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Targeting expression of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation

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Transforming growth factor-β1 (TGF-β1) possesses highly potent, diverse and often opposing cell-specific activities, and has been implicated in the regulation of a variety of physiologic and developmental processes. To determine the effects of *in vivo* overexpression of TGF-β1 on mammary gland function, transgenic mice were generated harboring a fusion gene consisting of the porcine TGF-β1 cDNA placed under the control of regulatory elements of the pregnancy-responsive mouse whey-acidic protein (WAP) gene. Females from two of four transgenic lines were unable to lactate due to inhibition of the formation of lobuloalveolar structures and suppression of production of endogenous milk protein. In contrast, ductal development of the mammary glands was not overtly impaired. There was a complete concordance in transgenic mice between manifestation of the lactation-deficient phenotype and expression of RNA from the WAP/TGF-β1 transgene, which was present at low levels in the virgin gland, but was greatly induced at mid-pregnancy. TGF-β1 was localized to numerous alveoli and to the periductal extracellular matrix in the mammary gland of transgenic females late in pregnancy by immunohistochemical analysis. Glands reconstituted from cultured transgenic mammary epithelial cells duplicated the inhibition of lobuloalveolar development observed *in situ* in the mammary glands of pregnant transgenic mice. Results from this transgenic model strongly support the hypothesis that TGF-β1 plays an important *in vivo* role in regulating the development and function of the mammary gland.

Key words: breast/lactation deficiency/TGF-β1/transgenic mice/WAP

Introduction

Over the past decade, considerable attention has been given to the group of closely related polypeptides that constitute the transforming growth factor-β (TGF-β) superfamily (Sporn and Roberts, 1989, 1992; Moses *et al.*, 1990). Members of this expanding family, which include the TGF-βs, inhibins, activins, mullerian inhibiting substance, bone morphogenetic proteins, and products of the Vg-1 gene and the decapentaplegic gene complex, have been implicated in a diverse array of regulatory functions through positive and negative modulation of cell growth and differentiation (Barnard *et al.*, 1990; Massague, 1990; Roberts and Sporn, 1990). Three isoforms of TGF-β have been cloned from mammalian cDNA libraries: TGF-β1, -β2 and -β3. TGF-β1, the best characterized and the prototype of the TGF-β family, is biologically active as a 25 kDa, disulfide-bonded homodimer whose sequence is highly conserved in evolution and whose specific, high-affinity receptors are present on virtually all cells (Lin *et al.*, 1992; Massague, 1992). TGF-β1 is secreted from a variety of cell types as a relatively high-molecular-weight, biologically latent precursor (L-TGF-β1), which contains its pro-region (latency associated peptide LAP) associated non-covalently with the mature dimeric peptide (Lawrence *et al.*, 1984; Keski-Oja *et al.*, 1988). Upon release of the mature dimer from this complex, activated TGF-β1 can interact with its receptor (Flaumenhaft *et al.*, 1992). The conversion from the latent to active form may constitute an important mechanism by which TGF-β1 activity is regulated *in vivo*. It has been demonstrated that a modified form of TGF-β1, engineered by replacing cysteine residues 223 and 225 in the pro-region with serine residues (Brunner *et al.*, 1989), is secreted in an active form.

The widespread distribution of TGF-β1 and its receptors, together with the highly conserved nature of TGF-β1, are consistent with an important role in normal cellular function. The bifunctional effects of TGF-β1 on the growth of cells in culture are striking and variable, depending on the cell type and environment. For example, TGF-β1 can stimulate the proliferation of cells such as NRK and AKR-2B cells in soft agar or fibroblasts derived from early human fetuses (Roberts *et al.*, 1985; Hill *et al.*, 1986), but it can also potently inhibit the proliferation of many epithelial, endothelial, hematopoietic, neuronal, fibroblastic and transformed cells (Wakefield and Sporn, 1990).

TGF-β1 appears to play a role in embryonic development. Studies using immunohistochemical and *in situ* hybridization methods have localized TGF-β1 to many embryonic cell and tissue types, including those associated with morphogenetic processes (Heine *et al.*, 1987; Lehnert and Akhurst, 1988). Moreover, TGF-β1 is associated with tissues of mesenchymal origin including cartilage, connective tissue and bone, and probably plays an important role in the remodeling process associated with limb buds, teeth, facial bones and the valves of the heart (Heine *et al.*, 1987). Platelet-derived TGF-β1 plays a central role in wound healing, which is believed to include processes similar to those associated with embryogenesis (Assoian and Sporn, 1986). Picogram amounts of TGF-β1 can attract macrophages and fibroblasts toward the wound, and stimulate these

cells to produce growth factors and extracellular matrix proteins, respectively (Wahl *et al.*, 1987; Sporn and Roberts, 1989). Furthermore, mice carrying a null mutation in both alleles of the TGF-β1 gene die before 3 weeks of age, and apparently can die *in utero* as well, demonstrating an essential role for this peptide in embryonic and/or postnatal development and viability (Shull *et al.*, 1992; Kulkarni *et al.*, 1993).

The mammary gland is amenable to detailed analysis of development because a significant portion of this process occurs postnatally. Furthermore, the murine mammary gland experiences repeated cycles of growth, development and regression during a time period that permits detailed study. Developmental roles of TGF-β are reflected in its apparent involvement in the maturation and function of the mammary gland (Robinson *et al.*, 1991; Silberstein *et al.*, 1992). Implants containing TGF-β1, -β2 or -β3 protein have been introduced into the mammary glands of subadult virgin mice directly in front of the mammary end buds so that the effect of the peptide on the ductal development of the gland might be studied (Silberstein and Daniel, 1987; Robinson *et al.*, 1991). The implants locally and reversibly inhibited ductal growth, suggesting that TGF-β may help regulate ductal penetration of the fatty stromal tissue in juvenile mice. In contrast, introduction of similar TGF-β implants into hormonally- or pregnancy-induced mammary glands failed to overtly affect lobuloalveolar development (Daniel *et al.*, 1989). Furthermore, TGF-β inhibited β-casein expression in HC11 mammary epithelial cells and in mammary explants from mid-pregnant mice, providing evidence for a role in regulating functional differentiation and lactogenesis (Mieth *et al.*, 1990; Robinson *et al.*, 1992).

The widespread use of transgenic mouse technology has provided valuable and novel insights into a variety of complex systems (Hanahan, 1989; Merlino, 1991). We decided to re-examine the possible *in vivo* role of TGF-β1 in lobuloalveolar development by targeting expression of a TGF-β1 transgene to the pregnant mammary gland. Transgenic mice were generated harboring a fusion transgene consisting of the porcine TGF-β1 cDNA (Kondaiah *et al.*, 1988) inserted into the first exon of the mammary-specific, pregnancy-responsive whey-acidic protein (WAP) gene (Burdon *et al.*, 1991). The TGF-β1 cDNA used in this construct was modified at two cysteine residues (Samuel *et al.*, 1992) to encourage secretion of the active form of TGF-β1. Under the influence of the WAP promoter, production of TGF-β1 was directed to the pregnant mouse mammary gland, permitting analysis of its physiological effects *in vivo*. Such experiments demonstrated clearly that mice expressing the WAP/TGF-β1 transgene were impaired in alveolar function and exhibited a deficiency in lactation.

