 Microscope connected to a Hamamatsu
C5810 CCD device. Cells with nuclear fluorescent intensity above a
certain threshold in Adobe Photoshop were counted as positive and
expressed as a percentage of total MEC nuclei. Fluorescent images of
polarity markers were captured using a Zeiss LSM 510 confocal micro-
scope. A single Z section was digitally processed using Adobe Photo-
shop. Active TGF-β staining was processed as described previously (7).
To quantify the mean TGFβ intensity per cell, a small, circular mask was
placed on the basal side of epithelial nuclei in the DAPI channel using
SigmaScan Pro5 (Systat Software, Inc.). The masks were then transposed
onto the corresponding fluorescein isothiocyanate channel. A mean inten-
sity under each mask was derived in each field. These values were normal-
ized against background to calculate a mean intensity per cell for each
treatment group. A two-tailed t test was used to determine significance.

Tissue Extracts—Mammary glands were collected and pooled from
mice treated for 2 days with E + P and used to enrich for MECs as
described by Pullen and Streuli (20). Briefly, the tissues were minced
and digested at 37 °C for 1 h in Dulbecco’s modified Eagle’s medium/F-12
media (Invitrogen, Carlsbad, CA) containing collagenase (2 mg/ml;
Roche Applied Science) and hyaluronidase (100 units/ml; Sigma), fol-
lowed by washes with Dulbecco’s modified Eagle’s medium/F-12 media.
Whole cell extracts (WCEs) were prepared from enriched MECs after
lysis with RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40,
0.5% deoxycholate, 0.1% SDS, Roche complete protease inhibitor mix-
ture, 1 mM NaF, 0.1 mM sodium vanadate, 0.1 mM sodium molybdate).
Nuclear and cytoplasmic extracts were prepared from enriched MECs
using the NE-PER kit according to the manufacturer’s instructions
(Pierce). The purity of these extracts was determined by Western blot
analysis (data not shown) using antibodies against IRS-1 (cytoplasmic)
and phospho-Smad2 Ser465/467 (nuclear). Protein concentrations were
determined using the BCA assay from Pierce.

Immunoprecipitation and Western Blotting—Immunoprecipitation
assays were performed using whole cell RIPA extracts from enriched
MECs. Forty µg of extract was incubated overnight at 4 °C with 4 µg of
primary antibody and protein A/G PLUS agarose (Santa Cruz Bio-
technology). Precipitated immune complexes, whole MEC extracts, and
nuclear and cytoplasmic MEC extracts were run on 12% Tris-glycine
SDS-PAGE gels and transferred to polyvinylidene difluoride mem-
branes. After blocking with 5% nonfat dry milk in Tri-buffered saline +
0.1% Tween 20 (TBST), blots were incubated overnight at 4 °C with
primary antibodies diluted 1:1000, including p27 (Ab-2; NeoMarkers),
phospho-Lck (Upstate Biotechnology, Charlottesville, VA),
phospho-p27 (Thr187), E-cadherin (Zymed Laboratories Inc., South
San Francisco, CA), phospho-Erin-Radixin-Moesin (ERM) (Cell Sig-
aling Technology), smooth muscle α-actin (A14; Sigma), keratin 5 (Covance,
Richmond, CA). Immunoperoxidase staining was detected using the
Vectastain Elite ABC and the diaminobenzidine substrate kits accord-
ing to manufacturer’s instructions (Vector Laboratories, Burlingame,
CA). Nuclei were counterstained with Harris hematoxylin. Immunoflu-
orescence staining was detected using the appropriate Texas Red- or
Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene,
OR), and nuclei were counterstained with 4’,6-diamidino-2-phenylin-
dole (DAPI; Vector Laboratories). Immunofluorescence for the acti-
vated form of TGF-β1 (chicken polyclonal; R&D Systems, Minneapolis,
MN) was performed on frozen sections as described previously (7).

