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Physiology

Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution

(bax/Stat/lactogenic hormones/glucocorticoids)

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ABSTRACT **Programmed cell death (PCD) of mammary alveolar cells during involution commences within hours of the end of suckling. Locally, milk accumulates within alveolar lumens; systemically, levels of lactogenic hormones fall. Four experimental models were used to define the role of local factors as compared with systemic hormones during the first and second stages of involution. In three models, milk release was disrupted in the presence of systemic lactogenic hormones: (*i*) sealing of the teats, (*ii*) mammary gland transplants that cannot release milk due to the absence of a teat connection, and (*iii*) inactivation of the oxytocin gene. The ability of systemic hormones to preserve lobular-alveolar structure without blocking PCD was illustrated using a fourth transgenic model of lactation failure. During the first stage of involution, local signals were sufficient to induce alveolar PCD even in the presence of systemic lactogenic hormones. PCD coincided with bax induction, decreased expression of milk proteins, block of prolactin signal transduction through Stat5a and 5b, and activation of Stat3. The two stages of mammary gland involution are regulated by progressive gain of death signals and loss of survival factors. This study demonstrates that genetic events that occur during the first reversible stage are controlled by local factors. These mammary-derived death signals are dominant over protective effects related to systemic hormone stimulation.**

During involution a coordinated process of alveolar programmed cell death (PCD) and lobular-alveolar remodeling restructures the mammary gland. Simple removal of the suckling stimulus triggers this process. With loss of suckling, milk accumulates within alveolar lumens, and levels of systemic lactogenic hormones fall (1). Mammary gland involution goes through two distinct stages. In the first stage, alveolar cells undergo PCD, but there is no remodeling of the lobular-alveolar structure. During the second stage, the lobular-alveolar structure of the gland is obliterated as proteinases degrade basement membrane and extracellular matrix (ECM) (2). The two stages exhibit characteristic changes in gene expression or activity. First-stage changes include up-regulated expression of sulfated glycoprotein-2 (2, 3), tissue inhibitor of metalloproteinases-1 (1, 2, 4–6), interleukin-1β converting enzyme (2, 7), cell cycle control proteins (c-Jun, JunB, JunD, c-Fos, and c-Myc) (4, 5), and decreased expression levels of milk protein genes (2, 8). Second-stage changes include increased expression levels of matrix metalloproteinases gelatinase A and stromelysin-1 (1, 2) and serine protease urokinase-type plasminogen activator (2, 9).

PCD of individual alveolar cells during the first days of involution is correlated with increased expression levels of the death inducers bax and bcl-x_{short} (bcl-x_S) as compared with bcl-x_{long} (bcl-x_L), all members of the bcl-2 family (8, 10–12). Importantly, this phase of PCD is p53-independent (8). Changes in activity of two Stat family members accompany mammary gland involution: decreased activity of the prolactin-signaling molecules Stat5a and Stat5b (13, 14) and activation of Stat3 (14).

In this study we examined the role of local as compared with systemic factors during the two stages of involution. Specifically, we asked whether local or systemic factors control the onset of PCD, bax induction, and the phosphorylation state of Stat5a, 5b, and Stat3. The role of hormonal stimuli during involution was examined using suckling to maintain physiological levels of lactogenic hormones and by injection of exogenous glucocorticoids (6, 12). Previous studies using exogenous administration of glucocorticoids or other individual lactogenic hormones have yielded some conflicting results. One group reported that glucocorticoids inhibit PCD by interfering with AP-1 function (1), whereas another found that alveolar cell PCD occurs in the presence of glucocorticoids (2).

Four mouse models were selected to differentiate effects of local versus systemic factors. In the first model, teat sealing disrupted milk delivery to the pups. In the second model, the use of transplanted glands prevented milk release. These glands develop normally, but have no teat connection to deliver milk. In the third model, milk ejection was impaired due to oxytocin deficiency (oxy $-/-$ mice). The oxy $-/-$ mice carry a homozygous targeted disruption of the oxytocin gene and can breed normally, but there is no lactation due to failure of milk ejection (15). Another lactation failure model [whey acidic protein-simian virus 40 T antigen (WAP-TAg) transgenic mice] showed that lactogenic hormones can preserve lobular-alveolar structure even in the presence of extensive PCD. WAP-TAg mice carry a transgene that targets TAg expression to mammary alveolar cells. There it triggers PCD and inhibition of milk protein synthesis (8, 11). Finally, removing and replacing pups on nontransgenic dams for different times determined the reversibility of the first stage of involution.

During the first reversible stage of involution, we found that local factors are sufficient for induction of PCD, bax, and

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Abbreviations: PCD, programmed cell death; WAP, whey acidic protein; TAg, simian virus 40 T antigen; ECM, extracellular matrix; Ab, antibody.