Results

Generation of non-lactating WAP/TGF-β1 transgenic mice

The DNA construct used to generate the transgenic mice was specifically designed to express biologically active TGF-β1 in pregnant and lactating females. The porcine TGF-β1 cDNA was ~1.35 kbp in length and included its own 3′ polyadenylation signal sequence. Previously, site-directed mutagenesis had been used to replace cysteine residues at 223 and 225 with serine residues so that TGF-β1 would be secreted in a biologically active form, and not bound to the LAP (Brunner *et al.*, 1989; Samuel *et al.*, 1992). To efficiently target expression to the lactating mammary gland, the modified TGF-β1 cDNA was placed under the regulatory influence of the mouse WAP gene. In order to effect the highest possible expression of the TGF-β1 transgene, the entire mouse WAP gene was used in this study (Burdon *et al.*, 1991). The WAP gene consists of four exons and the first exon contains a unique *Kpn*I site residing just 5′ of its first ATG (Figure 1A) into which the modified TGF-β1 was inserted. The translational stop codon of the TGF-β1 cDNA was followed in frame by two additional stop signals, ensuring that WAP RNA sequences could not be translated into some form of TGF-β1−WAP fusion protein. The WAP gene also contained its own polyadenylation signal in the fourth exon (Figure 1A).

The 8.1 kbp WAP/TGF-β1 fragment was isolated and microinjected into the pronuclei of single-cell FVB/N mouse embryos. Mice from the resulting litters were tested for the presence of the intact transgene by hybridization to a porcine TGF-β1 cDNA probe (Figure 1B). Four positive founder mice were generated, Wβ-2, Wβ-6, Wβ-9 and Wβ-12, all of which transmitted the transgene to their offspring. When the two female founder mice were impregnated by non-transgenic males, Wβ-6 was able to support her pups to weaning; however, the first litter of the Wβ-9 female founder died shortly after birth. Milk was greatly reduced or absent in the stomachs of these Wβ-9-derived pups, suggesting that the Wβ-9 founder was incapable of normal lactation. Thereafter, all subsequent Wβ-9 litters were successfully rescued by cross-fostering to other lactation-competent females. The WAP/TGF-β1 transgene in Wβ-9 was transmitted in typical Mendelian fashion, and all F_1 and F_2

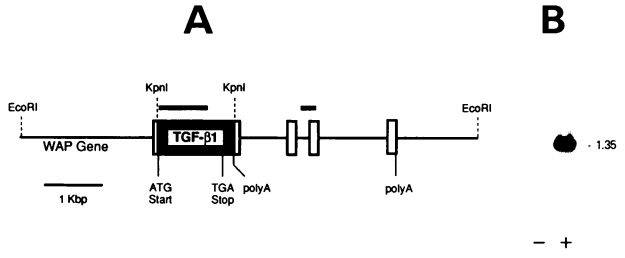

Fig. 1. Transgene structure. (**A**) Schematic representation of the 8.1 kbp *Eco*RI−*Eco*RI DNA fragment microinjected into the nuclei of FVB/N embryos to generate transgenic mice. The WAP gene is shown with 5′ flanking region (left), four exons (white boxes) and polyadenylation signal (right). A 1.35 kbp modified porcine TGF-β1 cDNA was inserted into the *Kpn*I site within the first WAP exon. Previously, cysteine residues at 223 and 225 had been replaced with serine residues, resulting in secretion of the active form of TGF-β1 (Samuel *et al.*, 1992). The TGF-β1 polyadenylation signal, and the translational start and stop signals, are as indicated. Two additional in-frame translational stop codons are located immediately 3′ relative to the first TGA stop signal, ensuring that WAP RNA sequences could not be translated into protein. The DNA sequence at the 5′ and 3′ ends of the TGF-β1 cDNA were found to be unchanged in the transgenic mice relative to the original plasmid construct, indicating that this portion of the transgene structure was not altered upon integration into the mouse genome. The two black bars above the transgene indicate the position of DNA probes used for DNA:RNA hybridization. One was used to detect mouse WAP RNAs (right), and a second was specific for porcine TGF-β1 RNA (left). (**B**) Southern blot analysis of offspring of the Wβ-9 founder mouse using the full-length porcine TGF-β1 *Kpn*I−*Kpn*I cDNA fragment as hybridization probe. Digestion of genomic DNA with *Kpn*I generated the expected 1.35 kbp band specific for the TGF-β1 transgene (+).

female progeny from these subsequent litters continued to exhibit a non-lactation phenotype. F_1 female progeny from the Wβ-2 and Wβ-12 male founders were also tested for their ability to nurture their pups. None of the post-partum F_1 females from the Wβ-2 founder showed any aberrant phenotype, whereas about half of the F_1 females from the Wβ-12 line demonstrated an inability to lactate. The transgene was routinely transmitted from the Wβ-12 founder to 75% of its offspring, suggesting that the transgene may have integrated into two separate chromosomal sites in this founder. However, this could not be confirmed readily because the transgene copy number was approximately the same (between 10 and 20 copies per diploid genome) in all positive progeny of Wβ-12.

Expression of the WAP/TGF-β1 transgene in the mammary gland

Total RNA was prepared from a variety of organs from post-partum non-lactating representatives of the Wβ-9 and Wβ-12 lines of transgenic mice, and analyzed for transgene expression by Northern blot hybridization using a porcine-specific probe. Figure 2 shows that a strong hybridization signal was evident only in the mammary gland of an F_2 female of line Wβ-12, with a much weaker signal in the spleen. Analysis of an F_1 female from line Wβ-9 indicated that transgene expression was also high in the mammary gland, and low but detectable in muscle and brain.

The expected transgene-specific TGF-β1 transcript of ~1.5 kb was not found in any of the tissues; instead, two larger porcine TGF-β1 transcripts were observed (Figure 2). One was ~4.4 kb, consistent with the size of a full-length unprocessed, intron-containing WAP/TGF-β1 hybrid primary transcript. The smaller processed transcript was ~2.4 kb. These data suggest that transcription had continued through the TGF-β1 polyadenylation signal and terminated in the fourth exon of the WAP portion of the transgene. This notion was supported by two hybridization experiments. A radiolabeled WAP genomic DNA fragment containing both exonic and intronic sequences hybridized to both the 4.4 and 2.4 kb RNAs, while a polymerase chain reaction (PCR)-amplified radiolabeled probe specific for the first intron of the WAP gene hybridized to the larger transcript, but not the smaller one (data not shown).

