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Transforming growth factor β3 induces cell death during the first stage of mammary gland involution

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SUMMARY

Involution of the mammary gland following weaning is divided into two distinct phases. Initially, milk stasis results in the induction of local factors that cause apoptosis in the alveolar epithelium. Secondly after a prolonged absence of suckling, the consequent decline in circulating lactogenic hormone concentrations initiates remodeling of the mammary gland to the virgin-like state. We have shown that immediately following weaning TGFβ3 mRNA and protein is rapidly induced in the mammary epithelium and that this precedes the onset of apoptosis. Unilateral inhibition of suckling and hormonal reconstitution experiments showed that TGFβ3 induction is regulated by milk stasis and not by the circulating hormonal concentration. Directed expression of TGFβ3 in the alveolar epithelium of lactating mice using a β-lactoglobulin promoter mobilized SMAD4 translocation to the nucleus and caused apoptosis of these cells, but not tissue remodeling. Transplantation of neonatal mammary tissue derived from TGFβ3 null mutant mice into syngenic hosts resulted in a significant inhibition of cell death compared to wild-type mice upon milk stasis. These results provide direct evidence that TGFβ3 is a local mammary factor induced by milk stasis that causes apoptosis in the mammary gland epithelium during involution.

Key words: Mammary gland, Involution, Apoptosis, TGFβ3, SMAD, Stat3, Mouse

INTRODUCTION

During development of the mammary gland, the ductal system that emanates from the nipple penetrates into the mammary fat pad such that it becomes filled with a minimally branched structure. Once estrous cycling begins, these branches become decorated with secondary branches. However it is only during pregnancy that full development is achieved. During this state, the branches further arborize and the lobular alveolar structures that produce the milk proteins develop and decorate the ductal ends. These structures are maintained following parturition providing suckling continues but upon weaning the glands rapidly regress to the virgin-like state in a process known as involution (Imagawa et al., 1994; Topper and Freeman, 1980).

It is well established that the overall development of the mammary gland is regulated by a complex interplay of ovarian, adrenocortical and pituitary hormones (Topper and Freeman, 1980). However, it is becoming increasingly apparent that many of the diverse effects of these hormones are mediated through the local synthesis of growth factors, including epidermal growth factor (Fisher and Lakshmanan, 1990), transforming growth factor α (Derynck, 1992), fibroblast growth factor (Basilico and Moscatelli, 1992; Fisher and Lakshmanan, 1990), transforming growth factor β (TGFβ; Roberts and Sporn, 1992b), insulin-like growth factors (Daughaday and Rotwein, 1989) and colony stimulating factor 1 (CSF1; Pollard and Hennighausen, 1994). These exert their effects either directly on the mammary epithelial cells or indirectly through the stroma or mesenchyme to allow for proper mammary gland development. Involution is also regulated by an interaction between circulating hormones and locally synthesized factors. In fact, involution has two distinct phases (Li et al., 1997; Lund et al., 1996). The first, regulated by locally produced but unidentified factors, is defined by alveolar epithelial cells undergoing programmed cell death (apoptosis) and is reversible following a re-application of the suckling stimulus. The second stage during which the lobuloalveolar structures are remodeled is irreversible and occurs only after the continuous removal of the pups results in a fall in circulating pituitary hormone concentrations.

In our previous studies, we observed that mice homozygous for an inactivating mutation in the CSF1 gene (*Csfm^{op}*) had a defect in branching morphogenesis but not alveolar development during pregnancy and, despite the expression of milk proteins, these mice failed to feed their pups (Pollard et al., 1991). Consequently, at day 1 postpartum (D1PP), a rapid collapse of the dense lobuloalveolar structures occurs (Pollard and Hennighausen, 1994). CSF1 is the major growth factor regulating cells of the mononuclear phagocytic lineage (Tushinski et al., 1982). Consequently *Csfm^{op}*/*Csfm^{op}* mice have a paucity of macrophages in most but not all tissues (Cecchini et al., 1994; Wiktor-Jedrzejczak et al., 1990).

Macrophages normally surround the outgrowing ductal branches but these cells are relatively absent in the CSF1 null mutant (Gouon-Evans et al., 2000) leading to the suggestion that these macrophages provide factors involved in regulating or facilitating ductal morphogenesis. In an attempt to define these factors, we focused upon the transforming growth factor β family (McCune et al., 1992; Roberts and Sporn, 1992b) that are thought to be involved in the processes of ductal outgrowth and the spacing of the secondary branches.

Currently, there are three mammalian isoforms, TGFβ1, TGFβ2 and TGFβ3 (Roberts and Sporn, 1992a). These have been shown to be expressed at different stages of mammary gland development, although none of these transcripts is detected in lactating glands (Pelton et al., 1990; Robinson et al., 1991). Implants of TGFβ1 (Daniel et al., 1989) and TGFβ3 into subadult mammary glands inhibit ductal outgrowth (Silberstein and Daniel, 1987). Consistent with these observations, transgenic mice expressing a dominant-negative form of the TGFβ receptor showed hyperplasia in the mammary gland during development (Gorska et al., 1998). Similarly, ectopic expression of TGFβ1 using a MMTV enhancer/promoter region in transgenic mice showed inhibition of ductal growth but not alveolar outgrowth during pregnancy (Pierce et al., 1993). However, when TGFβ1 was ectopically expressed using the pregnancy-specific whey-acidic protein (WAP) promoter (Kordon et al., 1995), lobuloalveolar development was impaired resulting in mammary glands that were unable to support lactation. Despite the effects on branching morphogenesis and mammary gland development following mis-expression of TGFβ1, in the present studies, we find that expression of TGFβ1 and TGFβ2 is similar in the mammary glands of *Csfm^{op}/Csfm^{op}* mice compared to wild-type mice. However, we detected a dramatic increase in TGFβ3 expression in the alveolar epithelium at the first day postpartum in *Csfm^{op}/Csfm^{op}* mice. This elevated expression is caused by the failure of lactation since it can be mimicked in wild-type mice by withdrawal of pups. Using unilateral teat sealing and hormonal reconstitution experiments, we have established that TGFβ3 expression is regulated by milk stasis and not by changes in the hormonal environment. This expression precedes apoptosis and, in transgenic experiments when TGFβ3 is expressed in the alveolar epithelium of lactating mice, is sufficient to cause cell death. Furthermore, transplantation of whole mammary tissue containing a mutation in the TGFβ3 gene into wild-type mice resulted in an inhibition of apoptosis upon milk stasis despite normal ductal outgrowth and expression of milk proteins. These results indicate that TGFβ3 is a local mammary-derived signaling factor synthesized in response to milk stasis that induces apoptotic cell death during the first phase of involution.

MATERIALS AND METHODS

Animals and treatments

Osteopetrotic *Csfm^{op}/Csfm^{op}* mice and littermate controls +/+ and +/*Csfm^{op}* (wild-type) were bred and maintained in an isolated unit at the Albert Einstein College of Medicine animal house as described previously (Pollard et al., 1991). Mice were fed ad libitum with powdered chow and infant milk formula (Enfamil). At 10 days of age, *Csfm^{op}/Csfm^{op}* were distinguished from wild-type mice by the absence of incisors. Mice were designated day 1 postpartum (D1PP) the morning of delivery.

C57/B6 mice heterozygous for a null mutation in the TGFβ3 gene (Proetzel et al., 1995), a kind gift from Dr T. Doetschman, University of Cincinnati, Cincinnati, Ohio, were interbred to obtain litters containing TGFβ3 null pups for transplantation of mammary gland rudiments. Pups were collected on the day of birth, their mammary glands removed under the dissecting microscope and placed into cold DME medium before being transplanted into 3-week-old C57/B6 females whose fourth mammary glands had been removed completely and their nipples cauterized as described (DeOme et al., 1959; Lewis et al., 1999). A single TGFβ3$^{+/-}$, TGFβ3$^{+/+}$ or TGFβ3$^{-/-}$ mammary gland was transplanted on either side of the host. The genotype of the TGFβ3 allele was performed using PCR of 1 μl of DNA extracted from the neonate using primers (5′-TGGGAGTCATGGCTGTAACT-3′ and 5′-CACTCACACTGGCAAGTAGT-3′) as described (Proetzel et al., 1995). The wild-type allele, 400 bp, and mutant allele, 1300 bp, were separated on an agarose gel.

To assess the effect of pituitary hormones in vivo, D1PP *Csfm^{op}/Csfm^{op}* and +/*Csfm^{op}* females whose pups had been removed for 10 hours on the morning of birth, were injected with 100 μl of prolactin (10 μg/mouse) (Sinha et al., 1972), or oxytocin (10 U/mouse) (Higuchi et al., 1985), or the two in combination, or metoclopramide (125 mg/kg/day) (Bussen et al., 1996; Durant et al., 1995) or bromocriptine (2.5 mg/mouse) (Durant et al., 1995). In each case, comparable groups were injected with saline as a control.

