Nulliparous CCAAT/Enhancer Binding Proteinδ (C/EBPδ) Knockout Mice Exhibit Mammary Gland Ductal Hyperlasia

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CCAAT/Enhancer binding proteins (C/EBPs) are a family of nuclear proteins that function in the control of cell growth, death, and differentiation. We previously reported that C/EBPδ plays a key role in mammary epithelial cell G_0 growth arrest. In this report, we investigated the role of C/EBPδ in mammary gland development and function using female mice homozygous for a targeted deletion of C/EBPδ (C/EBPδ −/−). C/EBPδ −/− females develop normally and exhibit normal reproductive and lactational performance. Adult nulliparous C/EBPδ −/− females, however, exhibit mammary epithelial cell growth control defects. The mean number of mammary ductal branches is significantly higher in adult nulliparous C/EBPδ −/− females compared with C/EBPδ +/+ (wild-type control) females (66.8 ± 5.2 vs 42.9 ± 6.3 branch points/field, $P < 0.01$). In addition, the mean total mammary gland cellular volume occupied by epithelium is significantly higher in adult nulliparous C/EBPδ −/− females compared with C/EBPδ +/+ controls (29.0 ± 1.4 vs 20.4 ± 1.3, $P < 0.001$). Our results showed that the BrdU labeling index was significantly higher in mammary epithelial cells from nulliparous C/EBPδ −/− females compared with C/EBPδ +/+ controls during the proestrus/estrus (4.55 ± 0.70 vs 2.14 ± 0.43, $P < 0.01$) and metestrus/diestrus (6.92 ± 0.75 vs 3.98 ± 0.43 $P < 0.01$) phases of the estrus cycle. In contrast, the percentage of mammary epithelial cells undergoing apoptosis during both phases of the estrus cycle did not differ between C/EBPδ −/− and C/EBPδ +/+ females. The increased epithelial cell content and proliferative capacity was restricted to the nulliparous C/EBPδ −/− females as no differences in mammary gland morphology, ductal branching or total epithelial content were observed between multiparous C/EBPδ −/− and C/EBPδ +/+ females. These results demonstrate that C/EBPδ plays a novel role in mammary epithelial cell growth control that appears to be restricted to the nulliparous mammary gland. Exp Biol Med 228:278–285, 2003

Key words: C/EBPδ; mammary epithelial cell; involution; estrus cycle; nulliparous

Tissue development and morphology is controlled by a balance of cell division, quiescence, and programmed cell death. In adult animals, most tissues and organ systems are composed of cells that have exited the cell cycle and exist in a quiescent, or G_0-like growth arrest state (1). Despite the importance of G_0 growth arrest in cell and tissue biology, little is known about the regulation and function of G_0 growth arrest-specific genes. Genes that encode proteins that block cell cycle progression or facilitate cell cycle exit into G_0 growth arrest are generally classified as tumor suppressor genes (2). Loss of tumor suppressor gene function by mutational inactivation, promoter methylation silencing, or gene deletion is a common early event in tumor development (2, 3).

The mammary gland is a novel organ system that completes its morphological development almost entirely during postnatal life (4, 5). In the adult female, the mammary gland undergoes repeated cycles of cell division, quiescence, and differentiation (6, 7). The relationship between cyclic changes in circulating ovarian steroid hormones and the mammary gland has been well documented; however, the molecular mechanisms mediating mammary gland hormonal responses are incompletely understood and continue to be investigated (8).

We previously reported that C/EBPδ, a member of the CCAAT/enhancer binding protein (C/EBP) family of nuclear proteins, functions in mouse and human mammary epithelial cell G_0 growth arrest *in vitro* (9–13). In addition, we showed that C/EBPδ gene expression is induced during

This work was supported in part by the National Institutes of Health National Center for Research Resources Grant RR00114 (to A.G.), the National Institutes of Health Grant 57607-09 (to J.D.) and National Cancer Institute Grant P30 CA16058 (to Ohio State University Comprehensive Cancer Center).

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Received July 2, 2002.
Accepted November 1, 2002.

1535-3702/03/2283-0278$15.00

the initial "reversible" phase of mouse mammary gland involution *in vivo* (14, 15). C/EBPδ is one of five C/EBP family members that have been identified, including C/EBPα, C/EBPβ (also called CRP2, NF-IL6, LAP, AGP/EBP, IL-6BP, or NF-M), C/EBPγ, C/EBPδ (CRP3, NF-IL6β, CELF), and C/EBPε (16). C/EBP family members are expressed in a tissue-specific manner and function in a wide range of cellular activities, including the regulation of cell growth and differentiation (16). C/EBPs function in the control of cell growth and differentiation by a variety of biochemical mechanisms, including transcriptional activation of downstream target genes, such as GADD45γ (17); repression of E2F-DP-mediated S phase transcription (18); and protein–protein interaction with cell cycle regulatory proteins, such as pRb, p107, p21, and cdk2 (19–21).