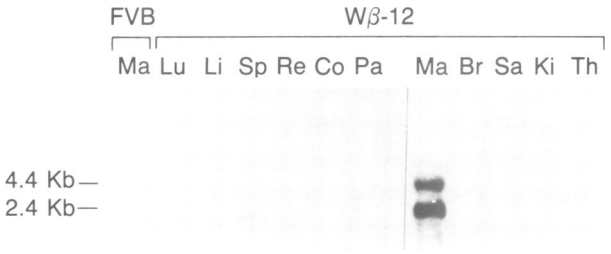

Fig. 2. Northern blot analysis of TGF-β1 transgene expression in tissues of a 19-day pregnant female from line Wβ-12. All samples, containing 15 μg of total tissue RNA, were electrophoretically fractionated on 1% agarose, transferred to nitrocellulose and hybridized to the porcine-specific TGF-β1 probe shown in Figure 1A. As a negative control, mammary tissue was taken from a 19-day pregnant non-transgenic FVB/N female (left). Ma, mammary; Lu, lung; Li, liver; Sp, spleen; Re, reproductive organs (ovary + uterus); Co, colon; Pa, pancreas; Br, brain; Sa, salivary gland; Th, thymus. Sizes of porcine-specific TGF-β1 RNAs are shown on the left.

Correlation of expression of the WAP/TGF-β1 transgene with the inability to lactate

Representative female offspring from each TGF-β1 line were mated and at full term the viability of the pups was noted. The mothers were killed and their mammary glands analyzed for the expression of porcine TGF-β1 RNA transcripts by Northern blot hybridization. Figure 3 shows that expression of the WAP/TGF-β1 transgene was high in the mammary glands of one mother from line Wβ-9 and one from Wβ-12, neither of which could lactate. In contrast, transgene expression was very low or undetectable in three lactating mothers, including representatives of lines Wβ-2 and Wβ-6, and one F_1 female from line Wβ-12 which exhibited no obvious phenotypic abnormality of the mammary gland. These results clearly demonstrate a complete concordance between expression of the WAP/TGF-β1 transgene and inability to lactate. They also lend credence to the notion that the Wβ-12 founder possessed two transgene integration sites, only one of which was sufficiently active to induce the non-lactation phenotype.

Expression of the TGF-β1 transgene inhibits pregnancy-induced lobuloalveolar development

To determine the temporal pattern of expression of the WAP/TGF-β1 transgene throughout pregnancy, mammary tissue was removed from virgin females and from mice at 7, 15, 17 and 19 days of pregnancy. Mice used in this study were 5-week-old non-transgenic FVB/N and transgenic Wβ-9 females. Total RNA was prepared from mammary glands from all time points and subjected to Northern blot analysis using the porcine-specific TGF-β1 probe. It was evident that the WAP/TGF-β1 transgene was expressed in

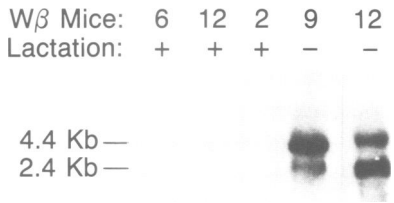

Fig. 3. Expression of the WAP/TGF-β1 transgene correlates with a lactation-deficiency phenotype. Northern blot hybridization was used to analyze RNA samples obtained from 19-day pregnant mammary tissues from members of four lines of transgenic mice (Wβ-2, Wβ-6, Wβ-9 and Wβ-12). Female members that failed to productively lactate and that could not support a litter are indicated by a (−), while females that were capable of normal lactation are indicated by a (+). Shown are two F_1 generation full-term females from line Wβ-12, one of which could lactate while the other could not; it is likely that the Wβ-12 founder contained two separately integrated transgenes, only one of which expressed porcine TGF-β1. The lane representing a non-lactating female from line Wβ-12 (far right) was taken from a separate experiment, shown in Figure 2. Sizes of porcine-specific TGF-β1 RNAs are shown on the left.

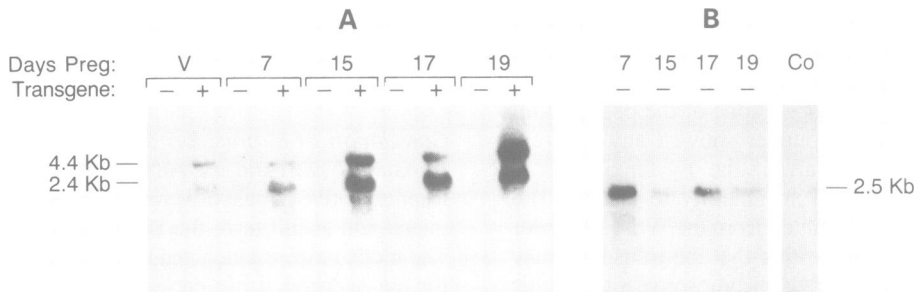

Fig. 4. Expression of the WAP/TGF-β1 transgene is induced at mid-pregnancy. Mammary tissue was removed from non-transgenic FVB/N and transgenic Wβ-9 females that were either virgins (v), or at various stages of pregnancy (shown at top). (**A**) Transgene expression was assessed by Northern blot hybridization using the porcine-specific cDNA probe (Figure 1). A (+) indicates that the mouse was transgenic, while a (−) indicates a non-transgenic control animal. Sizes of porcine-specific TGF-β1 RNAs are shown on the left. (**B**) Expression of the endogenous mouse TGF-β1 gene at the same stages of pregnancy was assessed by Northern blotting using a mouse-specific TGF-β1 precursor probe and stringent hybridization conditions. The size of the endogenous mouse TGF-β1 RNA is shown on the right. Co is a negative control of porcine spleen total RNA demonstrating failure of the mouse probe to cross-hybridize with porcine TGF-β1 RNA under these conditions.

the virgin mammary gland, albeit at low levels, indicating that the transgenic WAP promoter was active in the absence of pregnancy (Figure 4A). At 7 days of pregnancy, expression of the transgene was slightly increased, with much higher expression at 15−19 days of pregnancy (Figure 4A). This is in contrast to expression of the endogenous TGF-β1 in non-transgenic glands, which is clearly reduced at 19 days relative to 7 days of pregnancy (Figure 4B).

At each time point of this study, an inguinal mammary gland was removed from each female transgenic Wβ-9 and control FVB/N mouse for whole-mount analysis (Figure 5). The transgenic and non-transgenic mammary glands of the virgin and 7-day pregnant mice were not overtly different (Figure 5A−D). However, between day 15 and 19, coinciding with increased expression of the TGF-β1 transgene, alveolar development of the transgenic mammary gland was greatly inhibited compared to the robust growth demonstrated by the non-transgenic glands (Figure 5E−J). Alveoli in the transgenic gland were less plentiful and appeared as smaller clusters. In contrast, the ductal epithelium in WAP/TGF-β1 mice had filled the fat pad with a typically dense network of ducts. However, end buds with numerous mitotic figures persisted in transgenic females, even at 19 days of pregnancy. These structures were not seen in age-matched 6-week-old non-transgenic control females beyond day 7 of pregnancy.