To block milk efflux, two drops of Nexaband liquid (Veterinary Products Laboratories, Phoenix, AZ) was applied directly to the nipple of the 4th inguinal gland of +/*Csfm^{op}* dams at D1PP and the glue allowed to dry for a few seconds before return of the dam to the pups (Li et al., 1996). At the end of the experiment, the efficacy of the sealing was established by histology that showed milk accumulation in the alveolar lumens and ducts of the sealed gland compared to the open relatively empty lumens in the contralateral gland.

RNA analysis

TGFβ1-TGFβ3 probes for northern blot were obtained from Dr S. W. Qian, Laboratory of Chemoprevention, National Cancer Institutes, Bethesda. Total mRNA was isolated as previously described (Pollard and Hennighausen, 1994) and 20 μg aliquots were separated by formaldehyde agarose gel electrophoresis prior to transfer to nylon membrane using the Turbo blotter method (Schleicher and Schull, Inc., Keene, New Hampshire). The membranes were prehybridized for 3 hours at 45°C and allowed to hybridize at 42°C overnight with [^{32}P]dCTP-labeled probes. The membranes were washed twice in 1× SSC and 0.1% SDS for 15 minutes and twice in 0.25% SSC and 0.1% SDS for 15 minutes at 45°C before exposure to audioradiographic film.

Histology, TUNEL assay, immunohistochemistry and in situ hybridization

Mammary glands were fixed overnight in 10% neutral buffered formalin for the TUNEL assay and in periodate-lysine-2% paraformaldehyde-0.05% glutaraldehyde (PLPG) fixative for immunohistochemistry. Tissues were dehydrated through graded alcohols to xylene, embedded in paraffin and sectioned at 5 μm for immunohistochemical staining and stained with Hematoxylin and Eosin. Programmed cell death was detected by the TUNEL assay using the ApopTag Peroxidase kit (Oncor, Gaithersburg, Maryland) as described in the manufacturer's instructions. The specificity of the TUNEL assay was confirmed by omitting the terminal deoxynucleotidyl transferase from the reaction, a treatment that resulted in the failure to detect positive cells and the parallel assessment of the manufacturer's positive control section. For immunohistochemistry, rabbit polyclonal antibodies against 350-375 carboxyl terminal amino acids of human TGFβ3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Following incubation with the primary antibody and washing, detection of the specific antibody binding was performed using an avidin-biotin/peroxidase kit (Vector

Laboratories Inc., Burlington, CA) as described. To ensure specificity, the primary anti-TGFβ3 antiserum (1 mg/ml) and a tenfold excess of TGFβ3 peptide (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated together for 30 minutes at room temperature prior to application to the section. SMAD4 and phosphorylated-Stat3 immunohistochemical staining was performed using a polyclonal goat antibody raised against the peptide corresponding to the carboxyl terminus of the human SMAD4 (Santa Cruz Biotechnology, Santa Cruz, CA) or a phospho-specific Stat3 (Tyr705) rabbit polyclonal antibody (New England Biolabs, Beverly, MA) following the manufacturer's suggested protocol.

For in situ hybridization, digoxigenin-labeled TGFβ3 cRNA probes were prepared using the DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's protocol. Mammary glands were fixed in 4% paraformaldehyde in PBS and prepared for sectioning. 5 μm sections were deparaffinized, rehydrated and washed twice in PBS for 5 minutes prior to treatment with proteinase K in Tris-EDTA for 20 minutes at 37°C. The sections were refixed in 4% paraformaldehyde and again washed twice in PBS for 5 minutes prior to acetylation of the tissues (0.25% v/v acetic anhydride, 1.5% v/v triethanolamine and 0.42% v/v concentrated HCl) for 10 minutes at room temperature. The sections were washed twice for 5 minutes and dehydrated in a series of alcohols before being prehybridized in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml of yeast tRNA, and 500 μg/ml of herring sperm DNA) at room temperature for 2 hours and hybridized in the hybridization buffer with 200-400 ng/μl of DIG-labeled TGFβ3 probe at 60°C overnight. The next day, the sections were quickly washed in 5× SSC, then in 50% formamide/2× SSC for 30 minutes at 60°C, in TEN (10 mM Tris pH 8.0, 1 mM EDTA and 500 mM NaCl) for 10 minutes at 37°C, in 2× SSC for 30 minutes at 60°C, and finally twice in 0.2× SSC at 60°C. Digoxigenin-labeled TGFβ3 probe was detected with anti-digoxigenin-alkaline phosphatase antibody (1:5000 dilution) following with NBT/BCIP treatment for color developing as described in the manufacturer's instruction.

Generation of TGFβ3 transgenic mice

A 1.2 kb TGFβ3 cDNA was amplified from Bluescript KSII+ plasmid containing TGFβ3 from Dr S. W. Qian, Laboratory of Chemoprevention, National Cancer Institutes, Bethesda, by PCR using specific primers with addition of *Kpn*I restriction sites. The amplified product was inserted into the RiPA plasmid (Hanahan, 1985) containing a hybrid IgE-SV40 intron and SV40 poly(A) at the *Kpn*I site. The TGFβ3 cDNA together with the IgE intron and SV40 poly(A) was amplified by PCR using primers with additional *Xba*I restriction sites. The amplified product was inserted into pBJ58 plasmid (a kind gift from Dr John Clark, Roslin Institute, Edinburgh) at the *Xba*I site downstream of β-lactoglobulin promoter (Simons et al., 1987). A *Not*I fragment containing the β-lactoglobulin promoter, hybrid intron, TGFβ3 cDNA and SV40 poly(A) was used to make transgenic mice using conventional methods. Founders were determined by Southern blotting of tail DNA for integrated TGFβ3 cDNA. After breeding of the founders to establish lines, high expressing founders were identified by analysis of total mammary gland RNA at D1PP for TGFβ3 mRNA expression by northern blot. These mice were mated and mammary glands at D1PP-D3PP isolated for analysis of the effects of the TGFβ3 overexpression on cell death.