Studies using gene deletion ("knockout") mice have demonstrated that C/EBPs play critical roles in the development and function of specific cell types and organ systems, particularly reproductive tissues. C/EBPβ, for example, plays an essential role in ovarian function, ovulation, and female reproductive biology (22–24). In addition to defects localized to the ovary, C/EBPβ −/− females also exhibit blunted mammary ductal development, reduced mammary lobuloalveolar proliferation, and defective mammary epithelial cell differentiation that is independent of ovarian function (23, 24).

Previous reports have identified relatively subtle, tissue-specific abnormalities in C/EBPδ −/− mice (25, 26). For example, adipocyte differentiation is slightly impaired in C/EBPδ −/− mice (25). In addition, C/EBPδ −/− mice exhibit alterations in learning and memory that result in a selectively enhanced contextual fear conditioned response (26). The current study investigated mammary gland development and function in C/EBPδ −/− female mice. The results indicate that adult nulliparous C/EBPδ −/− females exhibit excessive mammary ductal branching and increased total mammary epithelium compared with nulliparous C/EBPδ +/+ females. The increased mammary ductal branching and total mammary epithelium results from increased proliferation of mammary epithelial cells during the normal estrus cycle. Reproduction, lactation and mammary gland involution are essentially normal in C/EBPδ −/− females. These results indicate that C/EBPδ functions in the maintenance of mammary epithelial cell growth control in the nulliparous mammary gland.

Materials and Methods

Animals. Generation of C/EBPδ knockout (−/−) mice has been previously described (26). Pups born from C/EBPδ heterozygote (+/−) matings were genotyped by Southern blot and/or PCR using primers specific for C/EBPδ and the neomycin selection cassette (26).

Postlactational Mammary Gland Morphology. All litters were standardized to 6 pups/dam at day one postpartum. Pups were removed after 8–10 days of lactation and dams were sacrificed. Mammary glands were fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin and eosin (three C/EBPδ +/+ [control] and three C/EBPδ −/− mice per time point). Mammary gland sections were evaluated by a Veterinary Pathologist (A.P.G.).

Northern Blot Analysis. Total RNA was isolated from fresh and snap frozen mammary tissue using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) as previously described (14, 15). Twenty micrograms of total RNA was electrophoresed on a 1.2% agarose gel, transferred to a nylon membrane hybridized with random primer ^{32}P-labeled probes, washed, and autoradiographed. Mouse C/EBPα, C/EBPβ, and C/EBPδ cDNA probes were generously provided by Dr. Steven L. McKnight (UT Southwestern Medical Center, Dallas, TX) and testosterone repressed prostate message-2 (TRPM-2) probe was a generous gift of Dr. M. Tenniswood (W. Alton Jones Cell Science Center, Lake Placid, NY). Gas-1 was a generous gift from Dr. Claudio Schneider (Cosorzio Interuniversiatrio Biotecnologie, Trieste, Italy), and IGFBP-5 oligonucleotide probes were synthesized from published sequence information. Northern blots were exposed and signals detected using a Molecular Dynamics 445 SI Phosphorimager. Signals were quantified using Molecular Dynamics Imagequant version 4.2 and loading correction performed from ethidium bromide stained 18S rRNA (3 mice/time point).

Mammary Gland Whole Mounts. Whole mounts of the right fourth mammary gland were performed on 6-, 9-, 10-, 12-, 16-, 26-, and 28- to 30-week old, virgin female mice as described (22). Digital images of virgin female whole mounts were captured and the Bioquant NOVÅ Imaging System (R&M Biometrics, Nashville, TN) was used to quantify total gland area and epithelial area within the gland distal to the lymph node. Mammary ducts (epithelium) were selected using the video capture array. Duct arborization was evaluated manually by counting the number of branch points ('forks') on five randomly chosen 10× fields per mammary gland mount.

Estrus Cycle Studies. Five-week-old virgin female mice were housed two C/EBPδ +/+ and two C/EBPδ −/− per cage (eight C/EBPδ +/+ and eight C/EBPδ −/− total). Estrus cycle stage was determined by vaginal cytology and confirmed by post sacrifice vaginal histology (27). Estrus cycling was documented for 3 weeks to ensure similar cycle lengths between C/EBPδ +/+ and C/EBPδ −/− females. At selected time points between 10–30 weeks of age, C/EBPδ +/+ and C/EBPδ −/− females were sacrificed in proestrus-estrus, and C/EBPδ +/+ and C/EBPδ −/− females were sacrificed in diestrus (metestrus-diestrus; four mice/genotype/time point).