Image Capturing, Counting, and Statistical Analyses—An Olympus
BX40 light microscope connected to a MagnaFire digital camera was
RESULTS

Cell Cycle Progression Is Blocked in C/EBPβ-null Mammary Epithelial Cells—To mimic the burst of proliferation observed during early pregnancy, mice were treated acutely with E + P for 2 days, resulting in ~15% of MECs entering S-phase in wild type mice (3). Previous studies demonstrated a 10-fold decrease in proliferation, as measured by BrdUrd incorporation, in mammary glands from C/EBPβ-null mice after 2 days of E + P treatment (3). These results suggested a block in the G1/S phase transition. To better define the stage at which cell cycle progression was blocked, additional proliferation markers were analyzed. The expression of Ki67, phospho-histone H1, and phospho-histone H3 were examined by immunohistochemistry and compared with the 2-h BrdUrd pulse using mammary tissues from WT and C/EBPβ-null mice after 2 days of E + P treatment (3). These results suggested a block in the G1/S phase transition. To better define the stage at which cell cycle progression was blocked, additional proliferation markers were analyzed. The expression of Ki67, phospho-histone H1, and phospho-histone H3 were examined by immunohistochemistry and compared with the 2-h BrdUrd pulse using mammary tissues from WT and C/EBPβ-null mice after 2 days of E + P treatment (3). Using immunohistochemical staining, the percent of BrdUrd-positive cells showed a 10-fold decrease in C/EBPβ-null MECs, similar to previous results obtained by indirect immunofluorescence (3). The nuclear Ki67 antigen is expressed in proliferating cells during all phases of the active cell cycle but absent in resting (G0) cells (22). In response to E + P, ~27% of MECs from WT glands were positive for Ki67, compared with fewer than 6% of MECs from C/EBPβ-null glands (Fig. 1B). Hormone treatment resulted in 24% of WT MECs expressing phospho-H1 versus 2% of C/EBPβ-null MECs positive for this marker (Fig. 1B). Histone H3, when phosphorylated on serine 10, is used as a marker of mitosis and chromatin condensation (24). Similar to the other markers, a significant decrease in phospho-H3 staining was observed in C/EBPβ-null MECs as compared with WT (Fig. 1B). Since both S phase and mitotic markers were equivalently affected in C/EBPβ-null mammary cells, these results suggested that the null cells are either unable to enter the cell cycle from G0 or never progressed past the G1-S transition in response to hormone treatment.

Increased Activation of TGF-β Correlates with Block in Proliferation but Does Not Alter Polarity—TGF-β1 was recently shown to co-localize with steroid hormone receptors (6) and is a potent inhibitor of epithelial cell proliferation (reviewed in Ref. 4). We hypothesized that there might
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FIGURE 2. Alterations in TGF-β signaling, but no difference in epithelial cell polarity, between WT and C/EPBβ-null mammary glands. A, immunofluorescent staining for the active form of TGF-β1 (green) was performed on frozen mammary gland sections from WT and C/EPBβ-null mice treated for 2 days with E + P. Images were captured by confocal microscopy, with DAPI-stained nuclei shown in blue. Representative images are shown from three animals per genotype. Quantitation of mean fluorescent intensity per cell is presented on the scatter plot, with the points representing individual animals, and the blue bar representing the mean of all samples. Error bars show the S.E., and a statistically significant difference was observed between WT and C/EPBβ-null mean values (p < 0.0001; indicated by the blue asterisk). B, 15 μg of MEC WCE from WT or C/EPBβ-null mammary glands (2 days with E + P) were loaded per lane and blotted using a phospho-Smad2 antibody. The blots were reprobed first with a Smad2 antibody and then with an ERK antibody as a loading control. Quantitation of relative densitometric intensity for Smad2 and phospho-Smad2, normalized to the loading control, is shown in the bar graph with error bars showing the S.E. C, immunofluorescence staining for phospho-ERM (green) and smooth muscle α-actin (red) was performed on paraffin-embedded mammary gland sections from WT and C/EPBβ-null mice (2 days with E + P), and images were captured by confocal microscopy. DAPI-stained nuclei are shown in blue. Dactal lumens (Lu) are indicated. Bar, 10 μm. D, immunofluorescence staining for E-cadherin (red) and keratin 5 (green) was performed on paraffin-embedded mammary gland sections from WT and C/EPBβ-null mice (2 days with E + P), and images were captured by confocal microscopy. DAPI-stained nuclei are shown in blue. Bar, 10 μm.