RESULTS

TGFβ3 is expressed immediately upon the cessation of lactation

We had shown previously that mice homozygous for an

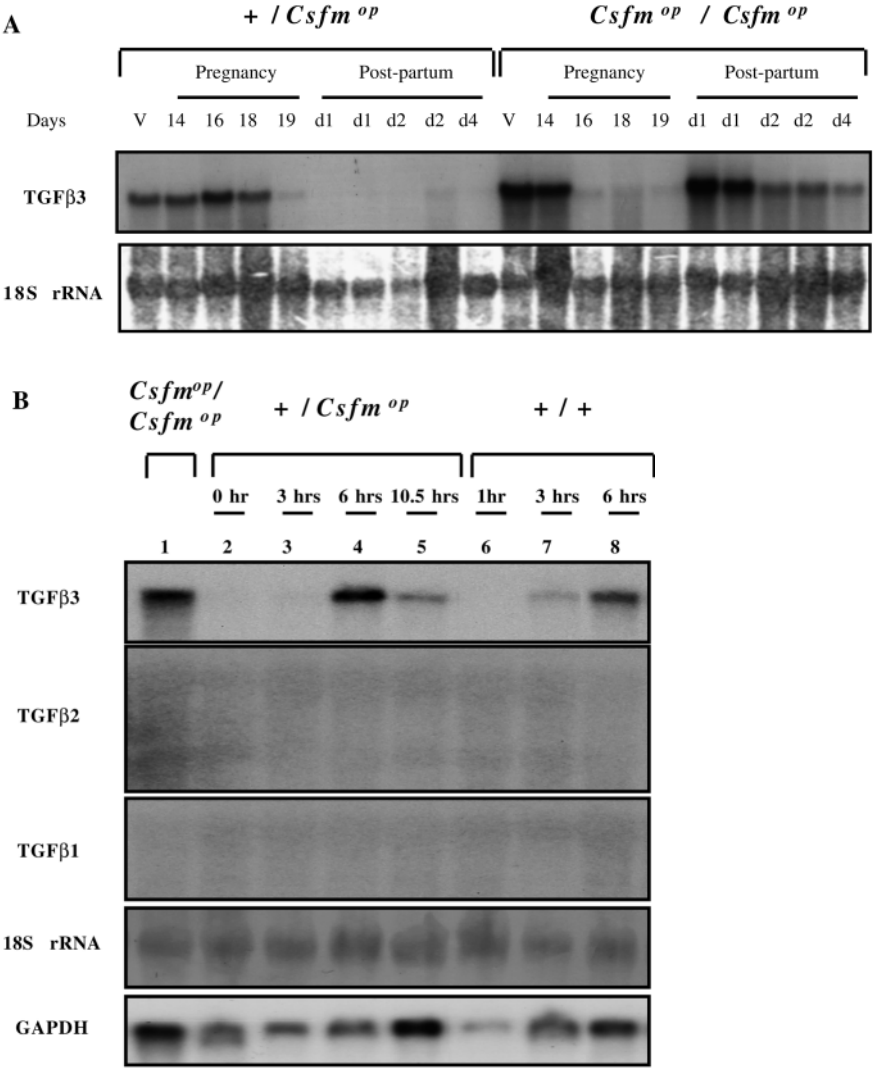

Fig. 1. Expression of TGFβ family transcripts during pregnancy and postpartum. (A) Northern blot analysis of TGFβ3 expression in mammary glands of *Csfm*^{op}/*Csfm*^{op} and +/*Csfm*^{op} mice through day 14 to 19 of pregnancy and immediately postpartum. 20 μg of RNA isolated from individual mammary gland was separated, transferred to filters and probed with [^{32}P]dCTP-labelled TGFβ3. V, virgin; d1, d2 and d4PP, the number of days postpartum. (B) Northern blot of a time-course study of mammary gland RNA isolated on D1PP and probed with TGFβ1, TGFβ2, and TGFβ3 probes. Lane 1, mammary gland from *Csfm*^{op}/*Csfm*^{op} dam; lane 2, +/*Csfm*^{op} dam with pups; lanes 3-5, pups were removed from +/*Csfm* ^{op} dams for 3 hours (lane 3), 6 hours (lane 4) and 10.5 hours (lane 5); lane 6-8, pups were removed from +/+ dams for 1 hour (lane 6), 3 hours (lane 7) and for 6 hours (lane 8). In both A and B, the membrane were stained with methylene blue in which the 18S-rRNA was shown as a control for RNA loading. In B, membranes were also probed with GAPDH as a loading control.

inactivating mutation in the CSF1 gene have a defect in branching morphogenesis in their mammary gland and are unable to nurture their pups (Pollard and Hennighausen, 1994). In an attempt to determine the molecular basis of this defect, we analyzed expression of TGFβ1, TGFβ2 and TGFβ3 mRNA in mutant and wild-type mammary glands during pregnancy and lactation. In wild-type mice, TGFβ3 mRNA was expressed at the highest level in non-pregnant mammary glands and through the first stages of pregnancy. In the later stages of pregnancy, this TGFβ3 mRNA expression declined until, following parturition and during lactation, it was expressed at very low levels (Fig. 1A). This is consistent with the pattern of expression described by Robinson et al. (1991). A similar pattern of expression was observed in *Csfm^{op}/Csfm^{op}* mice during pregnancy, except that the decline is more abrupt after day 14 of pregnancy, perhaps related to the premature maturation of the mammary gland in this mouse mutant (Pollard and Hennighausen, 1994). However, immediately postpartum in *Csfm^{op}/Csfm^{op}* mice there was a dramatic elevation of TGFβ3 mRNA expression to levels higher than detected in the virgin gland, followed by a gradual decline over the next 4 days (Fig. 1A). In contrast to this pattern of expression of TGFβ3 mRNA, TGFβ1 and TGFβ2 mRNA showed no differences in expression between wild-type and *Csfm^{op}/Csfm^{op}* mice (data not shown). Thus a single transcript of TGFβ1 was observed in virgin and pregnant mammary glands of both genotypes and this was downregulated at parturition. Four major transcripts of TGFβ2 were observed throughout pregnancy but at very low levels becoming undetectable during lactation. These patterns of expression are similar to that previously reported (Robinson et al., 1991).

Csfm^{op}/Csfm^{op} mice usually fail to nurture their pups (Pollard and Hennighausen, 1994). Thus, to determine whether the dramatic elevation of TGFβ3 mRNA observed in these mice postpartum was a direct consequence of the mutation or a secondary consequence of the lack of suckling, we removed the pups from +/*Csfm^{op}* dams at day 1 postpartum and measured TGFβ3 transcripts in their mammary glands. TGFβ3 mRNA expression was rapidly induced when pups were removed from +/*Csfm^{op}* or +/+ mice to levels comparable to that observed in *Csfm^{op}/Csfm^{op}* mice. This expression reaches a peak at 6 hours after pup removal (Fig. 1B). In contrast, over this time period neither TGFβ1 nor TGFβ2 transcripts could be detected (Fig. 1B) consistent with the patterns previously reported (Robinson et al., 1991) during lactation and suggesting that these proteins are not upregulated during the early phases of involution.

TGFβ3 is expressed in the mammary gland epithelium

In order to determine the site of TGFβ3 expression, we performed in situ hybridization and immunohistochemical staining for TGFβ3 in D1PP mammary glands. TGFβ3 antisense probe hybridized mainly to the alveolar epithelial cells of sections of mammary gland from *Csfm^{op}/Csfm^{op}* or +/*Csfm^{op}* mice whose pups had been removed (Fig. 2A). Hybridization was detected throughout the cytoplasm of the lobuloalveolar cells. In contrast to this expression in non-lactating mammary glands, significant hybridization was not detected in D1PP mammary glands from lactating mothers (data not shown). Hybridization was also not detected with a sense probe in *Csfm^{op}/Csfm^{op}* mammary glands (Fig. 2B) or in +/*Csfm^{op}* mice with pups removed or when the sections had been pretreated with RNAse (data not shown). This shows the specificity of the hybridization of TGFβ3 antisense probes.

Immunostaining with anti-TGFβ3 antibody showed strong staining in the epithelium of +/*Csfm^{op}* mammary glands from which the pups had been removed at D1PP (Fig. 2C). Most of the positive staining was confined to the cytoplasmic region of lobuloalveolar cells although some immunoreactivity was also found in the extracellular matrix and the fat pad. This probably represents secreted TGFβ3 because it did not correspond to mRNA expression. In contrast, only weak immunoreactivity

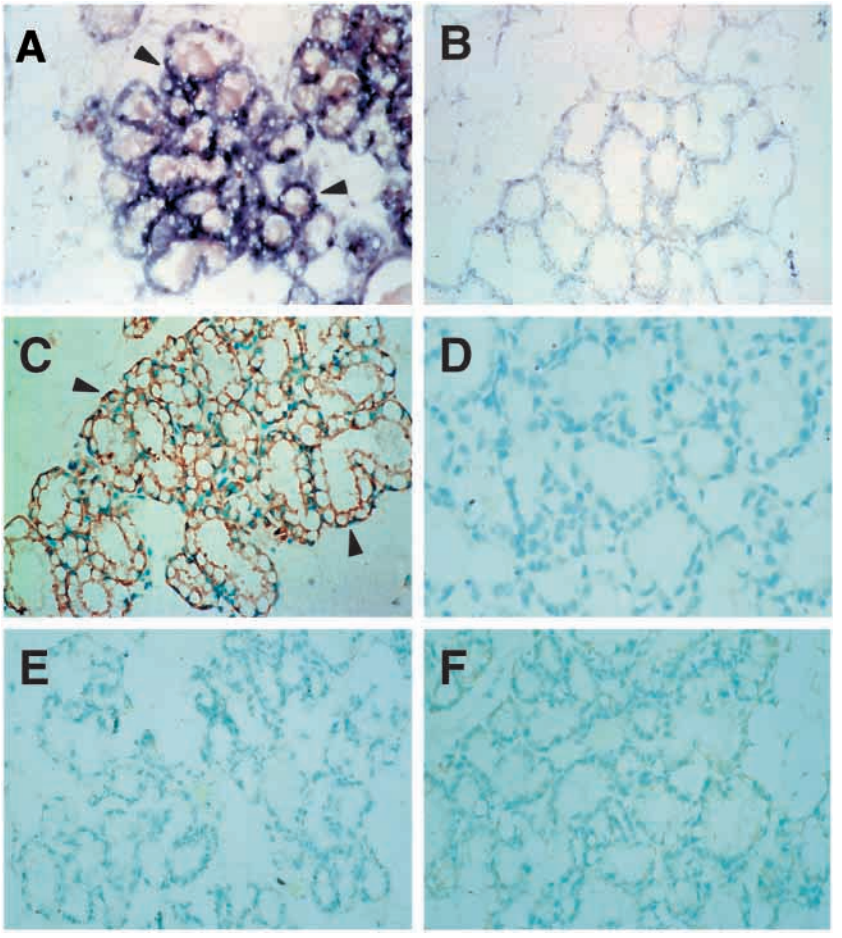

Fig. 2. In situ hybridization and immunohistochemistry for TGFβ3 in mammary glands at D1PP. (A,B) Sagittal sections of *Csfm^{op}/Csfm^{op}* involuting mammary glands hybridized with (A) antisense or (B) senseTGFβ3 probe. (C,D) Sections of +/*Csfm^{op}* mammary gland isolated from a mother that had not suckled for 9 hours (C) or still feeding (D) reacted with antibody specific for TGFβ3 protein. (E) Sections of +/*Csfm^{op}* mammary gland from a mother that had not suckled for 9 hours treated with IgG as a control. (F) +/*Csfm^{op}* mammary gland not suckled for 9 hours treated with the antibody specific for TGFβ3 protein but that had been immunodepleted with excess TGFβ3 peptide. Arrowheads in A and C indicate TGFβ3 positive epithelial cells.