***In Situ* Cell Proliferation and Apoptosis Studies.** Mice were injected with 5-bromo-2-deoxyuridine (BrdU) and mammary gland histologic sections were prepared and analyzed using a Zymed BrdU Detection Kit (Zymed Laboratories, Inc., South San Francisco, CA) according to the manufacturer's directions. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphos-

phate-digoxigenin nick end labeling (TUNEL) assay was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY) according to manufacturer's directions except using hematoxylin as a counterstain. Approximately 1000 nuclei were counted blind from each histological section for both assays.

Statistics and Data Analysis. Statistical analysis was performed using the GraphPad InStat and Prism software packages (GraphPad Software, San Diego, CA). Treatment mean differences were analyzed by Student's *t* test and multiple group analysis was assessed by one-way analysis of variance with posttest multiple mean comparisons performed by the Bonferroni test.

Results

Histologic Evaluation of Mammary Involution. We previously reported that C/EBPδ gene expression is highly induced during the initial "reversible" phase of mammary gland involution (14, 15). This initial phase of mammary gland involution is characterized by changes in mammary gland morphology and a shift in cellular gene expression from lactation-related genes to programmed cell death-related genes (14, 15). To investigate the influence of C/EBPδ on mammary gland involution and programmed cell death, mammary gland tissue was collected 0, 12, 24, 48, and 72 hr after the induction of involution by withdrawal of nursing pups. No significant differences in mammary gland morphology were observed during the onset and progression of involution between C/EBPδ wild-type controls (C/EBPδ +/+) and C/EBPδ knockout (C/EBPδ −/−) females (Fig. 1). In addition, there was no difference in the percentage of mammary epithelial cells undergoing apoptosis between C/EBPδ +/+ and C/EBPδ −/− females during involution (data not shown).

Gene Expression During Mammary Gland Involution. Although the loss of C/EBPδ may not alter overall histological changes during mammary gland involution, compensatory changes in gene expression may occur to maintain normal mammary gland function. To further assess the influence of C/EBPδ on mammary gland involution total RNA was isolated from lactating (0), 12-, 24-, 48-, 72-, and 120-hr involuting mammary glands from C/EBPδ +/+ and C/EBPδ −/− females. C/EBPα mRNA levels are relatively low in lactating and involuting mammary glands of both C/EBPδ +/+ and C/EBPδ −/− females (Fig. 2). Mammary gland C/EBPβ mRNA levels increase 2- to 4-fold from 0- to 72-hr postinvolution in both C/EBPδ+/+ and C/EBPδ −/− females (Fig. 2). In agreement with previous reports from our laboratory, mammary gland C/EBPδ mRNA levels increase significantly (approximately 4-fold) within 24 hr of the onset of involution in C/EBPδ +/+ females, then decline (14, 15). As expected, mammary gland C/EBPδ mRNA is undetectable in mammary glands from C/EBPδ −/− females (Fig. 2). TRPM-2 is a marker of mammary gland involution (14, 15, 28). Mammary gland TRPM-2 mRNA levels increase from 0- to 48-hr postinvolution in both C/EBPδ +/+ and C/EBPδ −/− females. The C/EBPδ −/− mammary gland TRPM-2 mRNA levels were significantly reduced compared with the C/EBPδ +/+ mammary gland TRPM-2 mRNA levels at 24-hr postinvolution induction. Mammary mRNA levels of gas1 (G_0 marker; Refs. 9, 10) and IGFBP-5 (apoptosis marker; Ref. 29) increase at 24- to 72-hr postinvolution in both C/EBPδ +/+ and C/EBPδ −/− females. The C/EBPδ −/− mammary gland IGFB-P5 mRNA levels were significantly reduced compared with the C/EBPδ +/+ mammary gland IGFBP-5 mRNA levels at 48-hr postinvolution induction. The physiological significance of the observed differences in TRPM-2 mRNA levels at 24-hr postinvolution and the IGFBP-5 mRNA levels at 48-hr postinvolution are unclear. Minimal morphological differences (Fig. 1) were observed between C/EBPδ +/+ and C/EBPδ −/− females during postlactational involution. No differences were observed between C/EBPδ +/+ and C/EBPδ −/− females at any of the selected time points for a series of involution-related pro-