Since the transgenic TGF-β1 RNA levels increased dramatically toward the end of pregnancy, immunohistochemical analysis was used to determine the topographical pattern of TGF-β1 localization. Sections of whole-mount glands from 15-day pregnant control and transgenic mice were incubated with TGF-β1 anti-CC(1-30-1) antibody. Figure 6 shows that positive staining for TGF-β1 was found in the cytoplasm and lumina of alveolar epithelial structures in the Wβ-12 lactation-deficient female (panels B and D). Similar intraepithelial staining was never found in sections from non-transgenic 15-day pregnant glands, although some TGF-β1 staining was evident at the basal surface of both alveolar and ductal structures (Figure 6A and C). In addition, strong TGF-β1-specific staining was associated with the periductal extracellular matrix of ductal structures in these 15-day pregnant transgenic mice (Figure 6F). In contrast, TGF-β1 was detected only weakly in the periductal matrix of the non-transgenic 15-day pregnant gland (Figure 6E).

Expression of endogenous milk proteins is suppressed in WAP/TGF-β1 mice

To assess the activity of the gene encoding the endogenous WAP milk protein in the mammary glands of pregnant WAP/TGF-β1 mice, total RNA was isolated from glands taken from Wβ-9 transgenic mice at different times during pregnancy and subjected to Northern blot analysis using a mouse WAP hybridization probe. Figure 7A shows that expression of the endogenous WAP gene was induced late in pregnancy in both control and transgenic mice. In control females, a sharp increase in the steady-state level of WAP RNA was detected by day 15 of pregnancy; in Wβ-9 transgenic females, a much more modest enhancement was observed on day 17 of pregnancy.

To characterize more precisely the development of the pregnant transgenic mammary gland, immunohistochemical analysis was used to localize the endogenous milk proteins, WAP and casein. Figure 8 shows that alveolar structures present in the mammary glands of late pregnant Wβ-12 transgenic mice exhibiting the lactation-deficient phenotype were capable of manufacturing and secreting both WAP and casein. Moreover, expression of these milk proteins was induced in these Wβ-12 mice at the same stage of pregnancy as control mice, suggesting that these genes were operating under normal hormonal control mechanisms (Figure 8). Staining for WAP and casein in the glands of late pregnant Wβ-9 transgenic mice was markedly diminished relative to Wβ-12 mice (data not shown).

Western analysis was employed to quantify WAP production in 19-day pregnant transgenic mice demonstrating the lactation-deficient phenotype. Figure 7B shows that WAP production was reduced in both Wβ-12 and Wβ-9 full-term mice to levels that were ~10- and 50-fold lower than in controls, respectively. Figure 7B also demonstrates that no WAP-specific proteins larger than those found in normal glands were being made in transgenic glands, indicating that no bizarre TGF-β1−WAP fusion proteins were being produced in either Wβ-9 or Wβ-12 pregnant mice.

Transgenic mammary cells fail to develop into normal lobuloalveolar structures in reconstituted mammary glands

To assess the ability of mammary epithelial cells to recapitulate the developmental abnormalities observed *in situ*

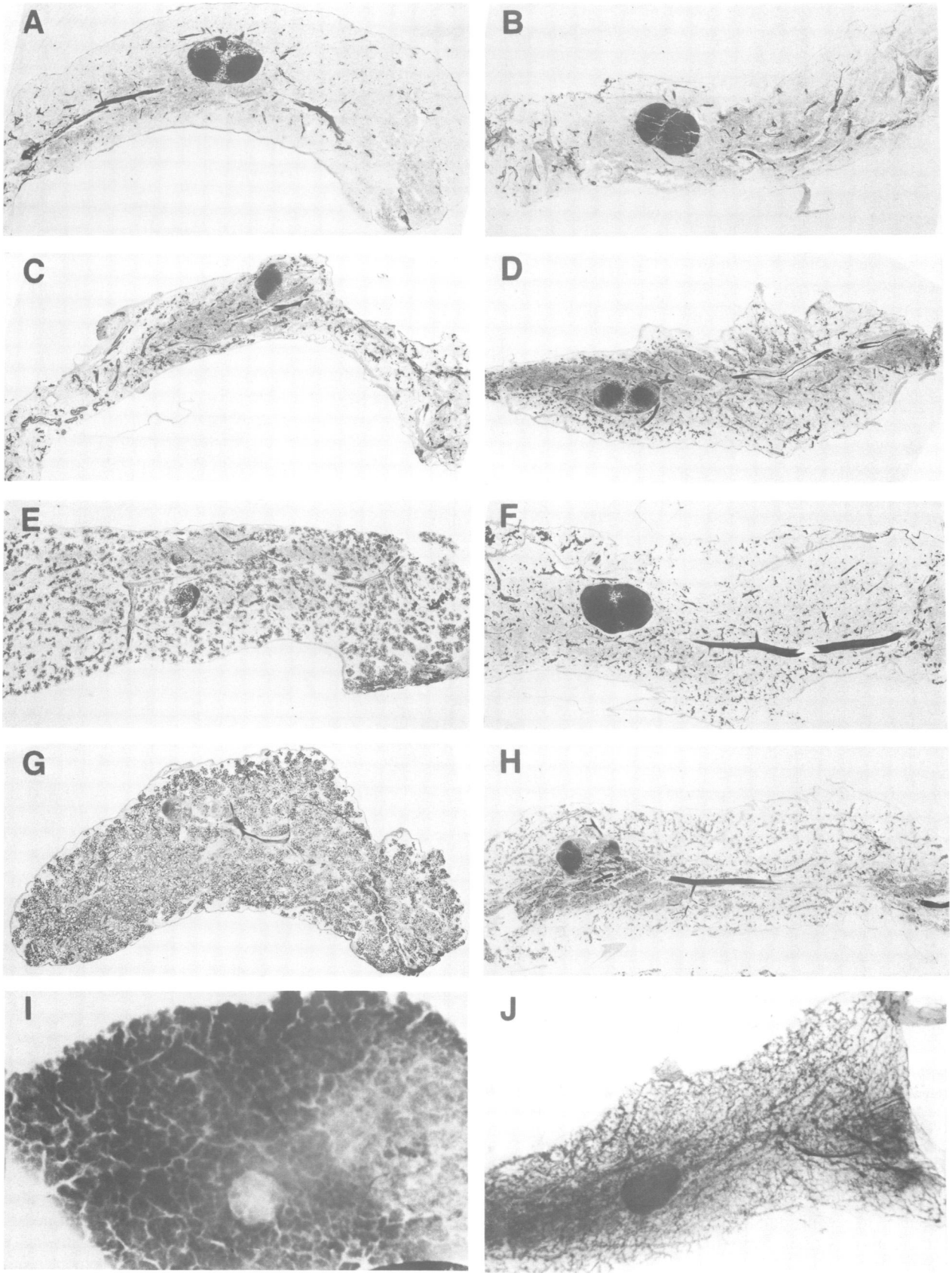

Fig. 5. Inhibition of pregnancy-induced lobuloalveolar development in WAP/TGF-β1 transgenic mice. Inguinal mammary glands (number 4) were removed from non-transgenic FVB/N (**A**, **C**, **E**, **G** and **I** at left) and transgenic Wβ-9 (**B**, **D**, **F**, **H** and **J** at right) females that were either virgins or at various stages of pregnancy. Mammary glands were fixed, stained and prepared as whole mounts. Glands were either examined directly as whole mounts (I, J) or used to generate thin sections (A–H). Mammary glands were from virgins (A, B), 7-day pregnant (C, D), 17-day pregnant (E, F) or 19-day pregnant (G–J) mice. Note the presence of the lymph node that characterizes the inguinal gland in mice. Magnification is 6.5×.