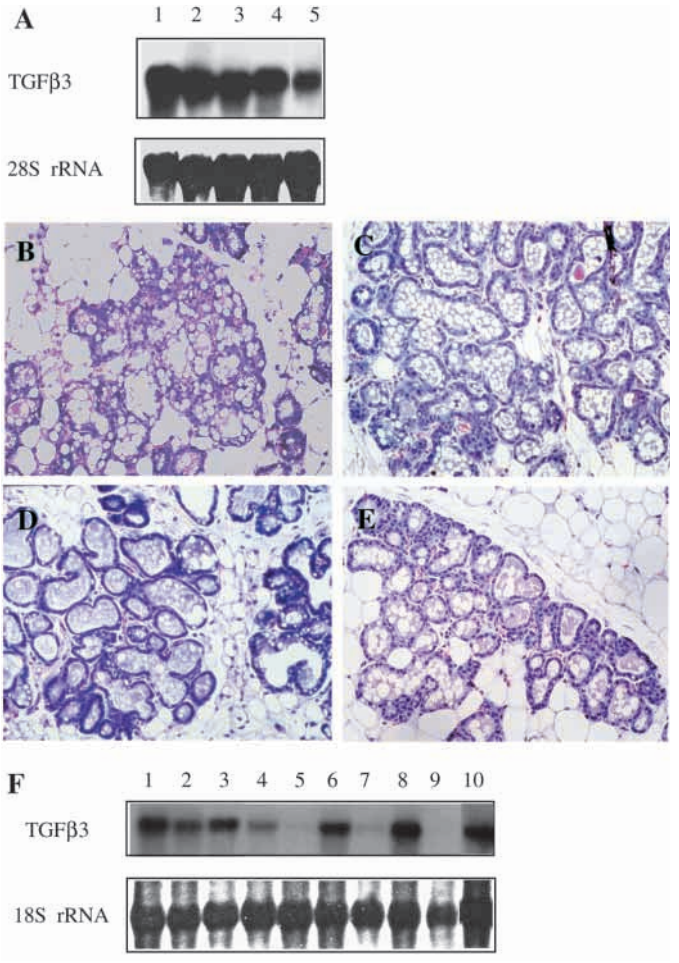

Fig. 3. TGFβ3 expression is regulated by milk stasis. (A) Northern blot analysis of TGFβ3 mRNA expression in *Csfm^{op}/Csfm^{op}* mammary glands at D1PP treated with lactogenic hormones. 20 μg of RNA from individual mammary gland was loaded and probed with [^{32}P]dCTP-labelled TGFβ3 probe. Lane 1, untreated *Csfm^{op}/Csfm^{op}* mammary glands at D1PP; lane 2-5 *Csfm^{op}/Csfm^{op}* mice at D1PP treated with oxytocin and prolactin, (lane 2), treated with oxytocin (lane 3), treated with prolactin (lane 4) and treated with metoclopramide (lane 5). (B-E) Histological sections of *Csfm^{op}/Csfm^{op}* mammary glands at D1PP stained with Hematoxylin and Eosin. *Csfm^{op}/Csfm^{op}* dam treated with saline (B), oxytocin (C), prolactin (D) and metoclopramide (E). (F) Northern blot analysis of +/*Csfm^{op}* mammary glands. Lanes 1-4, mammary glands from +/*Csfm ^{op}* dam at D1PP without pups for 9 hours. Dams were treated with saline (lane 1), oxytocin (lane 2), prolactin (lane 3), metoclopramide (lane 4). Lane 5-8, mammary glands from feeding +/*Csfm ^{op}* dam at D1PP; lane 5, unsealed inguinal gland for 1 day; lane 6, sealed inguinal gland for 1 day; lane 7, unsealed inguinal gland for 3 days; lane 8, sealed inguinal gland for 3 days. Lanes 9-10, mammary glands from feeding +/*Csfm ^{op}* dam at D1PP, treated with saline (lane 9) or with bromocriptine (lane 10). The membranes were stained with methylene blue in which the 18S or 28S-rRNA were shown as a control for RNA loading.

was found in lactating +/*Csfm^{op}* mammary glands at D1PP (Fig. 2D) and this was indistinguishable from control staining (Fig. 2E). The specificity of the immunostaining was confirmed because the positive staining detected in Fig. 2C was abolished by preincubation with a competitive peptide (Fig. 2F).

TGFβ3 expression is regulated locally by milk stasis

Lactation is regulated both by the pituitary hormones, prolactin and oxytocin, and locally derived signals (Sulman, 1970; Tucker, 1994). Removal of pups from lactating mothers results in a rapid decline in pituitary hormone secretion. Thus, we tested whether restoration of these hormones to mice that are not suckling suppressed TGFβ3 expression. In the first experiment, because of the rapid and dramatic induction of TGFβ3 in D1PP *Csfm^{op}/Csfm^{op}* mice, we treated these mice with doses of prolactin and oxytocin administered either singly or together at physiological concentrations (Higuchi et al., 1985; Sinha et al., 1972). However, none of these treatments had a significant effect on TGFβ3 mRNA level (Fig. 3A, lanes 1-4). We also treated these mice with metoclopramide, a dopamine type 2D receptor antagonist (Ben-Jonathan, 1985), that specifically releases prolactin from the pituitary (prolactin secretion is negatively regulated by dopamine) (Bussen et al., 1996; Durant et al., 1995). Under this treatment, there appeared to be a modest reduction in TGFβ3 mRNA expression but, nevertheless, TGFβ3 mRNA still showed a high level of expression that is considerably above that observed in lactating wild-type mothers (Fig. 3A, lane 5; Fig. 1A). Furthermore, daily treatment from

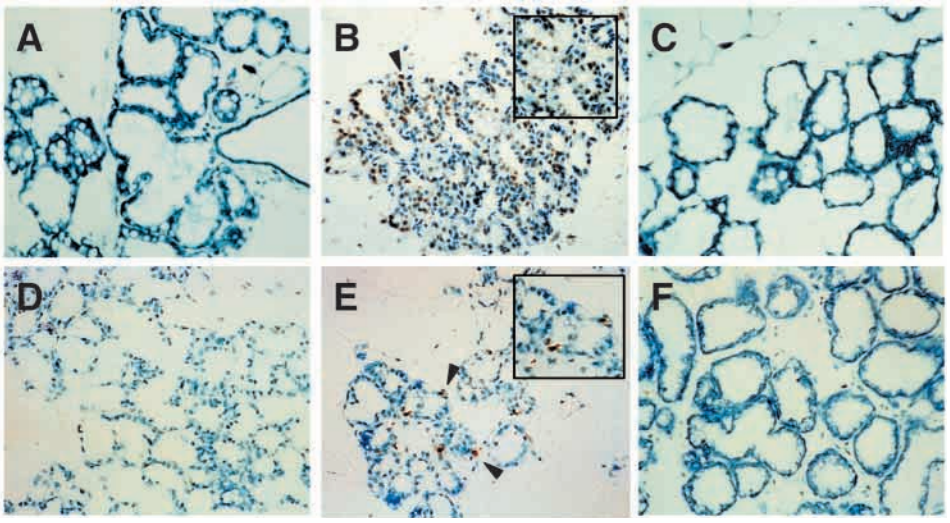

Fig. 4. Apoptosis in mammary glands at day 1 postpartum. Sagittal sections were analyzed for apoptosis using TUNEL staining. (A) +/*Csfm^{op}*; (B) *Csfm^{op}/Csfm^{op}*; (C) +/*Csfm^{op}* not suckled for 9 hours; (D) sealed mammary gland from +/*Csfm^{op}* feeding dam for 1 day; (E) sealed mammary gland from +/*Csfm ^{op}* feeding dam for 3 days; (F) contralateral gland from +/*Csfm^{op}* feeding dam for 3 days. Note that the *Csfm^{op}/Csfm^{op}* mammary gland is fully in the process of involution. Arrowheads point to apoptotic cells. Inset (B,E) shows a higher magnification of apoptotic cells.