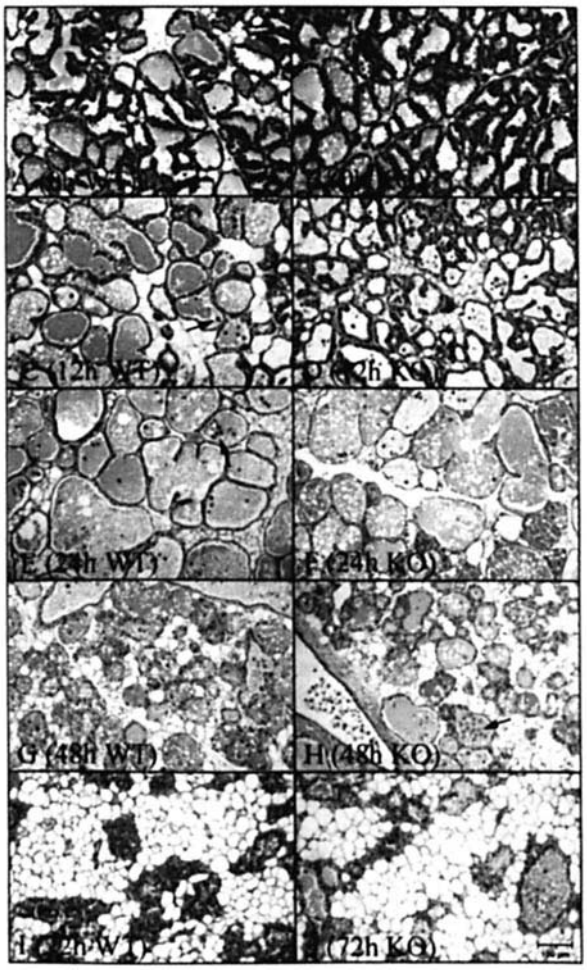

Figure 1. Histological analysis of involuting mouse mammary glands from C/EBPδ +/+ and C/EBPδ −/− females. Experimental time points: 0 hr: (A) C/EBPδ +/+, (B) C/EBPδ −/−; 12-hr involution: (C) C/EBPδ +/+, (D) C/EBPδ −/−; 24-hr involution: (E) C/EBPδ +/+, (F) C/EBPδ −/−; 48-hr involution: (G) C/EBPδ +/+, (H) C/EBPδ −/−; 72-hr involution: (I) C/EBPδ +/+, (J) C/EBPδ −/−. Arrows indicate presence of apoptotic epithelial cells in lumen.