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phosphorylation changes in Stat5a, 5b, and Stat3. Systemic hormone stimulation prevented progression of the gland into the second irreversible stage of involution, but did not block PCD.

MATERIALS AND METHODS

Mice and Procedures. *Teat sealing.* Female mice after the first pregnancy were analyzed. To prevent milk release, the teat of either the left or right fourth (inguinal) gland was sealed using the surgical adhesive agent Nexaband (Veterinary Products Laboratory, Phoenix). The remaining glands were left open. Mice were observed after sealing to ensure complete teat closure and uninterrupted nursing on open teats. Paired open and closed glands were harvested at 12, 24, and 48 h, and 3-, 6-, 8-, and 10-day time points after teat closure.

Number of mice for each timepoint. Two each were used for 12, 24, and 48 h; 13 for 3 days; and 17 total for 6-, 8-, and 10-day time points. Lactation duration before teat closure was between 2 and 17 days without significant differences on results after teat closure. There was no significant difference in results between the two mouse strains examined (NMRI and C57B6).

Mammary gland transplants. Mammary anlagen from 14.5-day-old female C57B6 embryos were dissected, placed on mesenchyme derived from the region of future mammary fat pad, and grown in DMEM with 10% fetal calf serum and 1% penicillin/streptomycin. After 24 h in culture, tissue aggregates that had formed were transplanted into the cleared fat pads of five 3-week-old virgin mice. Recipients were mated after 2 months and analyzed during and after their first pregnancy. Paired transplanted and nonmanipulated control glands were removed at 18 days pregnancy, and 1 day and 3 day lactation.

Oxy −/− mice. These mice contain a homozygous deletion of the oxytocin gene (15). Milk is retained in the alveolar lumens of these mice due to failure of milk ejection. Two first-pregnancy oxy −/−, and two oxy +/+ mice were analyzed. Serial litter replacement was used to maintain suckling. Litters expired every 24–36 h due to absence of milk. Glands were harvested at parturition or after 2 days of suckling.

WAP-TAg mice. The phenotype of WAP-TAg transgenic mice was described (8, 11). These mice cannot secrete milk proteins and enter into involution within 24 h of parturition (11). Eight female mice after the first pregnancy were analyzed. In 4 mice, serial litter replacement was used to maintain suckling for 3 days after parturition. Glands were harvested after 3 days of suckling (4 mice) or after 3 days of normal involution (4 mice).

Hydrocortisone acetate administration. First-pregnancy WAP-TAg mice were used after parturition and litter removal. Subcutaneous injections of hydrocortisone acetate (7.5 mg per mouse per day) (Merck, Sharpe and Dohme) were made on the day of parturition and the two following days (5 mice). Glands were harvested 24 h after the third injection. Mice that did not receive hydrocortisone served as controls (5 mice).

Extended suckling model. First-pregnancy NMRI mice were analyzed. Normal involution begins by 21 days lactation as pups wean themselves and eat solid food. Serial litter replacement was used to maintain systemic lactogenic hormone levels for 49–60 days (4 mice). Suckling was terminated at those time points due to unavailability of replacement litters, and glands were harvested. Mice that did not receive serial litter replacements served as controls. Glands were harvested 25 days postpartum (4 mice).

Reversibility of involution. Second- and third-pregnancy NMRI mice were analyzed. Pups were removed from the dams during an established lactation and restored to the dams either 2 or 3 days later. Lactation competence was assessed. Three dams received pups after 2 days, and two dams received pups after 3 days.

Histological Examination and *in Situ* Detection of PCD. Mammary gland specimens were fixed in 10% neutral formalin solution and embedded in paraffin using routine methods. Five-micrometer tissue sections were used for hematoxylin and eosin staining or for detection of PCD using Apoptag (Oncor) (8, 11).

Evaluation of *bax* and WAP Gene Expression by Northern and Western Blot Analyses. For Northern blot analyses, total RNA was isolated from individual mammary glands using acid/guanidium thiocyanate/phenol/chloroform extraction (16) and quantified on a spectrophotometer. Twenty micrograms of each sample was fractionated on a formaldehyde agarose gel, transferred to nylon membrane, and fixed on membrane by UV irradiation. Bax, WAP, and β-casein mRNA were detected using ^{32}P-labeled probes using described methods (8, 10, 11).