D1PP to D3PP with metoclopramide, or prolactin, or oxytocin did not restore the ability of *Csfm^{op}/Csfm^{op}* mice to feed their pups (data not shown). To ensure that the doses of hormone used produced physiological effects in our strain of mice, we prepared mammary glands from hormone-treated *Csfm^{op}/Csfm^{op}* mice for histology and stained them with H+E. In untreated mice, the milk proteins remained in swollen epithelial cells containing large vesicles that occlude many of the ducts (Fig. 3B). In contrast, in hormone-treated mice milk protein secretion into the alveolar lumen was observed and the alveolar epithelial cells were considerably flattened in a manner characteristic of a lactating gland (Fig. 3C-E). However, because these treatments did not stimulate nursing, milk protein was observed to build up in the alveolar lumens.

In the second experiment, we blocked the suckling stimulus in wild-type mice at D1PP by removing the pups followed by hormone or drug administration. In a similar fashion to that described above, these hormonal treatments did not significantly reduce TGFβ3 mRNA expression, which remains much higher than the control levels found in lactating mice (Fig. 3F, lanes 1-4). In contrast, treatment of lactating + /*Csfm^{op}* dams with bromocriptine (Durant et al., 1995), a drug that blocks prolactin release and induces the cessation of feeding, causes an increase in TGFβ3 mRNA expression relative to the level in the suckled gland (Fig. 3F, lanes 9-10).

To determine the effect of milk retention in the mammary gland and to differentiate local from systemic regulation of TGFβ3 expression, we sealed one of the fourth inguinal glands of +/*Csfm^{op}* lactating dams to prevent suckling from that nipple. The closed gland showed an induction of TGFβ3 mRNA expression that was not detected in the open contralateral side from which pups were able to suckle (Fig. 3F, lanes 5-8). More milk proteins were observed in the alveolar lumen of the closed glands than in the open glands when the mammary glands were removed from the animal for histology (data not shown). Interestingly, the TGFβ3 mRNA expression was maintained in the closed glands even when these inguinal glands were sealed for three days (Fig. 3F, lanes 7-8). Taken together, these results indicate that TGFβ3 mRNA expression is not regulated by the concentration of circulating pituitary hormones but is induced by milk stasis in the mammary gland.

TGFβ3 expression precedes cell death in the mammary gland

The rapid induction of TGFβ3 in the mammary glands of *Csfm^{op}/Csfm^{op}* mice following birth, or in wild-type mice when the pups are removed, suggests that TGFβ3 may play a role in involution. Cell death is a characteristic component of this process and, therefore, we assayed for cell death using the TUNEL assay. In *Csfm^{op}/Csfm^{op}* or +/*Csfm^{op}* mice, few apoptotic cells were detected at day 18 or 19 of pregnancy (data not shown) nor in +/*Csfm^{op}* mice during lactation (Fig. 4A). However, immediately postpartum in *Csfm^{op}/Csfm^{op}* mice, a dramatic elevation in apoptosis was observed with 4.4% of lobulo-alveolar cells being TUNEL positive (Fig. 4B) coincident with the rapid collapse of ductal architecture. In wild-type lactating mice, no apoptotic cells could be detected 6-10 hours following pup removal (Fig. 4C) even though TGFβ3 mRNA expression was at its peak (Fig. 1B). However, between 24 and 48 hours, there is a well-documented wave of apoptosis and ductal involution (Atwood et al., 1995; Li et al., 1997; Quarrie et al., 1996; see also Fig. 4E). This indicates that, in wild-type mice, the expression of TGFβ3 precedes apoptosis.

To distinguish local from humoral signals in the regulation of apoptosis, we determined the apoptotic index in the mammary gland whose teat had been sealed to prevent milk release and the contralateral open gland that was supporting lactation. The sealed gland showed a marked increase in apoptosis compared to the lactating gland in which virtually no apoptotic cells could be detected (<0.001%). This was already slightly elevated by D1PP (Fig. 4D) and dramatically by D2PP and D3PP (2.8% of cells TUNEL positive) (Fig. 4E). In contrast, the contralateral lactating gland displayed very little apoptosis (<0.001%) (Fig. 4F). Thus, the induction of both TGFβ3 and cell death is independent of the circulating hormonal status since this is normal in these mice because they are still suckling from the other mammary glands. We also examined this lack of pituitary hormonal responsiveness for the induction of apoptosis by administering prolactin or metoclopramide (to release prolactin) and oxytocin to C*sfm^{op}/Csfm^{op}* mice immediately after parturition. However, none of these hormonal treatments changed the apoptotic index in the mammary gland (data not shown) confirming that the induction of apoptosis was the result of local signals caused by milk stasis.

TGFβ3 induces cell death in the mammary gland epithelium

The expression of TGFβ3 in the epithelial cells of the mammary gland correlates with the induction of cell death. To determine whether this is a causal relationship, we examined the effect of expressing TGFβ3 in epithelial cells of the lactating mammary glands. To do this, we made a transgenic construct in which a TGFβ3 cDNA was placed under the regulation of the β-lactoglobulin promoter (Fig. 5A). This promoter was chosen because it is active specifically in the lobular-alveolar epithelial cells of the mammary gland at the end of pregnancy and through lactation (Harris et al., 1991; Simons et al., 1987). Transgenic mice were generated by conventional methodologies and 7 founders identified by Southern blot analysis of tail DNA with a TGFβ3 probe. Female progeny of these founders were mated and, after parturition, mammary glands were analyzed for TGFβ3 mRNA expression. Several founders showed high levels of TGFβ3 transcripts (Fig. 5B, lanes 3,4,5,6,13,14) and, of these, four were chosen for further analysis. In situ hybridization with an antisense TGFβ3 cRNA probe showed that the TGFβ3 mRNA expression was restricted to the alveolar epithelial cells where transcripts are detected at very high level (Fig. 5C). No hybridization was detected with the sense probe (Fig. 5D). To ensure that active TGFβ3 was synthesized, we analyzed the cellular localization of SMAD4 by immunohistochemistry using an anti-SMAD4 antibody. SMAD4 is actively translocated from the cytoplasm to the nucleus following TGFβ binding to its membrane receptor and thus, its localization provides an assay for TGFβ function (Kretzschmar and Massague, 1998). Control mice had only a few alveolar cells with nuclear association of SMAD4 with the vast majority showing signal in the cytoplasm (Fig. 6A). In contrast, in the TGFβ3 transgenic mice, many alveolar epithelial cells, but not stromal cells, of the lactating gland showed nuclear localization of SMAD4 (Fig. 6B). Therefore, we can conclude that biologically active TGFβ3 is synthesized in the mammary

gland epithelium of the TGFβ3 transgenic mice and that it activates transmembrane receptors localized on these alveolar epithelial cells.

The forced expression of TGFβ3 in the lactating mammary glands resulted in morphological changes that were consistent with the induction of cell death with apoptotic cells in the epithelial layer and some exfoliated apoptotic cells in the lumen of the acini. To confirm the induction of cell death by TGFβ3, we used the TUNEL assay to detect apoptotic cells of D1 to D3PP mammary glands. Compared to very low levels in control non transgenic mice (<0.001% TUNEL positive; Fig. 6C) dramatically elevated numbers of apoptotic cells were found in the alveolar epithelium of the TGFβ3 transgenic mice (approx. 1.6% of lobuloalveolar cells were TUNEL positive; Fig. 6D). All four founders tested showed substantially increased apoptosis when compared to control mice. This cell death was restricted to the alveolar epithelium in all founders.

Phosphorylation of the transcription factors, Stat3 correlates with the induction of cell death during the first stage of involution in the sealed mammary glands with Stat3 being phosphorylated in the sealed but not in the open mammary gland (Li et al., 1997). Unphosphorylated Stat3 is found in the cytoplasm of the cell and, upon appropriate receptor signaling, it becomes phosphorylated and translocates to the nucleus (Darnell et al., 1994; Ihle, 1995; Ihle and Kerr, 1995). Using immunohistochemistry with an antibody specific for phosphorylated Stat3, we determined its intracellular localization both in sealed and unsealed mammary glands and in those of the TGFβ3 transgenic mice. We observed nuclear localization of phosphorylated Stat3 in the alveolar but not other cells of the sealed but not the open gland at D1PP (Fig. 6G,H). A strong nuclear localization was also found in lobuloalveolar cells in the TGFβ3 transgenic mammary glands at D1PP which was not found in the non-transgenic mammary gland at the same stage (Fig. 6E,F). Thus, it is likely that one of the downstream effectors of TGFβ3 in the induction of cell death in the alveolar epithelium is phosphorylated Stat3.