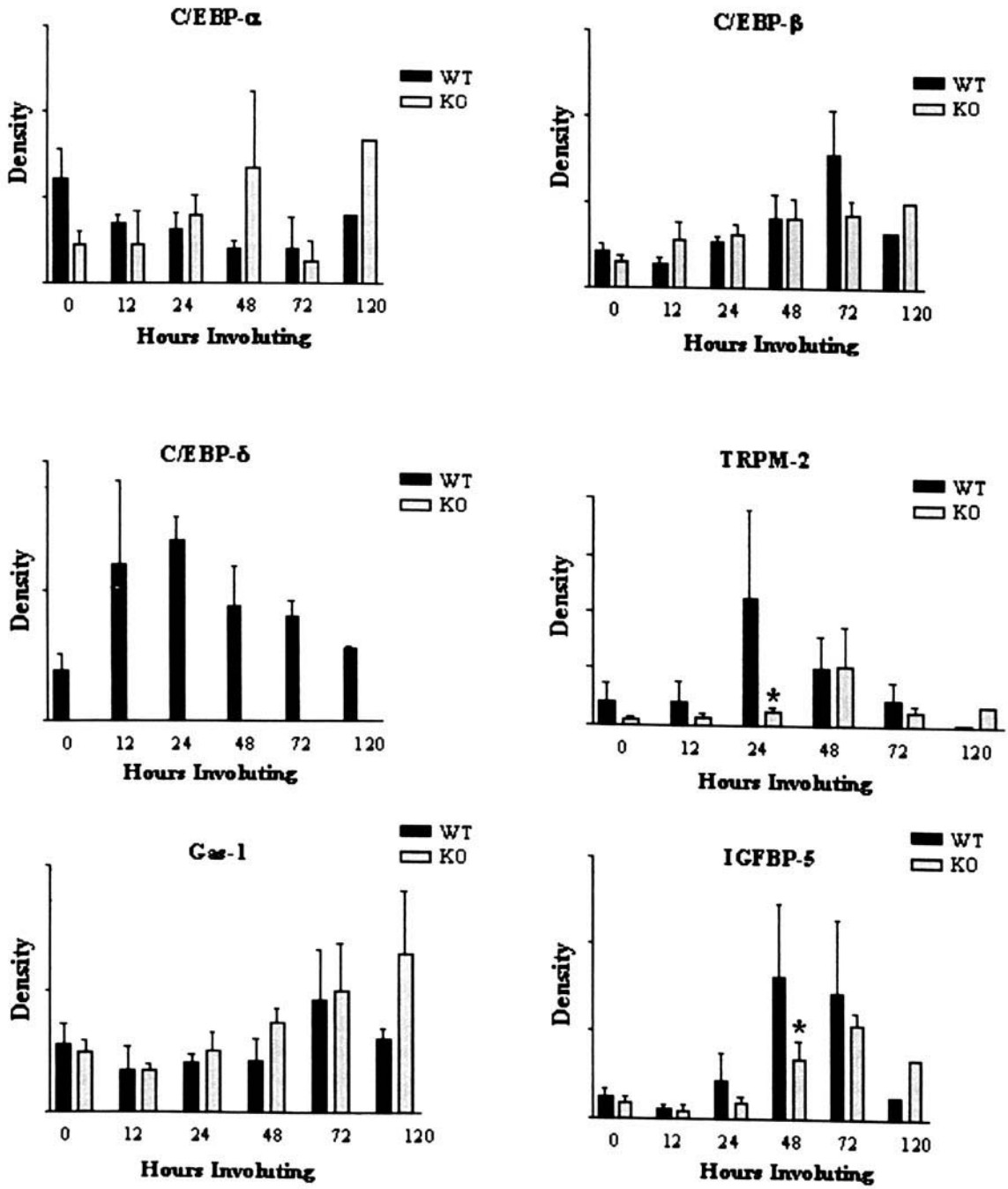

Figure 2. Northern blot analysis of total RNA isolated from involuting mammary glands from C/EBPδ +/+ and C/EBPδ −/− females. Mammary glands were isolated at selected time points after the induction of involution by pup removal. Total RNA was isolated and Northern blots sequentially probed with selected random primer labeled probes. Northern blot hybridization densities (three mice/time point) were quantitated and corrected for loading differences. The results are plotted as relative density units. The data are expressed as mean ± SEM ($^*P < 0.05$).

teins including C/EBPβ, p27, p21, p53, and phosphorylated (activated) Stat3 (data not shown).

Mammary Gland Whole Mounts: Analysis of Ductal Branch Points (Ductal Arborization and Total Mammary Epithelium). Mammary gland whole mounts were performed to assess mammary gland development in virgin females. At the earlier developmental time points (6–16 weeks of age), the ductal arborization (number of ductal branch points) and total amount of mammary gland epithelium were similar between C/EBPδ −/− and C/EBPδ +/+ females (data not shown). However, with increasing age (26 weeks of age) ductal arborization and the total amount of mammary gland epithelium increased in the age and parity (nulliparous) matched adult C/EBPδ −/− females (Fig. 3B and D) compared with C/EBPδ +/+ females (Fig. 3A and C).

Ductal arborization (branch points) and total mammary gland epithelial cell content was quantified using digital images and morphometry software. At 28–30 weeks of age C/EBPδ −/− females exhibited a greater degree of ductal

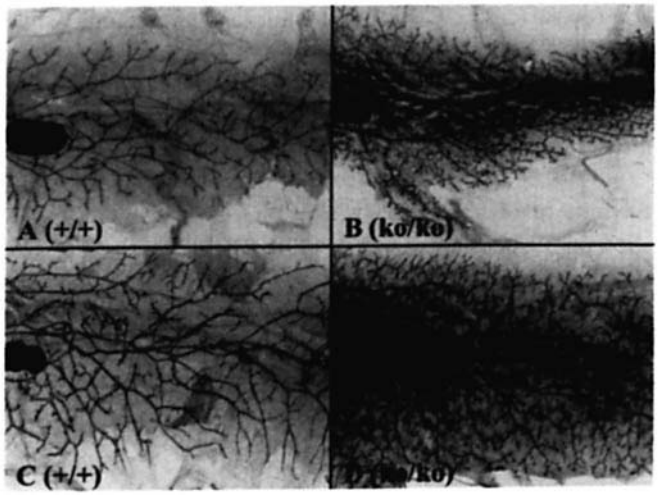

Figure 3. Whole mount analysis of mammary glands from adult (26 week old) nulliparous C/EBPδ +/+ and C/EBPδ –/– females. (A) and (C): mammary gland whole mounts from nulliparous C/EBPδ +/+ females. (B) and (D): mammary gland whole mounts from nulliparous C/EBPδ –/– (ko/ko) females.

arborization (66.8 ± 5.2 branch points/ field) compared with C/EBPδ +/+ females (42.9 ± 6.3 branch points/field; Fig. 4A). In addition, 28- to 30-week-old C/EBPδ –/– females exhibited a greater mean percentage of total gland area occupied by mammary epithelium (29% ± 1.4) compared with C/EBPδ +/+ females (20% ± 1.3; Fig. 4B).