For Western blot analyses, protein was extracted from fresh mammary gland tissue homogenized in lysis buffer (10 mM Tris·HCl, pH 7.6/5 mM EDTA/50 mM NaCl/30 mM $Na_4P_2O_7$/50 mM NaF/200 μM Na_3VO_4/1% Triton-X 100/1 mM phenylmethylsulfonyl fluoride/5 μg/ml aprotinin/1 μg/ml pepstatin A/2 μg/ml leupeptin) using a Polytron. Homogenate was incubated on a vertical rotator at 4°C overnight and extracts cleared by spinning in an Eppendorf centrifuge twice at 14,000 rpm at 4°C for 20 min. Forty micrograms of protein from each sample were fractionated on 14% Tris-glycine gels and transferred onto poly(vinylidene difluoride) membrane using a NOVEX Western blot apparatus (San Diego). After transfer and blocking with buffer (5% nonfat milk/20 mM Tris base, pH 7.6/137 mM NaCl) at 4°C overnight, the membranes were exposed to either 1:1,000 dilution of rabbit anti-Bax polyclonal antibody (Ab) (#493, Santa Cruz Biotechnology) or rabbit anti-WAP polyclonal Ab (11) for 1 h at 4°C, followed by exposure to 1:20,000 dilution of peroxidase-conjugated goat anti-rabbit IgG polyclonal Ab (Jackson ImmunoResearch). Proteins were visualized using the ECL detection system (Amersham). Blots were stripped by incubating in 62.5 mM Tris·HCl, pH 6.7/2% SDS/100 mM 2-mercaptoethanol for 30 min at 50°C, and reprobed using conditions described above.

Detection of Stat5a, Stat5b, and Stat3 Phosphorylation and Stat 5a/5b Heterodimerization by Immunoprecipitation and Western Blot Analyses. Proteins were extracted from frozen mammary tissue (−70°C). Eight mililiters of lysis buffer was added to each gram of tissue and homogenized using a Polytron as described above. Antibodies used were Stat5a and 5b (14), Stat3 (Santa Cruz Biotechnology), and monoclonal anti-phosphotyrosine Ab (4G10) (Upstate Biotechnology, Lake Placid, NY). Protein extracts (1.5 mg) were incubated with 1 μl of Stat5a, 2 μl of Stat5b, or 10 μl of Stat3 antisera for 30 min at 4°C on a vertical rotator. Protein A-Sepharose beads (Sigma) were added, and incubation continued overnight. Samples were washed three times with lysis buffer, resuspended in 2× sample buffer (250 mM Tris·HCl, pH 6.8/4% SDS/10% glycerol/2% 2-mercaptoethanol/0.006% bromophenol blue), boiled for 3 min, centrifuged briefly, and fractionated on an SDS/8% polyacrylamide gel. Proteins were transferred onto poly(vinylidene difluoride) membranes as described above. For Western blot, blots were blocked overnight with TBST (10 mM Tris, pH 8.0/150 mM NaCl/0.1% Tween 20) plus 2% BSA. Primary Ab diluted in TBST/2% BSA was incubated with blots for 1 h at room temperature. Antisera for Stat5a, Stat5b, and Stat3 were diluted 1:20,000, 1:10,000, and 1:2000, respectively. The anti-phosphotyrosine Ab was diluted to 0.2 μg/ml (1:5,000). After three washes in TBST, horseradish peroxidase-conjugated goat anti-rabbit (or mouse, depending on the primary Ab used) IgG diluted to 1:5,000 was added and incubated for another 30 min at room temperature. After four additional washes in TBST, proteins were detected using the ECL system as described above. Blots were stripped by incubating in 62.5 mM Tris·HCl, pH 6.7/2%

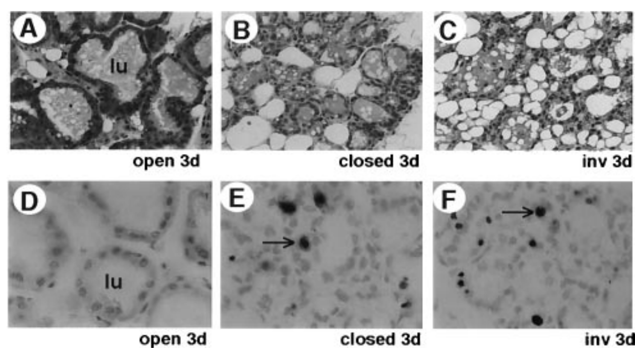

FIG. 1. Mammary-derived signals activate PCD during the first stage of involution. Histological analysis of mammary tissue from 3 days open (*A* and *D*) and closed glands (*B* and *E*) compared with normal 3 days involution (*C* and *F*). Hematoxylin and eosin staining (*A*, *B*, and *C*). *In situ* detection of PCD (*D*, *E*, and *F*). Arrows indicate cells undergoing PCD. Open, open teat; closed, Nexaband sealed teat; inv, normal involution after pup removal; 3d, 3 days; Lu, lumen.

SDS/100 mM 2-mercaptoethanol for 30 min with intermittent agitation at 65°C, and reprobed as described above.