be increased TGF-β expression in the C/EPBβ-null mammary gland correlating with the 2–3-fold increase in the number of cells expressing ERα and PR (3, 15), which might correlate with the decrease in proliferation. Frozen mammary gland sections from mice treated for 2 days with E + P were stained with an antibody specific for the active form of TGF-β1, as opposed to the latent form of TGF-β1 associated with the extracellular matrix (ECM). Immunostaining revealed up-regulation of active TGF-β1 in C/EPBβ-null mammary epithelial cells, as evidenced by the higher intensity of the fluorescent signal (Fig. 2A). In addition to more active TGF-β1-positive cells, there was increased expression per cell in the C/EPBβ-null MECs as compared with WT (Fig. 2A).

A functional read-out for TGF-β activity is the phosphorylation state of the signaling molecule Smad2. Phosphorylation at Ser465 and Ser467 by TGF-β type I receptor allows Smad2 to dimerize, interact with Smad4, translocate to the nucleus, and affect the transcriptional regulation of cell cycle genes involved in entry into S phase (25). Western blot analysis showed increased total levels of Smad2 in C/EPBβ-null as compared with WT MEC extracts (Fig. 2B), as well as higher levels of Smad2 phosphorylation (Fig. 2B), which correlates with the increased active TGF-β staining (Fig. 2A). The increase in Smad2 levels may be a result of the increase in the number of ERα-positive cells in C/EPBβ-null mammary glands (15), whereas the higher level of Smad2 phosphorylation may reflect the increase in active TGF-β1 expression.

Chronic TGF-β exposure often results in loss of epithelial cell polarity (5), which may account for the failure to respond to hormone-induced signals. Immunofluorescent staining for various polarity markers was performed and analyzed by confocal microscopy to see if a change in polarity had occurred in C/EPBβ-null mammary tissue. A polarity marker specific for the apical surface of epithelial cells is the phosphorylated form of ERM, a family of F-actin-binding proteins (reviewed in Ref. 26). Immunostaining for phospho-ERM (green) showed a discontinuous pattern that lined the lumen of ducts from WT mice, whereas a more uniform expression pattern was observed in C/EPBβ-null ducts (Fig. 2C). This distinction is most noticeable in the higher magnification images. Despite the difference in expression pattern, the protein was localized correctly in both genotypes. Co-immunostaining for smooth
between WT and C/EPBβ-null mice (Fig. 3A). This was confirmed by immunostaining of tissue sections, showing equal percentages of cyclin D1-positive nuclei in both WT and null MECs (Fig. 3B). In contrast, Western blot analysis for cyclin E demonstrated significantly reduced levels in C/EPBβ-null MEC extracts compared with WT (Fig. 3C). While a moderate decrease in expression was observed using cytoplasmic extracts, there was a more pronounced decrease in nuclear cyclin E levels. Therefore, the defect in cell cycle progression in C/EPBβ-null mammary glands appears to occur during late G1 phase coinciding with a reduction in cyclin E expression.

**Alterations in Cyclin-dependent Kinase Inhibitor Expression**—In addition to cyclins, which promote cell cycle progression, cyclin-dependent kinase inhibitors (CKIs) inhibit proliferation. The main CKIs associated with cyclin E are p21CIP1/Waf1 and p27Kip1. The abundance of p21 in the adult mammary gland is very low, and it may be expressed only in relatively quiescent mammary stem/progenitor cells (29). Accordingly, immunohistochemistry for p21 in WT and C/EPBβ-null mammary glands identified very few MECs that stained positive for p21 (data not shown). By Western blot analysis, p21 levels were detectable only after long exposure and were equivalent between WT and null MEC extracts (Fig. 4A). Levels of p27, however, were much higher than p21 in adult mammary tissue, and expression was readily detectable by both Western blot analysis and immunostaining. There was approximately a 2-fold increase in p27 expression in C/EPBβ-null MEC extracts as compared with WT (Fig. 4B). A slight increase in p27 was detected in the cytoplasmic fraction, but a more substantial increase in nuclear p27...
was observed in C/EBPβ-null MECs as compared with WT (Fig. 4B). Quantitation of immunohistochemistry also revealed a 2-fold increase in p27-positive nuclei in C/EBPβ-null as compared with WT (Fig. 4C).