Loss of TGFβ3 reduces the rates of cell death in the involuting mammary gland

To determine whether TGFβ3 is necessary for the milk-stasis induced cell death in the mammary gland, we transplanted TGFβ3$^{-/-}$ and wild-type mammary glands to wild-type hosts (Proetzel et al., 1995). Homozygous TGFβ3$^{-/-}$ pups die around parturition however, their mammary glands can be harvested and transplanted to syngenic hosts. A total of 60 mammary glands were transplanted into 30 young females at 3 weeks of age. After transplantation the mice were rested for a month to allow the transplanted mammary glands to grow out. The mice were then mated and killed 1 day after birth of their pups. Fourteen mice gave birth and these gave eleven TGFβ3$^{+/-}$ or TGFβ3$^{+/+}$ and three TGFβ3$^{-/-}$ transplanted mammary glands for analysis. To confirm the genotyping of the TGFβ3 null mutants, longitudinal sections of the mammary glands were stained for TGFβ3 by immunohistochemistry with an anti-TGFβ3 antibody. Strong staining was detected in the TGFβ3$^{+/-}$ or TGFβ3$^{+/+}$ mammary glands (Fig. 7A) while staining was absent in the TGFβ3$^{-/-}$ mammary glands (Fig. 7B). Parallel sections were analyzed for apoptosis using the TUNEL assay (Fig. 7C,D). Although apoptotic cells were detected in the TGFβ3$^{-/-}$ alveoli the percentage of cells undergoing apoptosis was significantly lower in the TGFβ3$^{-/-}$ mammary glands compared to the TGFβ3 wild-type mammary glands (Fig. 7E; *P* value 0.0012, Student's *t*-test).

Together, these data show that TGFβ3 is induced by milk stasis and is a physiological mediator of cell death during mammary gland involution.

DISCUSSION

The involution of the mammary gland following weaning involves a set of processes that result in a return to a virgin-like state. Experiments in mice and goats designed to block milk release without interfering with circulating lactogenic hormone concentrations, showed that the processes of apoptosis and the destruction of the lobuloalveolar structures that are characteristic of involution could be dissociated into separately regulated events (Feng et al., 1995; Jaggi et al., 1996; Quarrie et al., 1994). Experiments of this type argue for two stages of involution (Li et al., 1997; Lund et al., 1996). The first, dependent upon mammary-derived local signals, is induced as a consequence of milk stasis. During this phase, the alveolar cells undergo apoptosis but without substantial remodeling of the alveolar structures which are preserved by the circulating lactogenic hormones. This stage has been called protease-independent and is reversible for up to 2 days upon re-addition of the suckling stimulus (Li et al., 1997; Marti et al., 1997). It is characterized by alterations in the expression of cell cycle regulatory genes (e.g. c-jun, JunB, JunD, c-fos and c-myc), genes associated with apoptosis such as interleukin 1β converting enzyme (ICE), Bax and Bcl-X_s and downregulation of milk protein gene expression (Heermeier et al., 1996; Lund et al., 1996; Marti et al., 1994). The second stage, caused by the loss of circulating hormones, results in the destruction of the lobuloalveolar structures (Casey et al., 1996). This protease-dependent stage is marked by the expression of genes controlling the remodeling process such as the matrix metalloproteinases, gelatinase A, stromelysin-1, and the serine protease, urokinase-type plasminogen activator (Lund et al., 1996; Ossowski et al., 1979). Consequently, once initiated this stage is irreversible even upon re-addition of pups. In this study, we have identified TGFβ3 as one of the signaling molecule that initiates apoptosis during the first stage of involution.

The actions of TGFβs are very pleiotropic (Massagué et al., 1994). In some circumstances, they can either stimulate cell proliferation or cause cell death. In addition, they have profound effects on matrix remodeling. However, their best-documented role is in the inhibition of cell proliferation, particularly of epithelial cells. In the mammary gland, TGFβs display a complex pattern of expression suggesting different roles at different stages of development. During the period of active growth, all three isoforms are expressed in overlapping but distinct patterns, particularly in the terminal end buds (Robinson et al., 1991). During pregnancy, TGFβ2 and TGFβ3 transcripts are elevated with TGFβ1 being only modestly enhanced. Towards the end of pregnancy, the transcripts of all three isoforms are significantly reduced and following parturition during lactation their expression is dramatically reduced (Robinson et al., 1991). In the actively growing mammary glands addition of TGFβs using slow release pellets resulted in inhibition of branching morphogenesis and a regression of terminal end buds (Daniel et al., 1989; Silberstein

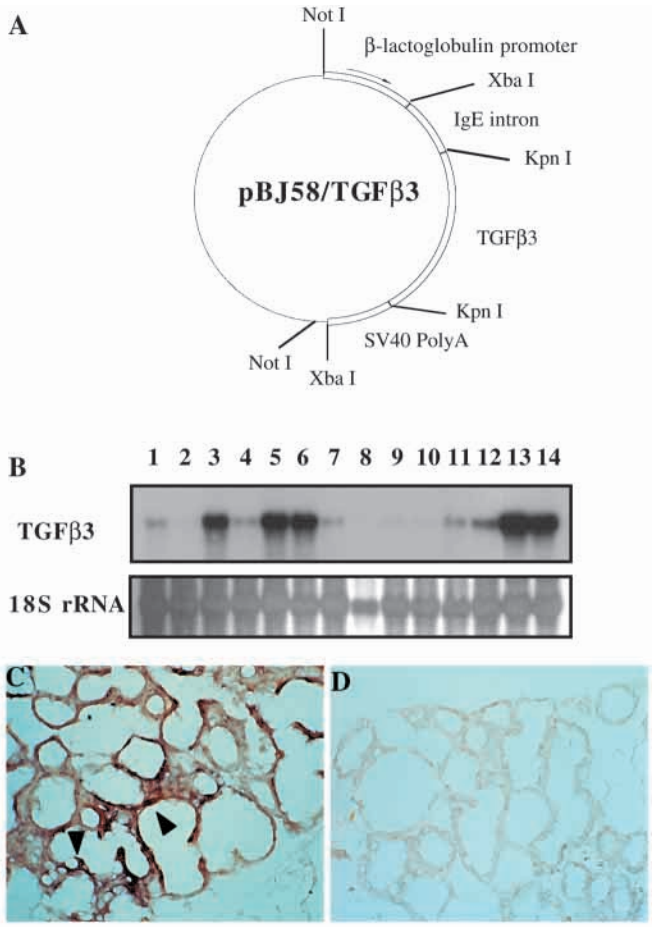

Fig. 5. Analysis of TGFβ3 transgenic mice. (A) TGFβ3 cDNA construct used for microinjection. 1.2 kb TGFβ3 cDNA was amplified from Bluescript KSII+ by PCR using primers with additional *Kpn*I restriction sites. The amplified product was inserted into RiPA plasmid at the *Kpn*I site between an IgE intron and SV40 polyadenylation site. TGFβ3 cDNA with IgE intron and SV40 poly(A) was amplified by PCR using primers with additional *Xba*I restriction sites. The amplified product was inserted into pBJ58 plasmid at the *Xba*I site downstream of β-lactoglobulin promoter. A Not I fragment containing the TGFβ3 cDNA was used to make transgenic mice. (B) Northern blot analysis of mammary glands from TGFβ3 transgenic mice feeding pups at D1PP. 20 μg of RNA from individual mammary gland was probed with [^{32}P]dCTP-labelled TGFβ3 probe. Lane 1-2, non-transgenic mouse: (lane 1) thoracic gland and (lane 2) inguinal gland. Lanes 3-4 and 11-12, 33-2 founder: (lanes 3,11), thoracic glands and (lanes 4, 12) inguinal glands. Lanes 5-6, 31-1 founder: (lane 5) thoracic and (lane 6) inguinal gland. Lanes 7-10 non-transgenic mice: (lanes 7, 9) thoracic glands and (lanes 8, 10), inguinal glands. Lane 13 and 14 30-4 founder: (lane 13) thoracic gland and (lane 14) inguinal gland. (C,D) In situ hybridization of the mammary gland from the 30-4 founder hybridized with an antisense probe (C) or sense probe (D). Arrowheads point to mammary epithelial cells expressing TGFβ3 mRNA.