Proliferation and Apoptosis During the Normal Estrus Cycle. Mammary epithelial cells undergo proliferation and programmed cell death in response to cyclic changes in systemic hormones during the estrus cycle (7). Defects in proliferative control or a suboptimal programmed cell death response could contribute to the excessive mammary ductal branching observed in the adult nulliparous C/EBPδ –/– mammary gland. Mammary epithelial cell proliferation was assessed in age-matched nulliparous C/EBPδ +/+ and C/EBPδ –/– female mice during the estrus cycle. In this analysis the proestrus/estrus stages were combined and designated as "estrus" and the metestrus/diestrus stages were combined and designated as "diestrus." During the "estrus" phase stage epithelial cell BrdU labeling (proliferation) was more than 2-fold higher in C/EBPδ –/– females compared with C/EBPδ +/+ females (4.55 ± 0.70 vs 2.14 ± 0.43; Fig. 5A). During the "diestrus" (proliferative) stage, mammary epithelial cell BrdU labeling index was also approximately 2-fold higher in C/EBPδ –/– females compared with C/EBPδ +/+ females (6.92 ± 0.75 vs 3.98 ± 0.43; Fig. 5A). Mammary gland serial sections were also evaluated for the appearance of apoptotic cells during estrus and diestrus. The number of mammary epithelial cells undergoing apoptosis was higher during estrus compared with diestrus, but there were no significant differences in TUNEL labeling indices between C/EBPδ +/+ and C/EBPδ –/– during either phase of the estrus cycle (Fig. 5B). There were no significant differences in BrdU labeling index or apoptosis in mammary glands from C/EBPδ +/+ and C/EBPδ –/– females during gestation/post partum-induced proliferation (data not shown).

Discussion

Previous work from our laboratory demonstrated that C/EBPδ functions in the initiation and maintenance of mouse and human mammary epithelial cell G_0 growth arrest *in vitro* (9–13). In this report we investigated the role of C/EBPδ in mammary gland development and function using the C/EBPδ –/– mouse. The results indicate that adult nulliparous C/EBPδ –/– females exhibit excessive mammary

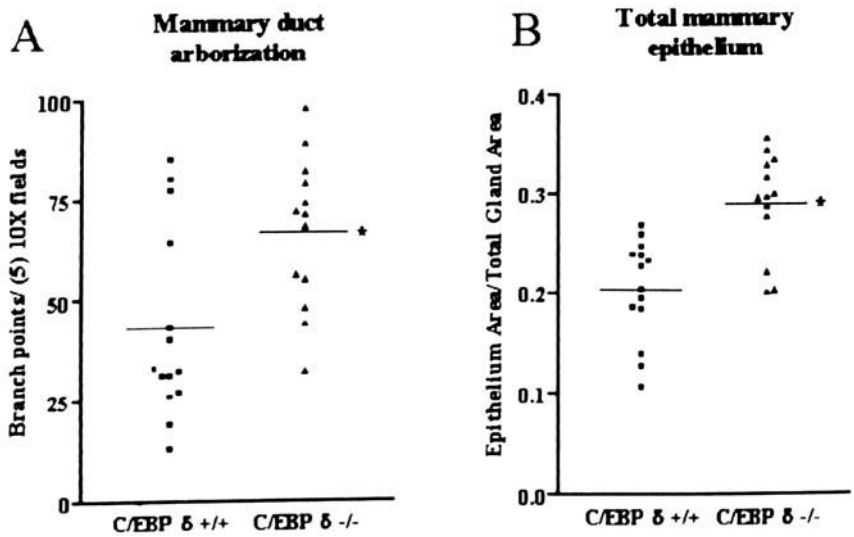

Figure 4. Quantitative analysis of mammary gland whole mounts from adult nulliparous C/EBPδ +/+ and C/EBPδ –/– females (28–30 weeks of age). (A) Mammary ductal arborization. The mean number of mammary ductal branches was significantly higher in nulliparous C/EBPδ –/– compared with C/EBPδ +/+ females (66.8 ± 5.2 vs 42.9 ± 6.3 branch points/five10× fields, $^*P < 0.01$). (B) Total mammary epithelium. The area of the total mammary gland whole mount occupied by mammary epithelium was calculated using morphometry software (see Materials and Methods section). The total mammary epithelium is expressed as a percentage of the total area of the mammary gland. The total mammary epithelium was significantly greater in nulliparous C/EBPδ –/– compared with C/EBPδ +/+ females (29.0 ± 1.4 vs 20.4 ± 1.3; $^*P < 0.001$).