RESULTS

Mammary-Derived Signals Were Sufficient for Induction of Alveolar PCD. In all models, failure of milk removal from the gland induced alveolar PCD in the presence of systemic lactogenic hormone stimulation (Figs. 1, 2, 3). Local signals were sufficient to induce PCD and the first stage of involution. In the closed teat model, the percentage of cells undergoing PCD (4.2%) was not significantly different from that in a normal 3-day involution gland (4.8%) (compare Figs. 1 *E* and *F*)(8). Maintenance of systemic hormone levels by suckling prevented the mice with closed teats from entering the second stage of involution. The critical time for lactation reversal during the first 3 days of involution was found to lie between 48 and 72 h. Lactation was restored to dams whose pups were removed for 48 h, but was not resumed in dams whose pups were removed for 72 h.

Induction of *bax* Gene Expression Was Solely Dependent upon Local Factors. The teat closure model was used to evaluate bax mRNA and protein expression in the presence of systemic lactogenic hormones. Full induction of both bax mRNA and protein was observed (Fig. 3 *A* and *B*) suggesting that local factors alone mediate the signaling pathway for *bax* expression during the first stage.

Local Factors Triggered Loss of Phosphorylation and Heterodimerization of Stat5a and Stat5b. The closed teat model was used to investigate whether Stat 5 activity was lost during the first stage of involution and to identify the mechanism (13, 14). Stat5a and 5b phosphorylation and heterodimerization were lost in closed, but not open, glands (Fig. 3*C*). Protein levels were unchanged. This indicated that local factors blocked Stat5 activity by reducing phosphorylation. Steady state levels of WAP (Fig. 3*B*), a milk protein whose *in vivo* expression is partially dependent on Stat5 activity (17), also were decreased in the closed glands.

Mammary-Derived Factors Stimulated Stat3 Phosphorylation. The teat closure model was used to examine activation of Stat3. Strong phosphorylation of Stat3 appeared in the closed gland by 12 h and persisted, although at slightly lower levels, through day 3 (Fig. 3*D*), indicating that local factors alone stimulate Stat3 activation during involution (14).

Maintenance of Systemic Lactogenic Hormones by Suckling Preserved Lobuloalveolar Structure Without Blocking PCD. Normally, mammary glands are remodeled by day 6 of involution (2). Typical histologic changes include obliteration of the alveolar lumens and collapsed lobules (Fig. 4*A*). Maintenance of systemic hormone stimulation by suckling blocked progression into the second stage of involution. In the 6-day closed glands, the alveolar lumens remained open, and the lobules were intact (Fig. 4*B*). Systemic hormone stimulation did not, however, block PCD (3.6%) (Fig. 4*C*).

A model of lactation failure, WAP-TAg transgenic mice, was used as a second approach to evaluate the role of suckling and systemic hormone stimulation on PCD and lobular-alveolar structure. WAP-TAg mice exhibit alveolar cell PCD and early involution due to lactation failure (8, 11). At 3 days postpartum, WAP-TAg glands normally exhibit collapsed and compressed alveoli (Fig. 5*A*), and there is no mRNA expression of the milk protein β-casein (Fig. 5*E*, lane 6). Serial litter replacement was used to test if maintenance of systemic lactogenic hormones through suckling would preserve lobular-alveolar structure or block PCD. Continuous suckling protected lobuloalveolar structure (Fig. 5*B*). The alveolar lumens remained open with intact lobules (Fig. 5*B*). β-casein mRNA expression persisted in the suckled glands (Fig. 5*E*, lanes 2–5). Consistent with the results from the closed teat model, hormonal stimulation did not block PCD (4.2%) (Fig. 5*C*).

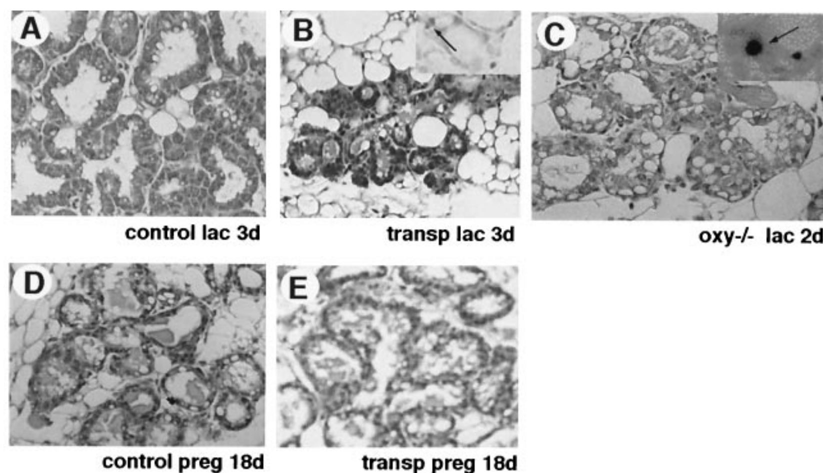

FIG. 2. Systemic hormone stimulation does not block alveolar PCD. Histological analysis of mammary tissue from 3 days postparturition transplanted (*B*) and nonmanipulated control (*A*) glands and 2 days postparturition oxytocin-deficient (oxy $-/-$) gland (*C*). Mammary gland development during pregnancy is normal in transplanted glands (*E*) as compared with control (*D*). Hematoxylin and eosin staining (*A*-*E*). *In situ* detection of PCD (*Insets* in *B* and *C*). Arrows indicate cells undergoing PCD. Control, nonmanipulated gland in a mouse receiving a transplant; lac 3d (2d), 3 (2) days postpartum lactation; transp, transplanted gland; preg 18d, 18 days pregnancy.