Multiple post-translational modifications modulate the activity and localization of p27 (reviewed in Ref. 30). Phosphorylation at Thr187 by cdk2 targets p27 for ubiquitin-mediated degradation and dissociates p27 from cyclin/cdk complexes, allowing cells to enter S phase (31). An antibody specific for p27 phosphorylated at Thr187 was used to perform immunoprecipitation experiments (lanes 3–6) from WT or C/EBPβ-null mammary glands (2 days with E + P) were loaded per lane and blotted using a p27 antibody. The blots were reprobed with an ERK antibody as a loading control. B, either 25 μg of MEC WCE (lanes 1 and 2) or 20 μg of MEC cytoplasmic/nuclear extracts (lanes 3–6) from WT or C/EBPβ-null mammary glands (2 days with E + P) were loaded per lane and blotted using a p27 antibody. The blots were reprobed with an ERK antibody to control for normalization. C, expression of p27 was analyzed by immunohistochemistry on mammary gland sections from 12-week-old WT or C/EBPβ-null mice after treatment for 2 days with E + P. Quantitation of p27-positive MEC nuclei is plotted in the bar graph, with error bars showing the S.E. A statistically significant difference was observed between WT and C/EBPβ-null (p < 0.001; indicated by the asterisk). Bar, 50 μm. D, expression of phospho-p27 (Thr187) was analyzed by immunofluorescence on mammary gland sections from 12-week-old WT or C/EBPβ-null mice after treatment for 2 days with E + P. Most of the brightly stained nuclei in the C/EBPβ-null section are myoepithelial cells (arrows) and not luminal epithelial cells. Bar, 50 μm. Quantitation of phospho-p27-positive MEC nuclei is plotted in the bar graph, with error bars showing the S.E. Statistically significant differences were observed between WT and C/EBPβ-null (p < 0.05; indicated by the asterisk).

DISCUSSION

These studies have demonstrated that loss of C/EBPβ results in the inability of mammary epithelial cells to respond to hormonal stimuli, as evidenced by decreased proliferation, and that multiple defects in cell cycle components contribute to this block. While many cell cycle studies have been performed using a limited number of breast cancer cell lines, relatively few have been done using mouse models of mammary gland proliferation. A key difference between these types of studies is that only direct effects of ERα/PR are observed in cell lines, whereas in mammary gland models allow indirect or paracrine effects on proliferation to be analyzed. In C/EBPβ-null mice, a uniform decrease in all proliferation markers tested indicates that cell cycle progression is blocked at exit from G0 or entry into S phase, which led us to examine proteins important for the G1/S transition, including cycins, CKIs, and cdk activity.

The most striking difference between WT and C/EBPβ-null mammary glands was the substantial increase in activated TGF-β, which may contribute to inhibition of cell proliferation but does not affect epithelial cell polarity. It was recently noted that steroid-receptor positive cells co-localize with active TGF-β1 and this may be the mechanism by which ERα/PR-positive cells are restrained from proliferating in response to hormonal stimulation (6). This finding is consistent with
the increase in the percentage of ERα/PR-positive cells and increased active TGF-β1 observed in mammary glands from C/EBPβ-null mice, correlating with decreased proliferation in response to E + P. Cell growth is inhibited by TGF-β-activated signaling through the Smad proteins, which may result in the transcriptional up-regulation of CKIs but down-regulation of c-myc and cdc25A expression. Some of these changes were evident in C/EBPβ-null mammary glands, including increased Smad expression and activity and a decrease in cdc25A expression.