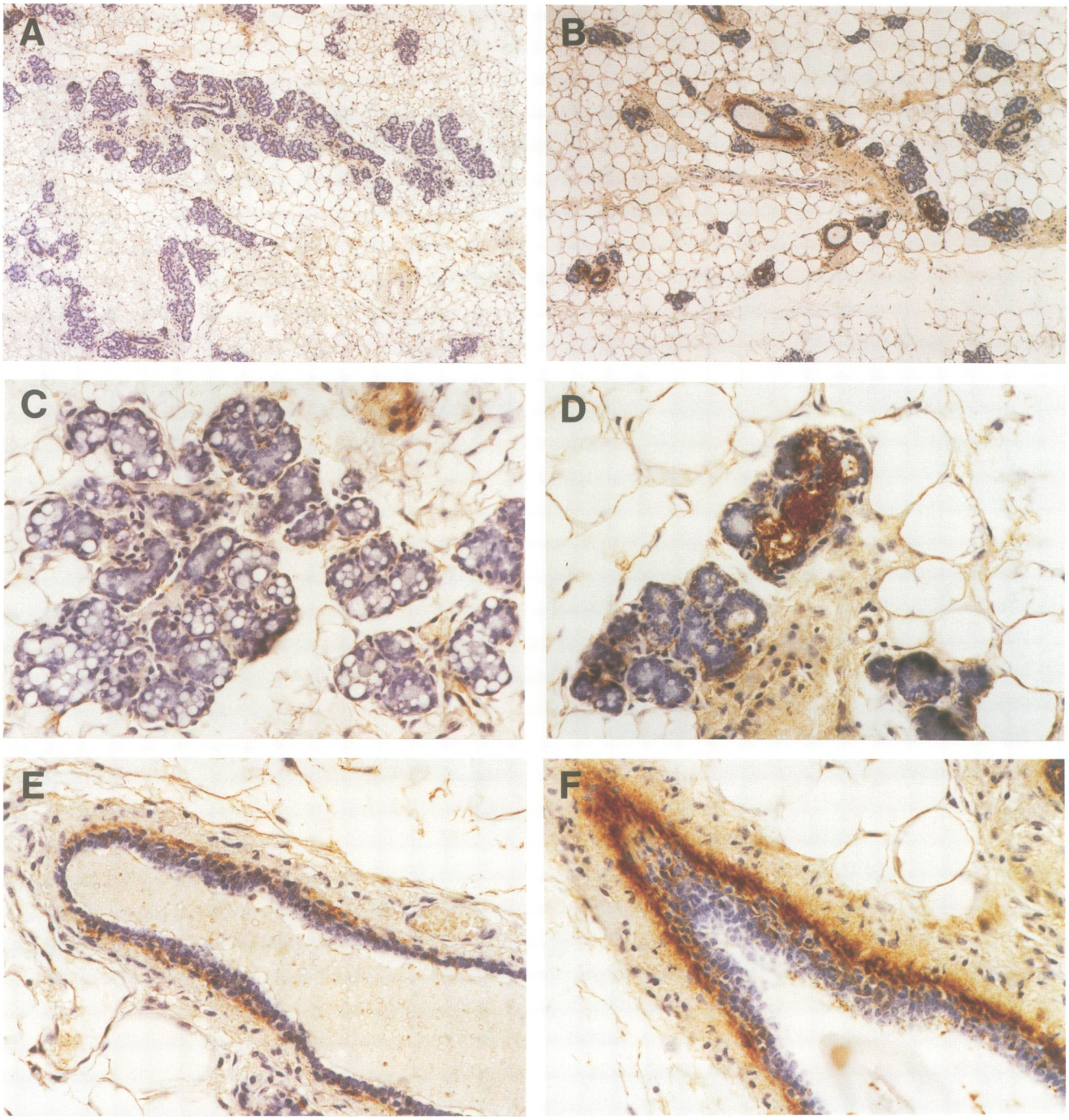

Fig. 6. Immunohistochemical localization of TGF-β1 in the transgenic mammary gland. Sections of whole-mount inguinal mammary glands from a non-transgenic control (**A**, **C** and **E**) and a lactation-deficient Wβ-12 (**B**, **D** and **F**) 15-day pregnant female were subjected to immunohistochemical staining using the TGF-β1 rabbit polyclonal anti-CC(1-30-1) antibody (Heine *et al.*, 1987; Flanders *et al.*, 1989). Note the positive staining for TGF-β1 in the (D) cytoplasm and lumina of alveolar epithelial structures, and (F) periductal extracellular matrix of the mid-pregnant Wβ-12 transgenic mammary gland. Magnification: (A and B) 100×; (C–F) 400×.

in the pregnant transgenic gland, cultured mammary cells were used to reconstitute the mammary gland in syngeneic hosts. Mammary tissue was removed from transgenic Wβ-9 and non-transgenic FVB/N virgin females, treated with collagenase overnight, and placed in culture. After 12 days in culture, mammary cells were injected into gland-free syngeneic inguinal fat pads. Subsequently, the host females were impregnated and allowed to proceed to full-term pregnancy and parturition. Thereupon, the injected fat pads and control samples of host gland were examined for alveolar development. Figure 9 shows that the reconstituted gland produced by the transgenic mammary epithelial cells duplicated the sparse lobuloalveolar development routinely observed *in situ* in the transgenic mammary gland at parturition (compare the whole mounts in Figure 9B with Figure 5J). In contrast, reconstituted glands produced by non-transgenic control mammary epithelial cells were indistinguishable from adjacent host glands (Figure 9A).