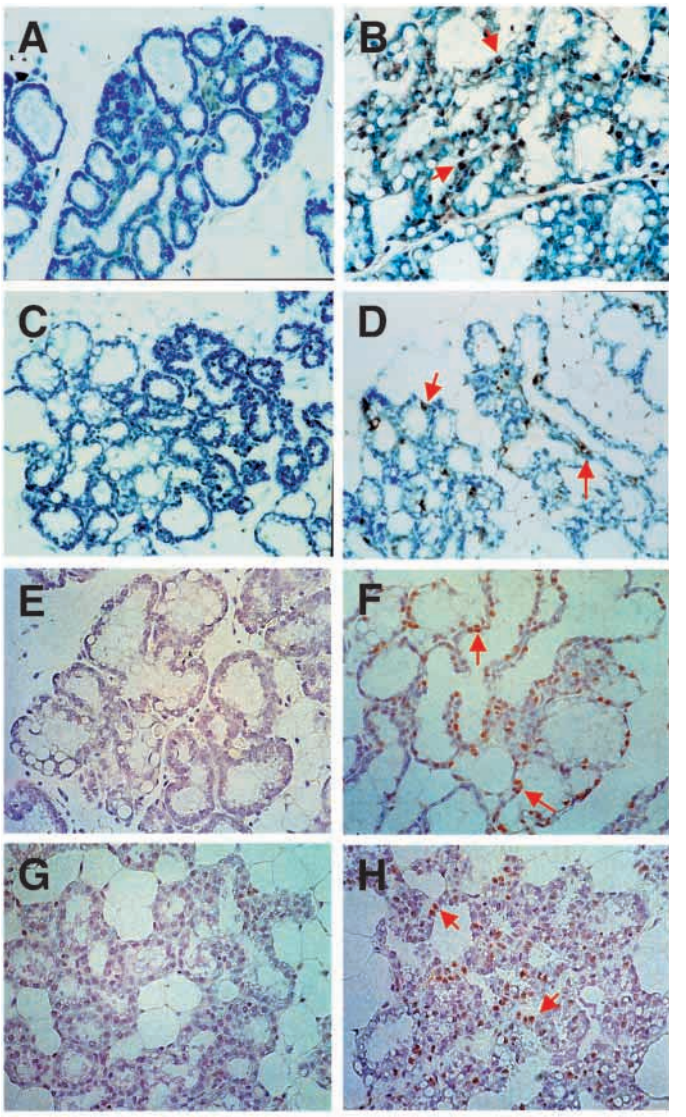

Fig. 6. TGFβ3 induces apoptosis and nuclear localization of Smad 4 and phosphorylated Stat3 in the epithelium of mammary glands at day 1 postpartum. (A,B) Immunohistochemistry for Smad4. (C,D) Mammary glands stained for apoptotic cells. (E-H) Mammary glands stained for phosphorylated Stat3. (A,C,E) Non-transgenic mammary gland and (B,D,F) TGFβ3 transgenic mammary gland. (G,H) Sections stained for phosphorylated Stat3 from (G) an unsealed gland of a feeding +/*Csfm*^{op} dam and (H) the sealed mammary gland from the same mother. Arrows in B, F and H point to positive nuclear localization signal while arrows in D point to apoptotic cells.

and Daniel, 1987). Expression of TGFβ1 during pregnancy in the mammary glands of transgenic mice using the MMTV promoter also reduced ductal branching (Pierce et al., 1993). Furthermore, the expression of a dominant-negative TGFβ receptor mutant in the mammary gland using the MMTV-LTR resulted in alveolar hyperplasia (Gorska et al., 1998). These experiments suggest that TGFβs inhibit ductal epithelial proliferation and maintain these cells in a quiescent undifferentiated state (Gorska et al., 1998). These data strongly argue for a role for TGFβs in the establishment of correct ductal spacing through the fat pad during development and pregnancy as well as in the differentiation of these structures into alveoli (Robinson et al., 1991). During pregnancy, TGFβs have also been proposed to prevent accumulation of milk proteins before lactation (Robinson et al., 1993). Thus, TGFβs inhibit milk protein expression in explant cultures of pregnant mammary glands (Robinson et al., 1993) and in hormone-

induced HC11 cells in culture (Mieth et al., 1990). Consequently the dramatic elevation of milk protein gene expression found at the end of pregnancy and during lactation is associated with the downregulation of all three isoforms of TGFβ during lactation (Robinson et al., 1991).

In our studies, we have shown that TGFβ3 mRNA and protein are rapidly induced within 3 hours in the lobuloalveolar epithelial cells following withdrawal of the lactating dam's pups. In contrast, TGFβ1 and TGFβ2 are not expressed at this time in either the lactating or the postweaning mammary gland. The induction of TGFβ3 following weaning is not regulated by a drop in circulating lactogenic hormone concentration because in teat-sealing experiments in which normal hormonal profiles are maintained, TGFβ3 is only expressed in the sealed gland and not in the contralateral feeding gland. Furthermore, exogenous administration of physiological concentrations of the pituitary hormones, oxytocin and prolactin, either alone or together, did not significantly effect TGFβ3 mRNA levels in the absence of suckling. In a tissue culture model where involution was caused by removal of lactogenic hormones, both TGFβ1 and TGFβ3 were induced over a longer time period (Atwood et al., 1995). However, this pattern of expression was not found in vivo at least over the period that overlaps with the induction of cell death. Thus, it can be concluded that, in the mammary gland in vivo, TGFβ3 but not TGFβ1 or TGFβ2 expression is regulated and rapidly induced by milk stasis.

In our experiments, we have shown that TGFβ3 is expressed in the mammary gland immediately following removal of a suckling stimulus. This expression precedes the apoptosis that is characteristic of the first stage of involution. TGFβs can, in addition to their effects on the cell cycle, also induce apoptosis. TGFβ1 can induce cell death in the liver (Strange and Roberts, 1996), after injection into the ventral prostate (Martikainen et al., 1990) and in several models of cancer both in vivo and in vitro (Hung et al., 1998; Lin and Chou, 1992; Schulte-Hermann et al., 1995). Other members of the TGFβ super-gene family are also well documented to have physiological roles in the induction of cell death during development. For example, Mullerian inhibiting substance (MIS) causes regression of the Mullerian ducts during male sexual development (Behringer et al., 1994; Price et al., 1979) while BMPs signal to induce apoptosis in rhombomeres 3 and 5 (Graham et al., 1994) and in the interdigit field of the chick limb (Yokouchi et al., 1996). Consequently we hypothesized that TGFβ3 might be the mammary-derived factor that physiologically signals to induce apoptosis in the alveolar structures during involution.

To test this hypothesis, we performed transgenic experiments whereby TGFβ3 was expressed from the β-lactoglobulin promoter in the lobuloalveolar epithelial cells of lactating mice. This promoter allowed TGFβ to be specifically expressed in the alveolar epithelium at this stage in a pattern similar to that found during involution (Simons et al., 1987) thus allowing us to identify stage-specific effects of TGFβ3. This expression pattern was confirmed for TGFβ3 by in situ hybridization. In the acini of TGFβ3 transgenic mice substantially increased numbers of apoptotic cells were detected both at the morphological level and by the TUNEL assay. TGFβ3 expression was sufficient to induce dramatic levels of programmed cell death in the lobular alveolar cells despite the maintenance of circulating hormones and the presence of suckling pups. Indeed in all founders the mammary glands remained functional, and responded to the circulating hormones by secreting milk in sufficient quantities to support the litter. This is reminiscent of the first stage of involution during weaning where cell death occurs but without destruction of the lobuloalveolar structures (Lund et al., 1996). But it also indicates that a suckling stimulus per se cannot over-ride the cell death signal.

In another transgenic experiment, where TGFβ1 was overexpressed in the mammary gland epithelium using a WAP promoter, the secretory epithelium failed to develop during pregnancy (Kordon et al., 1995). This failure was associated with the early senescence of mammary stem cells and a small (2- to 3-fold) increase in the rate of apoptosis in the ductal structures of virgin, pregnant and lactating mice. Apoptosis can also be observed in response to TGFβ1 in cultured mammary epithelial cells derived from c-myc overexpressing transgenic mice (Nass et al., 1996). Furthermore, in MDA-MB-435 human breast cancer cells, TGFβ1 treatment resulted in a significant enhancement of apoptosis following treatment of these cells with the apoptosis-inducing agent, vitamin E succinate (Yu et al., 1997). However, in the relatively normal mammary epithelial cell line, MCF-10A, TGFβ1 inhibits cell proliferation without inducing cell death (Iavarone and Massagué, 1997) and we have found a similar effect of TGFβ3 in this cell line (unpublished