While there was no apparent change in the expression levels or the number of cells positive for cyclin D1, a marked decrease in cyclin E expression was noted in MEC extracts from C/EBPβ-null mice, both in overall levels and especially in the proportion in the nucleus where the kinase exerts its activity. Data from Ewen and his colleagues (18) have suggested that C/EBPβ acts downstream of cyclin D1, independent of interactions with cdk, so it is not surprising that there was no change observed in cyclin D1 in C/EBPβ-null mammary glands. The full-length LAP isoform of C/EBPβ constitutively represses cyclin D1 target genes, and this repression is relieved by either overexpression of cyclin D1 or increased translation of the dominant-negative LIP isoform of C/EBPβ. While the deregulation of cyclin D1 and C/EBPβ LIP expression may be more relevant to breast cancer progression, expression of cyclin D1 target genes has also been reported to decrease in a mammary cell line derived from germ line deletion of all C/EBPβ isoforms (18). Cyclin D1 is not expressed in PR-positive cells (34) and only partially co-localizes with BrdUrd-positive MECs, so cyclin D1 does not appear to play a role in the decrease in proliferation observed in C/EBPβ-null mammary glands.

A well-established model for studying cell cycle regulation in vivo is partial hepatectomy to study liver regeneration in rodents. Loss of C/EBPβ expression or decreased C/EBPβ activity in models of liver regeneration also have been associated with decreased levels of cyclin E, without detectable changes in cyclin D1 expression (35, 36). The conclusion from these studies is that C/EBPβ appears to control cyclin E expression, either directly or indirectly, in agreement with the present results with respect to hormone-induced mammary gland proliferation.

While the best studied function of cyclin E is its association with cdk2, cyclin E has also been shown to act independently of cdk2 to promote DNA synthesis by associating with centrosomes (37). Overexpression of cyclin E results in accelerated entry into S phase and may lead to polyplody and genomic instability in cancer cells by this mechanism. In addition, homeotic genes have been shown to control the levels of cyclin E as an important step in regulating asymmetric division of neural stem cells in Drosophila (38). Therefore, it is interesting to speculate that loss of cyclin E expression in C/EBPβ-null mammary cells may have additional consequences, such as an alteration in cell fate.

Overexpression of cyclin D1 and cyclin E are common occurrences in primary breast cancers. While both amplification of the cyclin D1 locus and up-regulation of protein expression are found in tumors, the cyclin E locus is usually not amplified but does exhibit increased expression in ∼40% of breast cancers (reviewed in Ref. 39). It is also interesting to note that most cyclin D1-expressing tumors are ER-positive, but cyclin E-positive tumors often do not express ER. Expression of a low molecular weight form of cyclin E in breast tumors has been associated with poor prognosis (40). The truncated form of cyclin E has increased affinity for cdk2 and cannot be inhibited by CKIs. Breast tumors expressing low molecular weight cyclin E are usually not responsive to anti-estrogen therapies (41), underscoring the important role cyclin E normally plays in mediating hormone-induced mammary gland proliferation. Interestingly, cyclin A was expressed equally in both WT and C/EBPβ-null MECs (data not shown) implying that the decrease in cdk2 activity in the C/EBPβ-null gland is not due to alterations in cyclin A levels.

The cyclin-dependent kinase inhibitors are also critical regulators of cell cycle progression. While the role of p21 in regulating cell cycle progression has been studied extensively in cell lines, it appears that p21 levels are quite low in adult mammary gland tissue. It has been proposed that p21 may play a key role to control stem/progenitor cells proliferation but have little effect on cell cycle progression in the majority of MECs in the absence of DNA damage and p53 activation (29). In contrast, p27 is more widely expressed in the mammary gland and co-
localizes in steroid hormone receptor-expressing cells, which do not proliferate (42). Mammary glands from C/EBPβ-null mice exhibited a modest increase in the total level and number of p27-positive MECs. Of note was the retention of p27 in the nucleus where it may function to keep cyclin E/cdk2 complexes inactive. With less cyclin E and more p27 in the nucleus, the cdk2 kinase is minimally activated in C/EBPβ-null MECs. In turn, loss of cdk2 kinase activity results in decreased phosphorylation of p27 at Thr187, which is necessary for ubiquitin-mediated degradation, leading to stabilization of p27 (30).