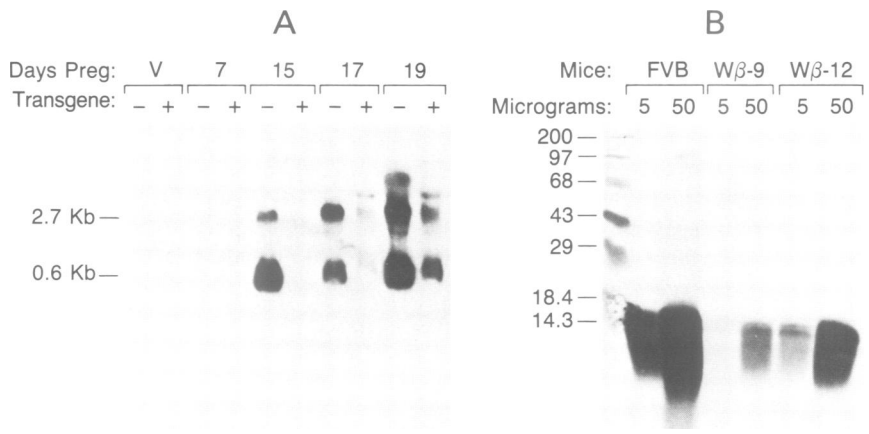

Fig. 7. Expression of the endogenous WAP gene is greatly reduced in transgenic mice. (**A**) Northern blot analysis of endogenous WAP gene expression in mammary glands from control (−) and Wβ-9 transgenic (+) mice that were either virgins (v) or at various stages of pregnancy (shown at the top). The WAP probe used for this hybridization is shown in Figure 1A and this RNA blot was prepared as described in Figure 4. Sizes of endogenous WAP RNAs are shown on the left. (**B**) Western blot analysis of endogenous WAP gene expression in control FVB/N, and in lactation-deficient transgenic Wβ-9 and Wβ-12 19-day pregnant mice. Proteins were removed from full-term mammary tissue by acid-ethanol extraction, and electrophoresed on an SDS−polyacrylamide gel as 5 and 50 μg samples. WAP was visualized by immunoperoxidase staining using an anti-WAP antibody (Shamay *et al.*, 1991). Sizes of protein standards are shown on the left.

Discussion

The mouse mammary gland provides an extremely useful system for determining the physiological effects of specific growth factors and modulators on development. The mammary gland goes through a period of rapid ductal proliferation at puberty, followed at pregnancy by proliferation of secretory alveolar structures which fill the stromal tissue. These lobuloalveolar structures persist until the end of lactation, at which time there is massive apoptosis in the gland characteristic of the involution process (Strange *et al.*, 1992). The rapid and precise execution of this complex set of processes undoubtedly requires the interplay of multiple hormone-dependent factors regulating cellular proliferation and differentiation (Forsyth, 1991).

The present study was concerned specifically with the *in vivo* influence of the multifunctional factor TGF-β1 on the development of the lactating mammary gland. We report here that the end point of development of the lactating mammary gland was dramatically different in transgenic mice overexpressing a WAP/TGF-β1 transgene. Transgene expression over the course of pregnancy was greatly enhanced in the last half of pregnancy; coincident with this, alveolar development and lactogenesis were strongly inhibited, resulting in a failure to lactate.

The precise mechanism by which lactation is compromised by expression of the WAP/TGF-β1 transgene has yet to be determined; however, our data suggest a number of intriguing hypotheses. Although alveolar structures in mammary tissue from 15- to 19-day pregnant transgenic mice were relatively sparse and organized in smaller clusters, nevertheless, they were capable of producing WAP and casein protein. This raises the possibility that the transgenic alveoli were fully functional, but were present in insufficient numbers to support normal lactation. TGF-β1 could be inhibiting the proliferation and expansion of small alveolar structures into larger secretory clusters, implicating TGF-β1 as a negative growth regulator. Alternatively, given the multifunctional nature of this cytokine, TGF-β1 may be capable of blocking the differentiation of alveolar stem cells overexpressing the transgene into functional glandular structures (Smith and Medina, 1988; Smith *et al.*, 1990). TGF-β1 has been implicated in the initiation of apoptosis in rodent hepatocytes (Oberhammer *et al.*, 1991, 1992) and may induce cell death in mammary cells as well. The alveolar stem cell compartment could be compromised either in the transgenic virgin gland, where transgene expression has been detected at a low level, or in the mid-pregnant gland, where transgene activity is highly induced.

TGF-β1 may also directly inhibit lactogenesis. This hypothesis is supported by reports in which TGF-β was shown to inhibit the hormone-induced expression of milk caseins in HC11 mouse mammary epithelial cells and in mammary explants from 14- to 15-day pregnant mice (Mieth *et al.*, 1990; Robinson *et al.*, 1992). In Wβ-12 and Wβ-9 19-day pregnant transgenic mice, endogenous WAP production was estimated to be reduced 10- and 50-fold, respectively. Moreover, immunohistochemical staining suggested that casein levels were reduced in Wβ-9 and Wβ-12 pregnant females as well. Whether lactogenesis is suppressed directly by TGF-β1 or indirectly through inhibition of the growth and development of lobuloalveolar structures remains to be determined; however, the two possibilities are not mutually exclusive.

A final possibility is that TGF-β1 may inhibit lobuloalveolar development by binding to and influencing adjacent stromal cells. Stromal cells affected by TGF-β1 might interact with otherwise normal epithelial cells in an altered manner, either directly or by altering the composition of the connective tissue matrix in which the alveolar cells grow. Changes in stromal−epithelial interactions typically induce profound developmental effects (Sakakura, 1987).

While lobuloalveolar development was impaired in the WAP/TGF-β1 transgenic mice, ductal proliferation and expansion appeared to be unimpeded. In contrast, in the first