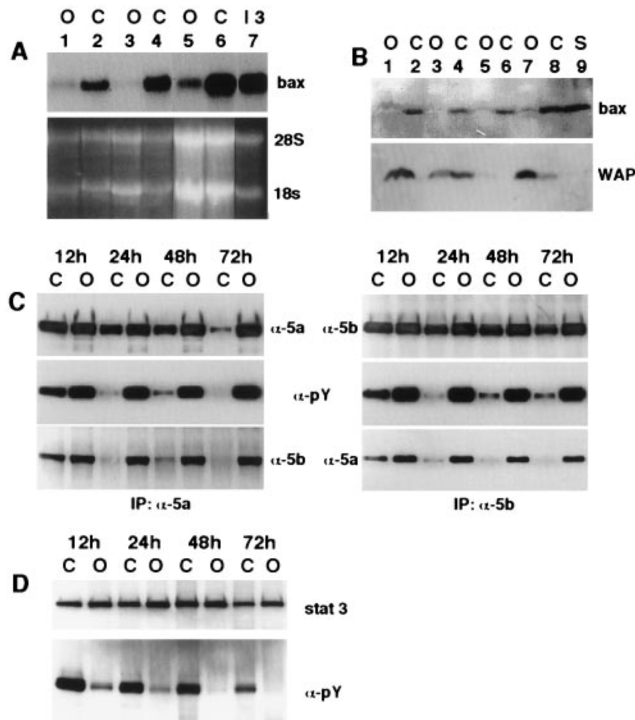

FIG. 3. Local stimuli are sufficient for induction of *bax* gene expression and phosphorylation changes in Stat5a/5b and Stat3. (*A*) Northern blot analysis of steady state levels of bax mRNA in open (O), closed (C), and normal involution day 3 (I3) glands. Loading control, ethidium bromide staining of 18S and 28S RNA. (*B*) Western blot analysis of steady state levels of bax and WAP protein in open (O) and closed (C) glands compared with spleen (S). Coomassie blue staining of the protein gel demonstrated equal loading of all samples. (*C*) Analysis of Stat5a and b protein levels, phosphorylation, and heterodimerization by immunoprecipitation and Western blot in open (O) and closed (C) glands at 12, 24, 48, and 72 h. α-5a, anti-Stat5a Ab; α-5b, anti-Stat5b Ab; α-pY, antiphosphotyrosine Ab. (*D*) Analysis of Stat3 protein levels and phosphorylation by immunoprecipitation and Western blot in open (O) and closed (C) glands at 12, 24, 48, and 72 h. α-3: anti-Stat3 Ab.

In a third experimental approach, glucocorticoid was administered to WAP-TAg mice to test if this single hormone had the same effect as suckling (1, 2). Similar to physiological stimulation, cells undergoing PCD persisted in concert with preservation of alveolar structure (Fig. 5*D*). Alveoli from glucocorticoid treated WAP-TAg mice were larger and contained more cells than the alveoli of suckled WAP-TAg mice. This suggested that high levels of exogenous glucocorticoids produce different cellular effects than physiologically elevated levels of endogenous glucocorticoid.

Two Pathways Provided by Suckling, Milk Removal and Systemic Lactogenic Hormone Stimulation, Are Both Necessary and Sufficient to Maintain Lactation. Serial litter replacement using normal mice was used to test if lobuloalveolar architecture and lactation could be maintained indefinitely by continuous suckling. During a normal lactation cycle, involution is triggered at approximately 3 weeks of age as the pups begin to wean themselves. By day 25 postpartum, the gland is in the process of being remodeled (Fig. 6*A*). In contrast, if continuous suckling is supplied by serial litter replacement, the alveoli remain widely open, the lobules remain intact, and milk production continues (Fig. 6*B*).