Together, up-regulation of TGFβ signaling, loss of cyclin E expression, increased p27, and decreased cdc25A protein levels lead to a loss of cdk2 kinase activity, preventing cells from entering S phase and proliferating in response to steroid hormones and prolactin. cdk2 kinase activity is required to complete the phosphorylation of Rb, and its other targets include p27, histone H1, Smad2, and C/EBPβ itself, all of which are altered in the C/EBPβ-null mammary gland. While cdk2 was thought to be essential for entry into S phase, cdk2-null mice are viable (reviewed in Ref. 43). Therefore, it appears that other CDKs can compensate, even though cdk2 is the main partner for both cyclin E and A. However, the lack of cdk2 activity is associated with the inhibition of hormone-induced proliferation in the mammary glands of C/EBPβ-null mice. C/EBPβ is a target of cdk2 kinase activity, but surprisingly C/EBPβ can be phosphorylated in cdk2-null mice (44). Hyperphosphorylation of Rb is also observed in cdk2 null mice at what were thought to be cdk2-specific sites, supporting the existence of a compensating kinase. cdk8 is found in an inhibitory mediator complex with C/EBPβ (45), and Ras signaling through mitogen-activated protein kinase induces phosphorylation of C/EBPβ, thought to result in a conformational change that removes cdk8 from the mediator complex. Importantly, mouse models of skin tumorigenesis have demonstrated a requirement for C/EBPβ in mediating Ras-induced transformation (46).

The proliferative events required for ductal elongation during puberty are relatively normal in C/EBPβ-null mammary glands, as well as in other knock-out models with a similar mammary gland phenotype, including cyclin D1, PR, PrlR, Stat5, and Ild2 null mice. However, the ability to respond to pregnancy hormones to induce lobluloalveolar differentiation is impaired in these mice. Normally the percentage of MECs containing steroid receptors is higher during puberty than in mature adult ducts, but this down-regulation event does not occur in C/EBPβ-, PrlR- and Stat5-null mammary glands (3, 16). Interestingly, proliferation of ERα-positive cells during puberty in either ducts or terminal end buds in Tgfb1+/− mice is not increased, in contrast to adult tissues (6).

It is still not known what sets up the correct steroid-receptor patterning after puberty and why several germ line knock-out mouse models have an increased percentage of steroid receptor positive cells. This is apparently not due to systemic hormonal deficiencies in knock-out mice, as C/EBPβ-null tissue transplanted to a WT host display the same phenotype. Thus, these effects on steroid receptor patterning appear to be cell autonomous. The fact that ductal elongation appears normal while alveolar differentiation is impaired in C/EBPβ-null mice helps support the existence of alveolar progenitor cells, which are distinct from ductal progenitors. Putative alveolar progenitor cells have been identified by using a pregnancy-driven Cre recombinase to mark cells that differentiate and remain in the mammary gland after involution (47). Cell cycle regulators, including TGFβ and p21, have been reported to be important for maintaining progenitor cells in a quiescent state (29, 48, 49). It may be that the block in hormone-induced proliferation is a function of the altered cell fate of alveolar progenitors.

Loss of C/EBPβ results in inappropriate steroid receptor patterning, which may lead to more cells expressing active TGFβ-β. The mechanisms responsible for activation of latent TGFβ-β remain to be established. The fundamental question of how steroid receptor patterning is established during puberty also remains to be determined. These studies, however, help support the concept that the dissociation of proliferation and ERα/PR/PrlR expression is a critical determinant of normal mammary gland development. In contrast, steroid receptor-positive cells often are proliferative in mouse models of mammary gland hyperplasia, such as p53−/− mice, and in precancerous lesions of the human breast, such as ductal carcinoma in situ, suggesting a switch from a paracrine to an autocrine response to proliferative stimuli (50, 51). cdc25A overexpression has been found in samples from primary breast tumors and was associated with poor survival in nearly half of these patients (52). Another study using human breast cancer cell lines demonstrated that deregulation of cdc25A expression was most likely post-transcriptional, with little change in mRNA levels (53). It is interesting to speculate that loss of TGFβ in precancerous breast lesions might result in increased cdc25A expression and/or stability, permitting steroid-receptor-positive cells to proliferate, in contrast to what is observed in C/EBPβ-null MECs. Understanding how steroid hormones contribute to normal mammary gland proliferation and how this regulation is disrupted in knock-out mouse models should help provide new insight into how cell cycle control is perturbed in breast cancer progression.

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