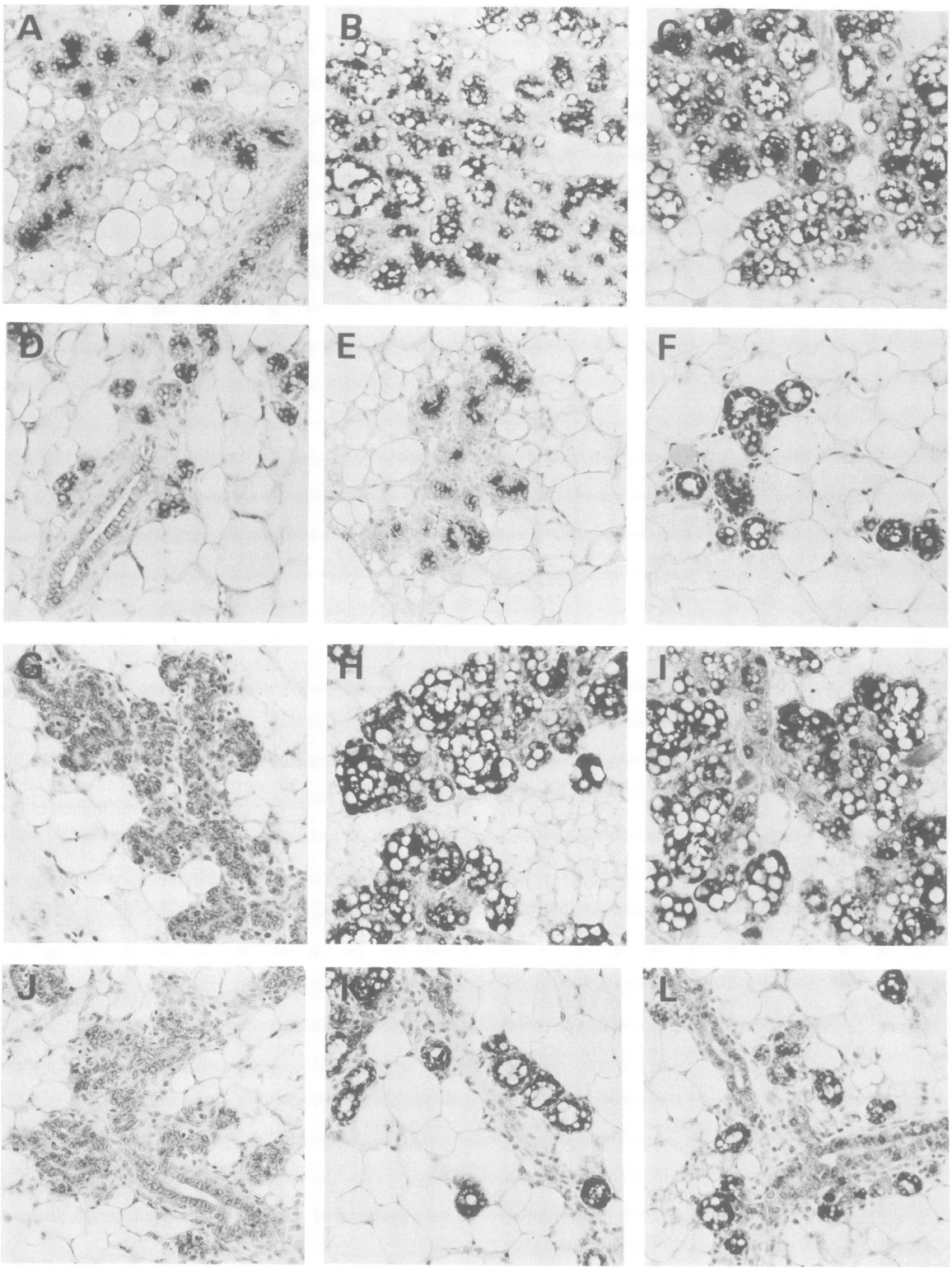

Fig. 8. The onset of WAP and casein synthesis is the same in control and lactation-deficient Wβ-12 transgenic mammary tissues. Shown are immunocytochemical analyses of casein (**A**–**F**, top two rows) and WAP (**G**–**L**, bottom two rows) gene expression in mammary tissue obtained from 7-day pregnant (A, D, G and J, left panel), 17-day pregnant (B, E, H and K, center panel) and full-term pregnant (C, F, I and L, right panel) female mice. Control mammary glands are shown in the first (A–C) and third (G–I) rows; Wβ-12 transgenic glands are shown in the second (D–F) and fourth (J–H) rows. Magnification is 200×.

studies to examine *in vivo* effects of TGF-β1 on mammary gland function, Daniel and co-workers have shown that implantation of slow-release pellets containing TGF-β1 into the mammary gland inhibited ductal elongation in virgin subadult mice (Silberstein and Daniel, 1987); however, lobuloalveolar development in pregnant mice was not overtly affected by the release of exogenous TGF-β1 (Daniel *et al.*, 1989). Differences in conclusions about the action of TGF-β1

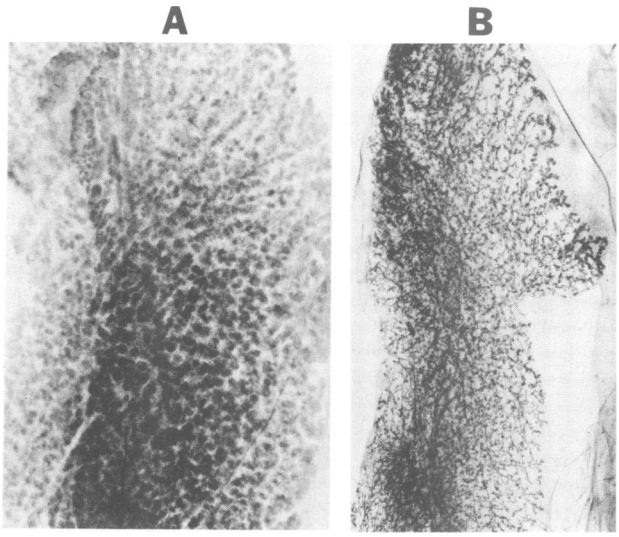

Fig. 9. Reconstituted glands produced by transgenic mammary cells duplicate the sparse lobuloalveolar development that characterizes transgenic mammary glands. Whole mounts of repopulated inguinal mammary fat pads from full-term pregnant FVB/N hosts are shown. **Panel A** shows the full lobuloalveolar development obtained with implants of cultured mammary cells from a non-transgenic virgin female. **Panel B** reflects the growth and development obtained with mammary cells from a Wβ-9 virgin female. Adjacent host glands that were not experimentally manipulated served as positive controls for lobuloalveolar development (data not shown). Magnification is 8.5×.

on ductal and alveolar development from studies using slow-release pellets and those using transgenic mice may be accounted for, in large part, by the tissue-specific targeting achieved via the WAP portion of the transgene. The transgene was designed to express TGF-β1 in alveoli during pregnancy-induced alveolar development, not in ductal cells during puberty-induced ductal growth and branching. Since the transgene encoded an activated form of TGF-β1, its sphere of influence was focused either on alveolar cells that express the transgene or on immediately adjacent cells (Wakefield *et al.*, 1990). In the transgenic model, alveolar cells were subjected to chronic autocrine stimulation throughout lobuloalveolar development, whereas in the pellet model stimulation was paracrine and limited to specific windows of time. Finally, it should be noted that severe retardation of alveolar development of the mammary gland in our transgenic mice was limited to the last third of pregnancy, a period that has not been examined using slow-release pellets (Silberstein and Daniel, 1987; C.Daniel, personal communication). In fact, normal and transgenic glands were morphologically indistinguishable up to day 13 of pregnancy.

Epithelial cells from cultured transgenic mammary glandular tissue were unable to produce fully differentiated lobular outgrowths when injected into the mammary fat pads of pregnant non-transgenic hosts, whereas non-transgenic mammary epithelial cultures were capable of full lobuloalveolar development under the same experimental conditions. These preliminary studies confirm that phenotypic changes in lobuloalveolar development associated with expression of the WAP/TGF-β1 transgene are intrinsic to the transgenic epithelial cell population.

The perturbation of lactogenesis and lactation observed in our transgenic mice suggests that TGF-β1 plays an important role in the development and function of the mammary gland; however, it must be noted that in a number of biological assays, TGF-β1, -β2 and -β3 all induced the same cellular response, and were interchangeable. This raises the possibility that under normal physiological conditions other TGF-β isoforms may be involved in regulating lactogenesis and the formation of alveolar structures. In fact, results from Robinson *et al.* (1991) suggest that either TGF-β2 or TGF-β3 are better candidates for true physiological agents; immunohistochemical analysis showed heavy staining for TGF-β3 in alveolar epithelial cells and TGF-β2 was detected at appreciable levels only during pregnancy (Robinson *et al.*, 1991, 1992).

This transgenic mouse study demonstrates that TGF-β1 strongly influences the growth and formation of the pregnant mammary gland, and supports the general notion that TGF-β1, or other closely related family members, functions *in vivo* as a developmental modulator. WAP/TGF-β1 transgenic mice should provide a useful model for detailed analysis of TGF-β1 function in lobuloalveolar development. Moreover, the potential for suppression of breast cancer by TGF-β1 (Wakefield and Sporn, 1990) can be assessed by studying the progeny of crosses between WAP/TGF-β1 mice with other transgenic animals expressing potent tumorigenic growth factors and oncogenes in the mammary gland.