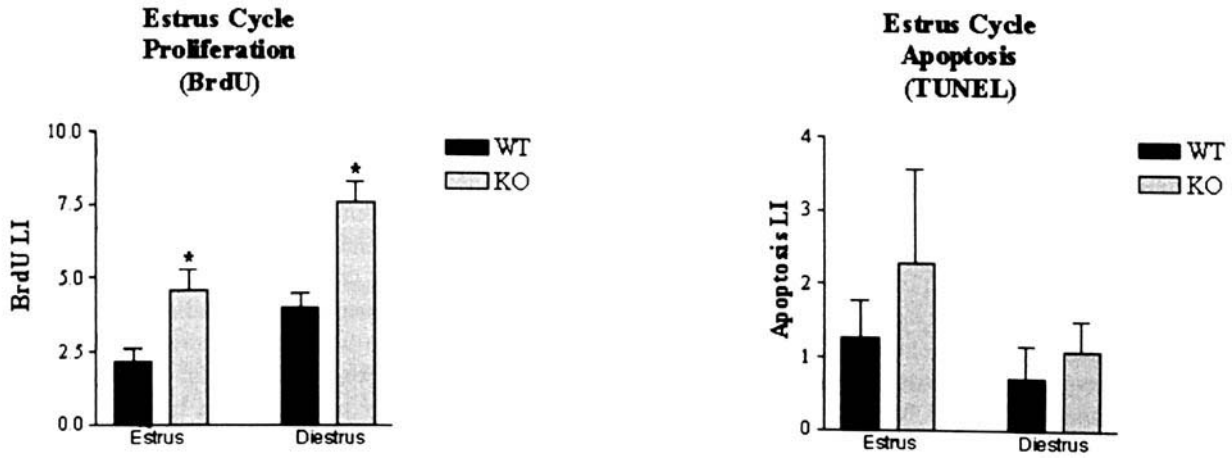

Figure 5. Estrus cycle analysis of mammary gland proliferation (BrdU) and apoptosis (TUNEL) in adult nulliparous C/EBPδ +/+ and C/EBPδ −/− females. (A) Mammary epithelial cell proliferation during the estrus cycle was assessed by BrdU labeling. Approximately 1000 nuclei were counted and the percentage of labeled nuclei expressed as BrdU labeling Index (BrdU LI; $^*P < 0.05$). (B) Mammary epithelial cell apoptosis was assessed by the TUNEL assay. Approximately 1000 nuclei were counted and the percentage of labeled nuclei expressed as Apoptosis labeling Index (Apoptosis LI; $^*P < 0.05$).

ductal branching and increased total mammary epithelium compared with nulliparous C/EBPδ +/+ females. The increased mammary ductal branching and total mammary epithelium results from constitutive proliferation of mammary epithelial cells.

The progressive accumulation of ductal branch points and total mammary epithelium observed in adult C/EBPδ −/− females could be the result of a variety of physiological factors, including constitutive elevation of growth-promoting steroid hormone levels. We investigated serum estradiol levels and found no significant difference between adult estrus synchronized C/EBPδ +/+ and C/EBPδ −/− females (data not shown). We next investigated the influence of the C/EBPδ −/− genotype on mammary epithelial cell growth during specific stages of the murine estrus cycle. In these experiments vaginal cytology was used to divide the estrus cycle into two general stages: estrus (including mice in proestrus/estrus) and diestrus (including mice in metestrus/early diestrus). In the rodent, the proestrus/estrus stage of the estrus cycle is characterized by the sequential rise and fall of serum estrogen followed by a rise and subsequent fall of serum progesterone levels. In contrast, the metestrus/early diestrus stage of the rodent estrus cycle is characterized by relatively low serum estrogen and progesterone levels (7). In agreement with previous reports, maximal proliferation (BrdU labeling) was detected during diestrus (6, 7). The proliferation rate (BrdU labeling) of mammary epithelial cells was about 2-fold higher in C/EBPδ −/− females compared with C/EBPδ +/+ females in both the diestrus and estrus stages of the estrus cycle. This suggests impaired G_0 growth control in the mammary epithelial cells of C/EBPδ −/− females that is not directly linked to changes in growth-promoting steroid hormones or estrus cycle status. In previous *in vitro* studies we developed a C/EBPδ antisense-expressing mouse mammary epithelial cell line (HC11/AS1) in which C/EBPδ levels were reduced to extremely low levels (10% of control levels; Ref. 9). HC11/AS1 mouse mammary epithelial cells expressing negligible levels of C/EBPδ exhibited high levels of proliferation in low serum, growth factor deprived media (9). In contrast, control mouse mammary epithelial cells expressing normal levels of C/EBPδ exhibited growth inhibition in low serum, growth factor-deprived media (9). These results indicate that loss of C/EBPδ alters the capacity of mammary epithelial cells to maintain the G_0 growth arrest state.