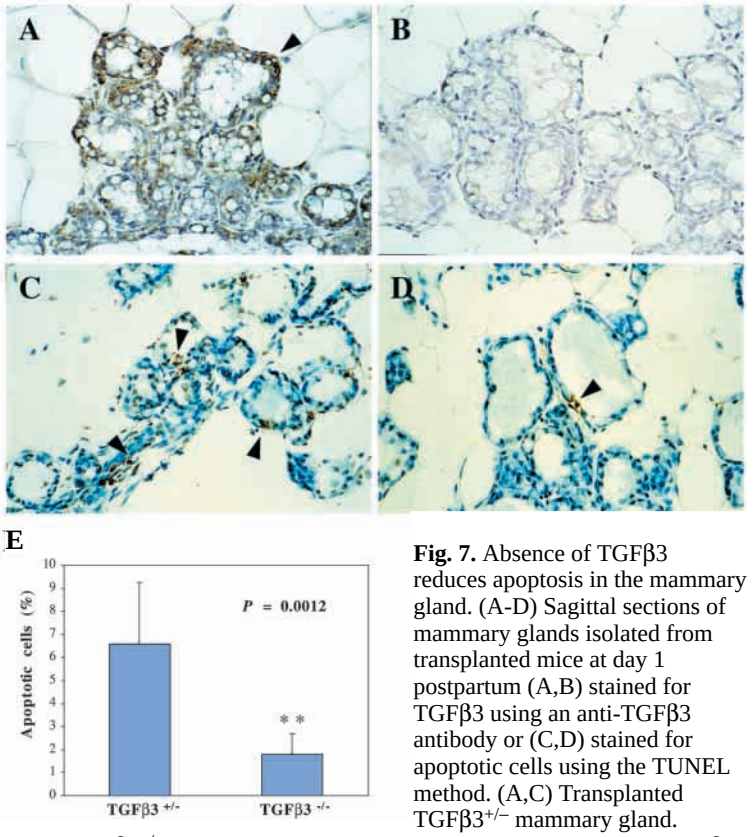

Fig. 7. Absence of TGFβ3 reduces apoptosis in the mammary gland. (A-D) Sagittal sections of mammary glands isolated from transplanted mice at day 1 postpartum (A,B) stained for TGFβ3 using an anti-TGFβ3 antibody or (C,D) stained for apoptotic cells using the TUNEL method. (A,C) Transplanted TGFβ3$^{+/-}$ mammary gland. (B,D) TGFβ3$^{-/-}$ transplanted mammary gland. Arrowheads point to TGFβ3-positive cells in A and apoptotic cells in C and D. (E) Quantitative analysis of the percent of apoptotic cells in the transplanted mammary glands of TGFβ3$^{+/-}$ or TGFβ3$^{-/-}$ mice. **TGFβ3$^{-/-}$ significantly different from TGFβ3$^{+/-}$ mammary gland (P=0.0012, Student's t-test).

results). In contrast to these contradictory results in cell culture experiments, the present experimental approach of expressing the specific isoform TGFβ3 in the lactating epithelium in a pattern consistent with that observed following weaning, strongly argues for a physiological role for TGFβ3 in the regulation of apoptosis during involution.

To further test the role of TGFβ3 in the induction of apoptosis in the mammary gland during involution, mammary glands were harvested from neonatal mice derived from matings of mice heterozygous for a null mutation in the TGFβ3 gene (Proetzel et al., 1995). TGFβ3 null mutant mice die immediately after birth. However, their mammary glands could be transplanted into syngeneic hosts where they developed in a similar fashion to those derived from the heterozygous or wild-type neonatal mice found in the same litter. This suggests that, although TGFβ3 is expressed during mammary gland development (Robinson et al., 1991), it is not required for this process. Once mature the transplanted recipients were mated and the transplanted and endogenous mammary glands harvested one day after parturition. Since the transplanted mammary glands have no connection to the nipple, they enter into the first stage of involution despite normal circulating hormones and lactation from the host thoracic glands. Analysis using the TUNEL assay revealed abundant cell death in wild-type or heterozygous mammary glands but not in the lactating host gland. This confirms previous studies on lactation in transplanted glands (Li et al., 1997) with normal development through pregnancy but with elevated levels of apoptosis postpartum. Analysis of the mammary glands derived from TGFβ3 null mutant mice however, showed that the percentage of cells undergoing apoptosis was significantly reduced compared to their wild-type counterparts. This confirms a causal role for TGFβ3 in the induction of cell death in the mammary gland during involution. However, apoptosis still occurs suggesting that there are other, redundant pathways involved in this process. This was not due to the compensatory induction of other TGFβs since we were unable to detect an up-regulation of these in the transplanted mammary glands (data not shown). Other candidates for regulators of cell death include the insulin-like growth factor-1, which may provide survival signals that counteract the death-inducing signals from molecules such as TGFβ3 and whose removal by expression of IGF binding protein 5 at involution enhances the rate of cell death (Chapman et al., 1999; Tonner et al., 1997).

The mechanisms by which TGFβs induce cell death as opposed to inhibiting cell proliferation are unknown although the characteristic players (Bcl family members and ICE enzymes) that mediate cell death are induced following TGFβ stimulation of the appropriate cell type (Chiarugi et al., 1997; Metcalfe et al., 1999; Rao and White, 1997). Recently, the MAP kinase signal transduction pathway has been implicated in TGFβ function by the identification of a TGFβ-associated MAP kinase kinase kinase (TAK1) and its activating factors TAB1 and TAB2. Interestingly, TAK1 has been found to be sufficient to cause cell death when ectopically expressed in early *Xenopus* embryos (Shibuya et al., 1998) suggesting that this pathway may have important roles in regulating mammalian cell death. However, the best-characterized downstream mediators of TGFβ signaling are the SMADs (Kretzschmar and Massagué, 1998). There are several stimulatory and two inhibitory SMADs documented. BMP2 and BMP4 appear to signal through SMAD1, while SMAD2 and SMAD3 signal from TGFβ or activin. In each case, these cooperate with SMAD4 and, in response to the agonist, these heterodimers move to the nucleus to participate in the transcriptional activation of agonist-induced genes (Derynck et al., 1998; Heldin et al., 1997; Moustakas and Kardassis, 1998; Yanagisawa et al., 1998). However, the roles for SMADs in the signaling pathways leading to cell death are not known. In the mammary gland alveolar epithelium, TGFβ3 acts in an autocrine fashion to induce apoptosis. It appears to involve, at least, the SMAD signaling pathway since SMAD4 was found to be nuclear associated in the cell population undergoing apoptosis in response to TGFβ3, in contrast to its cytoplasmic localization in the lactating gland. Moreover, it was previously shown that milk stasis induces an unknown mammary-derived growth factor that stimulates STAT3 phosphorylation (Li et al., 1997). While it is not known whether the JAK/Stat pathway is directly downstream of TGFβ signaling, our finding that mammary glands having overexpressed TGFβ3 in the lobulo alveolar epithelium also show nuclear localization of phosphorylated-STAT3 in these cells suggests that TGFβ3 may be involved in regulating STAT3 activity and that this transcription factor is an effector of TGFβ3 action. Interestingly, removal of STAT3 from the mammary epithelium using a conditional ablation method resulted in an inhibition of apoptosis in a similar fashion to that seen in the TGFβ3 null mutant mammary glands (Chapman et al., 1999). These data argue for TGFβ3 and STAT3 to be in a convergent pathway.

In mammary epithelial cells, apoptosis proceeds through a p53-independent pathway that involves the expression of the death-inducers, ICE, Bax and Bcl-X_S, and the stress response gene SGP-2 (Heermeier et al., 1996; Li et al., 1996; Lund et al., 1996). Indeed, in our case, we have observed that Bax is induced in mammary gland with TGFβ3 overexpression (data not shown). Targeted ablation of Bax resulted in a 20% reduction in cell death in the mammary gland during involution (Schorr et al., 1999), suggesting that Bax could also be downstream of TGFβ3 signaling. The expression of ICE can be induced by disruption of the integrin-mediated extracellular matrix attachment of mammary epithelial cells in culture (Boudreau et al., 1995; Lund et al., 1996). A similar inhibition of cell to matrix attachment induces cell death in a number of epithelial cell systems, a phenomenon termed ‘anoikis’ (Frisch and Ruoslahti, 1997; Gniadecki et al., 1998). Thus, TGFβ3 could act in the mammary gland in vivo through disrupting the matrix attachment of the lobuloalveolar epithelial cells. This is consistent with the role of TGFβ’s action on matrix remodeling in other organs (Erlebacher et al., 1998; Friess et al., 1998; Lehrmann et al., 1998; Lenz et al., 1998; Martin et al., 1998) and with the morphological changes seen in the lactating mammary glands of the TGFβ3 transgenic mice.

Previous studies have established that milk stasis induces a local factor that regulates cell death in the lobuloalveolar structures. In the present studies, we have shown that milk stasis specifically induces TGFβ3 at both the RNA and protein level within the alveolar epithelial cells. This TGFβ3, acting in an autocrine fashion, is sufficient to induce cell death of these cells but not to cause complete destruction of the alveolar structures. Furthermore, ablation of TGFβ3 expression in the mammary gland resulted in an inhibition of apoptosis. These data together provide compelling evidence that TGFβ3 is one

of the mammary-derived signaling factor that modulates the first phase of involution.

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