Materials and methods

Plasmids and preparation of DNA for microinjection

Transgenic mice were generated using an 8.1 kbp DNA fragment containing a 1.35 kbp porcine TGF-β1 cDNA (Kondaiah *et al.*, 1988) inserted into the first exon of the mouse WAP gene (Figure 1). The TGF-β1 cDNA had been modified near the initiator 5′ ATG translational start codon to more closely match the Kozak consensus sequence and improve translational efficiency, and at cysteine residues 223 and 225 to ensure secretion of biologically active TGF-β1 (Samuel *et al.*, 1992). This fragment was isolated from a WAP/TGF-β1 plasmid and separated from vector sequences by digestion with *Eco*RI followed by sucrose gradient centrifugation as previously described (Jhappan *et al.*, 1990; Merlino *et al.*, 1991).

Generation and maintenance of transgenic mice

Single-cell embryos were harvested from the inbred FVB/N strain of mice (Harlan Sprague Dawley Inc., Frederick, MD). The WAP/TGF-β1 fragment was microinjected into embryonic male pronuclei at a concentration of 0.5 μg/ml (Hogan *et al.*, 1986). Surviving embryos were transferred to the oviducts of pseudopregnant CD-1 foster mothers (Charles River). Isolation and analysis of genomic DNA from the resulting WAP/TGF-β1 transgenic mice was carried out by Southern blot hybridization (Hogan *et al.*, 1986; Jhappan *et al.*, 1990). Briefly, genomic DNA was prepared from tail biopsies of offspring, digested with *Kpn*I and resolved on an 0.8% agarose gel. Hybridization was to nick-translated porcine TGF-β1 cDNA probes using Quick-Hyb (Stratagene, CA). Since female WAP/TGF-β1 mice expressing the transgene were not capable of lactating, their pups were routinely fostered to lactating mothers and, wherever possible, male TGF-β1 were used to propagate the lines of transgenic mice. Genomic DNA from one non-lactating line, Wβ-9, was used to determine the sequence of the integrated transgene in the region adjacent to the *Kpn*I site into which the TGF-β1 cDNA was ligated.

RNA preparation and analysis

RNA was prepared from a variety of mouse tissues as previously described (Chomczynski and Sacchi, 1987). Briefly, tissues were quickly removed from killed animals and quick frozen on dry ice. Tissues were homogenized later in a 4 M guanidine isothiocyanate solution. The homogenates were acid-phenol extracted and total RNA was precipitated with isopropanol. Northern blot hybridization was used for all analyses of RNA samples. Expression of the WAP/TGF-β1 transgene was determined using a nick-translated 870 bp porcine-specific TGF-β1 cDNA probe derived by PCR amplification from the precursor region (Figure 1). Hybridization with the porcine probe was at 42°C overnight, and washing included a 1 h incubation at 67°C in 0.1 × SSC, 0.1% SDS. Expression of endogenous mouse TGF-β1 RNA was determined using a 682 bp mouse-specific PCR

fragment amplified from the precursor region of the mouse transcript (Derynck *et al.*, 1986). Hybridization with the mouse probe was at 52°C overnight. Fifteen micrograms of total RNA from each tissue were routinely used and hybridizations were as previously described (Jhappan *et al.*, 1990; Merlino *et al.*, 1991). Typically, ethidium bromide was added to RNA samples prior to electrophoresis to enhance staining. This permitted the evaluation of loading and transfer efficiency of each RNA sample. Size markers included 28S rRNA (4.7 kb), 18S rRNA (2.0 kb) and RNA ladders from Gibco-BRL.

Preparation of tissue for histology and immunohistochemistry

Tissues were fixed overnight in Bouin's solution (Sigma Chemical Co.) and then transferred to and stored in 70% ethanol. Routinely, mammary glands were prepared for whole-mount analysis. They were cleared of fatty tissue in acetone, stained in hematoxylin, cleared in xylene and stored in methyl salicylate or mounted on slides under permount. After photography of the whole mounts, histopathological analysis was performed on these mammary glands by embedding them in paraffin, thin-sectioning the blocks, and staining with hematoxylin and eosin.

Immunohistochemical staining

Five micron paraffin sections were deparaffinized and subjected to several blocking steps. Endogenous peroxidases were blocked for 10 min in 0.3% hydrogen peroxide in methanol followed by a final 15 min blocking in 20% goat serum and 0.1% bovine serum albumin (BSA). Blocking was followed by an overnight incubation at room temperature with the IgG fraction of the antibody. Sections were washed with phosphate-buffered saline (PBS), followed by the indirect immunoperoxidase antisera detection Elite kit (Vector Laboratories Inc.) protocol as described previously (Heine *et al.*, 1987). TGF-β1 was detected using the rabbit polyclonal anti-CC(1-30-1) antibody against extracellular TGF-β1 (Flanders *et al.*, 1989). Subsequent staining with hematoxylin was carried out on sections before mounting coverslips. Mouse salivary glands and lung sections served as positive controls for TGF-β1 localization. WAP and casein were localized on tissue sections as described previously (Burdon *et al.*, 1991). Additional controls included dilution of the primary antibody and substitution of normal rabbit serum for primary antibody.

Western blotting

Proteins were obtained from frozen transgenic and control mammary tissue by overnight acid-ethanol extraction at 4°C (Danielpour *et al.*, 1989). After extensive dialysis against 4 mM HCl, samples of 5 and 50 μg of extracted protein were lyophilized and fractionated on an SDS–15% polyacrylamide gel. Western blots were generated by standard techniques. After transfer to immobilon-P membranes (Millipore Corp.) and overnight blocking with 2% goat serum and 1% BSA, mammary proteins were exposed first to a 1:1000 dilution of rabbit anti-WAP polyclonal antibody for 45 min at room temperature (Shamay *et al.*, 1991), and second to 0.3 μg/ml peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Kirkegaard & Perry Laboratories) for 45 min at room temperature. WAP was visualized by development in hydrogen peroxide and 3,3′-diaminobenzidine.

Mammary gland cell culture and transplantation

Mammary glands were aseptically removed from 6- to 8-week-old virgin Wβ-9 transgenic and FVB/N non-transgenic mice at the same point in the estrus cycle to minimize hormonal influences. The glands were minced into 2 mm^2 fragments, treated with collagenase (1 mg/ml) overnight and placed in culture dishes in a 1:1 mixture of Ham's F12 and DMEM containing 1% fetal bovine serum and supplemented with insulin (4 μg/ml), dexamethasone (10^{-7} M) and prolactin (1 mg/ml). After 12 days in culture, mammary cells were trypsinized, washed, pelleted and injected into gland-free syngeneic fat pads that had been surgically rendered epithelium free (Smith *et al.*, 1991). After 10 days, the host females were impregnated and, following parturition, the glands were removed, fixed and examined as whole mounts (Smith and Medina, 1988).

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