The specific role of C/EBPδ in the control of mammary epithelial cell growth and ductal morphogenesis is unknown. Because C/EBPδ functions in the transcriptional activation of a wide range of genes, it is plausible that the loss of functional C/EBPδ may result in the subsequent loss of an essential gene in mammary epithelial cell growth control. Alternatively, C/EBPδ may also function in the repression of gene expression and the loss of functional C/EBPδ may result in the increased expression of a growth-promoting gene. Previous studies have shown that proliferating mammary epithelial cells located in the terminal end buds of normal cycling female mice express the progesterone receptor (30). In addition, studies using mammary gland-specific progesterone receptor knockout mice found that the progesterone receptor was required for mammary ductal side-branching and alveolar development (30, 31). Although the mouse progesterone receptor gene promoter lacks a consensus C/EBPδ binding site (32), it is possible that loss of functional C/EBPδ may directly or indirectly influence progesterone receptor gene expression. In addition, loss of functional C/EBPδ may bypass the progesterone receptor, and alter the expression of progesterone receptor down stream mediators, such as Wnt-4. Wnt-4 is an essential downstream mediator of progesterone-progesterone receptor signaling and a key intermediary in progesterone-driven mammary gland ductal morphogenesis (31, 33). In addition to hormonal receptors, intracellular signaling molecules and transcription factors, loss of functional C/EBPδ may influence mammary specific expression of se-

creted extracellular proteases such as Stromelysin (a matrix metalloproteinase). Overexpresion of stromelysin increases mammary ductal branching, resulting in a mammary gland phenotype that is similar to the nulliparous C/EBPδ −/− females (34).

The cells that are aberrantly proliferating in the mammary epithelium of C/EBPδ −/− females may include undifferentiated, mitotically active cap cells localized to the terminal end buds (35). Although cap cells are not typically present in the adult mammary gland, loss of C/EBPδ function during mammary gland development may prolong cap cell survival, increase cap cell growth, or decrease cap cell differentiation. Loss of C/EBPδ function (in combination with loss of C/EBPβ) reduces the capacity of fibroblasts to differentiate into adipocytes (36). Prolonged survival and/or impaired differentiation could result in a pool of mitotically active epithelial cells in the mammary glands of nulliparous adult C/EBPδ −/− females.

Recent studies have directly linked loss of C/EBPδ gene expression with the progression from normal mammary epithelium to increasingly malignant, metastatic, breast cancer (37, 38). Serial Analysis of Gene Expression (SAGE) studies investigated the comparative global gene expression profiles of normal human mammary epithelial cells and *in situ*, invasive and metastatic breast carcinomas (37). C/EBPδ was one of a limited number of genes (seventeen) that declined significantly in expression as cells progressed from normal mammary epithelium to metastatic breast cancer (37). Progressive loss of C/EBPδ expression with progressive development of an increasingly malignant breast cancer phenotype is consistent with a growth suppressor/tumor suppressor role for C/EBPδ. We recently observed that the SUM 52PE human breast cancer cell line does not express C/EBPδ (data not shown). Preliminary promoter methylation experiments indicate that the C/EBPδ gene promoter is heavily methylated (26/27 CpG dinucleotides) in the SUM-52PE cell line (data not shown). This suggests that loss of C/EBPδ gene expression occurs in human breast cancer-derived cells and that the mechanism of gene silencing (promoter methylation) is similar to that identified for other key tumor suppressor genes such as p16 (39). Taken together, these results indicate that C/EBPδ exhibits tumor suppressor functions in mammary epithelial cells. The tumor suppressor function of C/EBPδ, however, may not be restricted to the mammary epithelial cell as our lab has also demonstrated tumor suppressor activity for C/EBPδ in human prostate-derived cells (40).

Although we previously reported a dramatic (>100-fold) increase in C/EBPδ mRNA content during the initial "reversible" phase of mammary gland involution (14, 15) the present studies indicate that mammary gland involution is apparently normal in C/EBPδ −/− females. This suggests that the role of C/EBPδ during the initial phase of involution is nonessential, or that C/EBPδ is functionally replaced by redundancy in the mammary gland involution program. Loss of C/EBPδ, however, was not associated with a compensatory increase in expression of other C/EBP family members during the initial phase in mammary gland involution (Fig. 2 and data not shown).

Although differences between C/EBPδ −/− and C/EBPδ +/+ females were not detected during mammary gland involution, nulliparous C/EBPδ −/− females exhibited defective growth control during the normal estrus cycle. This suggests an uncompensated or essential role for C/EBPδ in growth control in the nulliparous mammary gland. Epidemiological evidence indicates that nulliparous women, and women who bear children later in life, are at increased risk for breast cancer (41). Although the protective effects of early pregnancy on breast cancer risk are poorly understood, it appears that exposure to elevated (pregnancy) levels of estrogen and progesterone can mimic the protective effects of pregnancy in experimental animals (41, 42). The nulliparous mammary gland may contain populations of cells with increased proliferative potential that may exhibit enhanced susceptibility to "genetic hits" with exposure to carcinogens (41, 42). Current studies are aimed at further characterizing the role of C/EBPδ in mammary epithelial cell growth control and tumor susceptibility.

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