DISCUSSION

Progressive Gain of Death Signals and Loss of Survival Factors. The two stages of mammary gland involution are

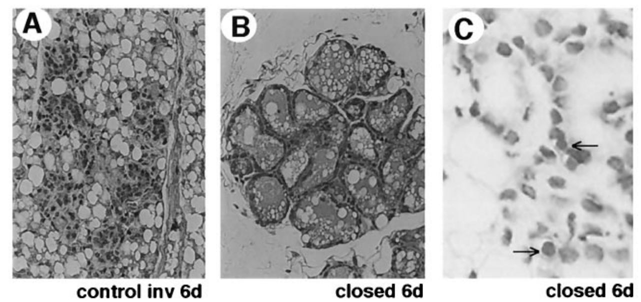

FIG. 4. Maintenance of lactogenic hormone stimulation blocks progression into the second stage of involution. Histological analysis of mammary tissue from 6 days normal involution (*A*) and 6 days closed glands with maintenance of suckling (*B* and *C*). Hematoxylin and eosin staining (*A* and *B*). *In situ* detection of PCD (*C*). Arrows indicate cells undergoing PCD. Control inv 6d, normal 6 days involution; closed 6 d, 6 days post teat closure.

controlled by progressive gain of death signals and loss of survival factors (Fig. 6*C*). The first stage of involution is controlled by local mammary-derived signals. Milk accumulation triggers increased expression of the death-inducing *bax* gene through a yet-undefined mechanism. The same stimuli mediate loss of Stat5a and 5b phosphorylation disrupting the principal pathway for prolactin signaling. Prolactin is a lactogenic hormone that can promote cell survival (9). Phosphorylation of Stat3 is low during lactation and increases sharply during the first stage of involution at a time when Stat5 phosphorylation is lost. It is not yet known if phosphorylated Stat3 plays a role in regulating alveolar PCD.

The second stage of involution is ushered in by the complete loss of survival factors due to decreased levels of systemic lactogenic hormones and activation of proteinase-dependent pathways (2). Ensuing disruption of basement membrane and extracellular matrix (ECM) results in remodeling of the gland to a state resembling the mature virgin. Systemic lactogenic

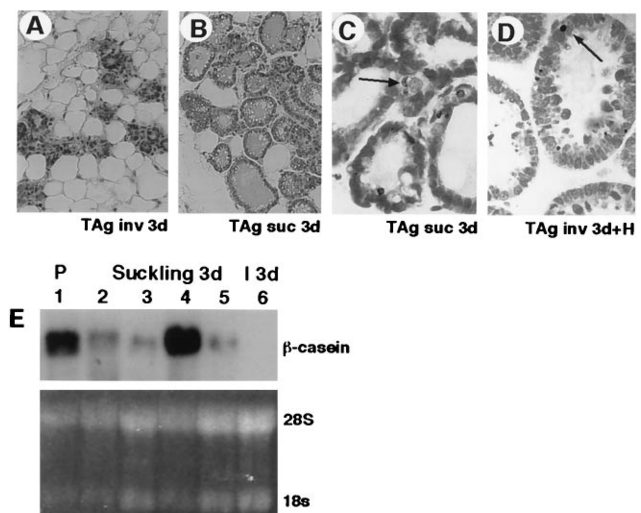

FIG. 5. Systemic hormonal stimulation by suckling or glucocorticoids alone preserve lobular-alveolar structure without blocking PCD. Histological analyses of mammary tissue from WAP-TAg transgenic mice in the absence (*A*) and presence (*B* and *C*) of suckling or after exogenous glucocorticoids (*D*). Hematoxylin and eosin staining (*A* and *B*). *In situ* detection of PCD (*C* and *D*). Arrows indicate cells undergoing PCD. TAg, WAP-TAg transgenic mouse; inv 3d, 3 days involution; suc 3d, 3 days suckling; +H, hydrocortisone injected mouse. (*E*) Northern blot analyses of β-casein expression in 3 days suckled TAg mice. Loading control, ethidium bromide staining of 18S and 28S RNA; P, day 18 pregnancy; suckling 3d, samples from individual TAg mice suckled for 3 days; I 3d, 3 days involution.

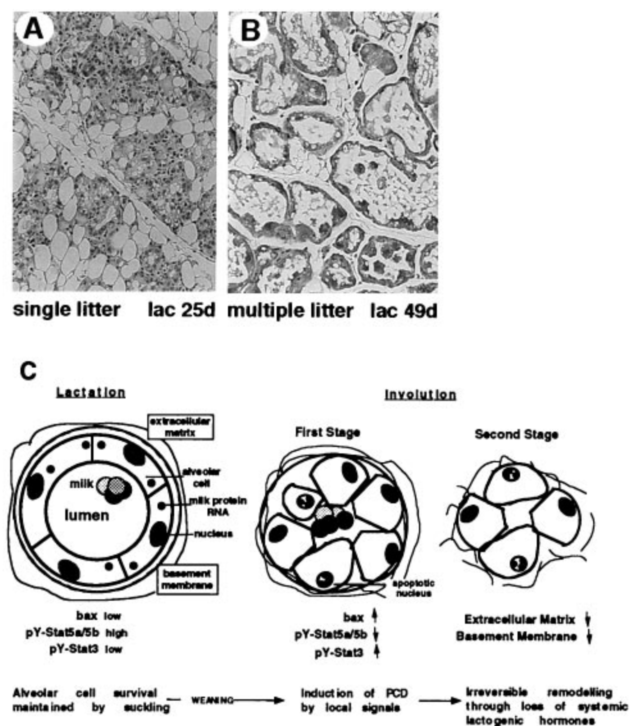

FIG. 6. Extended alveolar cell survival through continuous suckling. Hematoxylin- and eosin-stained sections of mammary tissue from 25 days postpartum mouse with a single litter (*A*) and 49 days postpartum mouse with serial litter replacement (*B*). lac 25d, suckling by a single litter for 25 days; lac 49d, suckling from multiple litters for 49 days. (*C*) Model of mammary gland involution illustrating progression through the first and second stages.

hormones are survival factors that persist through the first stage of mammary gland involution. Although these survival factors cannot overcome the dominant local PCD signals unleashed after removal of the suckling stimulus, they presumably prevent the gland from being remodeled (2, 7). Importantly, involution can be readily reversed during the first 48 h of involution, but not later.

Interplay Between Death and Survival Factors During Involution. The interplay between death and survival factors in the mammary gland is reminiscent of their role during embryonic development. Cavitation in the vertebrate embryo occurs by inducing PCD of inner ectodermal cells through a presumed short-range signal; the columnar cells that line the cavity are simultaneously rescued by interactions with basement membrane (18). Our model of the first stage of involution shares similarities with this period of vertebrate development. The death signals are locally induced and likely to act over a short distance. The ECM and basement membrane structures maintained by systemic hormones act as survival signals.

We propose that glucocorticoids and other systemic lactogenic hormones act as survival factors both during lactation and through the first stage of involution (Fig. 6*C*). During this first stage they act to protect lobular structure even as individual alveolar cells are lost to PCD. Physiologically, this means that the gland is prepared to rapidly restore lactation if suckling is renewed.

Increased bax protein levels are a credible death factor during the first stage. It is possible that either loss of Stat 5 phosphorylation or gain of Stat 3 phosphorylation are death signals; however, additional experiments are required to establish this. Stat5 phosphorylation can be controlled by both growth factor signaling (14) and cell adhesion molecules (19) and is required for normal development of the gland and lactation (17). Stat5-mediated gene control is modulated by the glucocorticoid receptor (20). This places it at a crossroad in signal transduction with a possible role as a survival factor in the mammary gland.

While this study has determined that the initial signal for bax induction and PCD is local and mammary-derived, we have not yet determined the identity of that signal. One hypothesis for the loss of Stat5 phosphorylation in the presence of systemic prolactin is that there are changes in cell receptor availability or activity. For example, this could occur secondary to either local changes in growth factors, accumulation of a specific protein (21), or to mechanical interruption of cell adhesion due to increased alveolar pressure (22).

Loss of Prolactin Signal Transduction During Involution. Prolactin has been linked to alveolar cell survival in some studies (23), but not in others (1). Prolactin signals to Stat5 through the long form of the prolactin receptor (24). Our experiments demonstrate that the prolactin signaling pathway is closed by local factors during the first stage of involution. This observation can be used to explain previous conflicting experimental results. Prolactin demonstrates a survival function when experiments are performed during lactation (23). At this time signaling pathways to Stat5 phosphorylation are available. Therefore, we can predict that loss of systemic prolactin would reduce phosphorylated Stat 5 levels. This may be why involution is then observed. Prolactin does not exhibit a survival function when replacement studies are performed during involution (1). In this case, we would predict that local mechanisms have closed the Stat5 pathway at a cellular level, and the prolactin signal can no longer be transmitted.

Physiological Roles for the First and Second Stages of Involution. The regulated two-stage process of mammary gland involution provides a balanced survival advantage for the species. For the benefit of existing pups, the gland is not lost immediately when suckling stops. Instead, mammary-derived factors trigger PCD of individual cells and limit milk production. Systemic survival factors promote restoration of milk production if pups resume suckling. However, if suckling is not renewed, systemic hormone levels drop and the mammary gland is dismantled. This prepares the organism for another round of reproduction and lactation.

Mammary Gland Involution as a Model for Tumor Therapy. Decreasing the frequency of PCD increases tumor size (25) and enhances oncogenesis (26). Increasing PCD frequency is a therapeutic goal for many cancer chemotherapies. However, the first stage of mammary gland involution teaches us that even extensive PCD cannot be equated with ablation of tissue. The survival signals generated, at least in part, from the intact ECM and basement membrane ensure that the alveolar cell compartment is not completely destroyed. On the other side, simply removing survival factors by attacking the integrity of basement membrane and ECM also can be problematic. Dysregulated expression of stromelysin-1, a metalloproteinase that normally degrades ECM during the second stage of involution, is associated with the appearance of PCD (27) and tumor development (28). These results suggest that a therapeutic approach based only on increasing death signals or simple removal of matrix survival factors may not be effective. Instead, our observations suggest that a two-fold approach that mimics the second stage of involution may be more effective. Addition of death factors may need to be accompanied by removal of survival factors.

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1. Feng, Z., Marti, A., Jehn, B., Altermatt, H. J., Chicaiza, G. & Jaggi, R. (1995) *J. Cell Biol.* **131,** 1095–1103.
2. Lund, L. R., Romer, J., Dohy-Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dane, K. & Werb, Z. (1996) *Development* **122,** 181–193.
3. Strange, R., Li, F., Saurer, S., Burkhardt, A. & Friis, R. (1992) *Development* **115,** 49–58.
4. Marti, A., Jehn, B., Costello, E., Keon, N., Ke, G., Martin, F. & Jaggi, R. (1994) *Oncogene* **9,** 1213–1223.
5. Marti, A., Feng, Z., Jehn, B., Djonov, V., Chicaiza, G., Altermatt, H. J. & Jaggi, R. (1995) *Cell Death Differ.* **2,** 277–283.
6. Talhouk, R. S., Bissell, M. J. & Werb, Z. (1992) *J. Cell Biol.* **118,** 1271–1282.
7. Boudreau, N., Sympson, C. J., Werb, Z. & Bissell, M. J. (1995) *Science* **267,** 891–893.
8. Li, M., Hu, J., Heermeier, K., Hennighausen, L. & Furth, P. A. (1996) *Cell Growth Differ.* **7,** 13–20.
9. Ossowski, L., Biegel, D. & Reich, E. (1979) *Cell* **16,** 929–940.
10. Heermeier, K., Benedict, M., Li, M., Nunez, G., Furth, P. A. & Hennighausen, L. (1996) *Mech. Dev.* **56,** 197–207.
11. Li, M., Hu, J., Heermeier, K., Hennighausen, L. & Furth, P. A. (1996) *Cell Growth Differ.* **7,** 3–11.
12. Quarrie, L. H., Addey, C. V. P. & Wilde, C. J. (1996) *J. Cell Physiol.* **168,** 559–569.
13. Schmitt-Ney, M., Happ, B., Hofer, P., Hynes, N. E. & Groner, B. (1992) *Mol. Endocrinol.* **6,** 1988–1997.
14. Liu, X., Robinson, G. W. & Hennighausen, L. (1996) *J. Mol. Endocrinol.* **10,** 1496–1506.
15. Young, W. S., Shepard, E., Amico, J., Hennighausen, L., Wagner, K.-U., LaMarca, M. E., McKinney, C. & Ginns, E. I. (1996) *J. Neuroendocrinol.* **8,** 847–853.
16. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
17. Liu, X., Robinson, G. W., Wagner, K.-U., Garrett, L., Wynshaw-Boris, A. & Hennighausen, L. (1996) *Genes Dev.* **11,** 179–186.
18. Coucouvanis, E. & Martin, G. (1995) *Cell* **83,** 279–287.
19. Streuli, C. H., Edwards, G. M., Delcommenne, M., Whitelaw, C. B., Burdon, T. G., Schindler, C. & Watson, C. J. (1995) *J. Biol. Chem.* **270,** 21639–21644.
20. Stocklin, E., Wissler, M., Gouilleux, F. & Groner, B. (1996) *Nature (London)* **383,** 726–727.
21. Wilde, C. J., Addey, C. V. P., Boddy, L. M. & Peaker, M. (1995) *Biochem. J.* **305,** 51–58.
22. Banes, A. J., Tsuzaki, M., Yamamotot, J., Fischer, T., Brigham, B., Brown, T. & Miller, L. (1995) *Biochem. Cell Biol.* **73,** 349–365.
23. Travers, M. T., Barber, M. C., Tonner, E., Quarrie, L., Wilde, C. J. & Flint, D. J. (1996) *Endocrinology* **137,** 1530–1539.
24. Wakao, H., Gouilleux, F. & Groner, B. (1994) *EMBO J.* **13,** 2182–2191.
25. Christofori, G., Naik, P. & Hanahan, D. (1994) *Science* **369,** 414–417.
26. Naik, P., Karrim, J. & Hanahan, D. (1996) *Genes Dev.* **10,** 2105–2116.
27. Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J. & Werb, Z. (1994) *J. Cell Biol.* **125,** 681–693.
28. Sympson, C. J., Talhouk, R. S., Bissell, M. J. & Werb, Z. (1995) *Perspect. Drug Discovery Des.* **2,** 401–411.