

Temporally regulated overexpression of parathyroid hormone–related protein in the mammary gland reveals distinct fetal and pubertal phenotypes

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Abstract

We have previously demonstrated that overexpression of parathyroid hormone-related protein (PTHrP) in the mammary glands of transgenic mice results in defects in ductal elongation and branching during puberty and in lobuloalveolar development during pregnancy. In addition, we have shown that PTHrP is necessary for the formation of the initial ductal tree during embryonic mammary development. In order to examine the effect of varying the timing of PTHrP overexpression on mammary development, we created tetracycline-regulated, K14-tTA/Tet^o-PTHrP double transgenic mice. In this report, we document that this ‘tet-off’ system directs transgene expression to the mammary gland and that it is fully repressed in the presence of tetracycline. Using these

mice, we demonstrate that transient overexpression of PTHrP before birth causes defects in ductal branching during puberty and that overexpression of PTHrP during puberty decreases the rate of ductal elongation. Furthermore, we demonstrate that if PTHrP overexpression is initiated after ductal morphogenesis is completed, lobuloalveolar development is unaffected. Finally, we demonstrate that the impairment in ductal elongation caused by PTHrP is associated with an increase in the basal rate of epithelial cell apoptosis in terminal end buds and a failure to increase end bud cell proliferation and decrease apoptosis in response to estrogen and progesterone.

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Introduction

The murine mammary gland forms in three distinct stages: during embryogenesis, during puberty, and during pregnancy/lactation (Daniel & Silberstein 1987, Sakakura 1987, Hennighausen & Robinson 1998). The embryonic phase begins with the budding of a bulb-shaped mammary anlage from the ventral epidermis between embryonic days 10 and 12. This mammary bud subsequently grows out into a corner of a specialized stromal compartment known as the mammary fat pad and, by birth, a rudimentary ductal tree of approximately 15–20 branches is formed. Little additional growth of the ducts occurs until the onset of puberty when they begin to extend and branch, eventually filling out the entire mammary fat pad. Growth occurs in a directed fashion away from the nipple region, and results from the formation of bulb-shaped structures called terminal end buds (TEBs) at the distal end of the epithelial ducts. These structures act as centers of epithelial proliferation, differentiation and apoptosis. At the end of puberty, when the ducts have reached the

borders of the fat pad, the TEBs regress and growth ceases. The next phase of mammary development occurs during pregnancy and only then does the fully differentiated, secretory epithelium form. As a result of hormonal changes, there is a proliferation of a large number of terminal ductules, followed by the development of lobuloalveoli. Towards the end of pregnancy, the alveolar epithelial cells become secretory and lactation is initiated at delivery. The cessation of lactation brings about massive apoptosis and remodeling of the lobuloalveolar structures, reverting the ductal architecture to one that resembles the virgin gland.

At all stages of its life cycle, the development of the mammary gland is regulated by systemic hormones and local epithelial–mesenchymal interactions (Daniel & Silberstein 1987, Sakakura 1987, Cunha 1994, Hennighausen & Robinson 1998). In fact, the effects of hormones on mammary development are, at least in part, mediated by the modulation of these local paracrine interactions (McGrath 1983, Daniel & Silberstein 1987, Hennighausen & Robinson 1998). Although the

importance of epithelial–mesenchymal interactions to mammary development has been appreciated for some time, the molecular mechanisms by which epithelial and mesenchymal cells communicate, and the ways in which systemic hormones affect their interactions, remain poorly understood.

One molecule that has recently been suggested to participate in epithelial–mesenchymal interactions is parathyroid hormone-related protein (PTHrP). PTHrP was originally identified as the tumor product responsible for the clinical syndrome of humoral hypercalcemia of malignancy (Philbrick *et al.* 1996, Strewler 2000). It derives its name from the fact that its gene and the parathyroid hormone (*PTH*) gene are both descended from a common ancestor. As a result, the two genes share sequence homology and structural characteristics that allow amino-terminal regions of PTH and PTHrP to signal through the same receptor, termed the type 1 PTH/PTHrP receptor (PTH-1R) (Philbrick *et al.* 1996, Strewler 2000). During development, PTHrP is produced by many epithelial structures, whereas the PTH-1R is often expressed on adjacent mesenchymal cells, suggesting a role for this ligand–receptor pair in epithelial–mesenchymal interactions (Lee *et al.* 1995, Wysolmerski & Stewart 1998). The examination of mammary development in several mouse strains engineered to either overexpress or underexpress PTHrP, PTH-1R, or both, has confirmed that this is the case during mammary development (Wysolmerski *et al.* 1995, 1998, Dunbar *et al.* 1999, Foley *et al.* 2001).

We have previously documented that PTHrP signaling is necessary for embryonic mammary development. If either the *PTHrP* or the *PTH-1R* gene is disrupted in mice, mammary epithelial buds form, but never give rise to a duct system (Wysolmerski *et al.* 1998, Dunbar *et al.* 1999, Foley *et al.* 2001). Instead, the epithelial cells revert to an epidermal cell fate and, at birth, no mammary epithelial structures remain. During embryogenesis, the *PTHrP* gene is highly expressed in the epithelial cells of the mammary bud, and the PTH-1R gene is expressed in the surrounding mesenchymal cells (Wysolmerski *et al.* 1998). Consistent with these patterns of expression, recent experiments have shown that PTHrP acts as a signal from the epithelium that is necessary for the formation of a mammary-specific mesenchyme. In PTHrP or PTH-1R knockout mice, no mammary mesenchyme forms (Dunbar *et al.* 1999, Foley *et al.* 2001). Alternatively, in transgenic mice that ectopically express PTHrP in the fetal epidermis, the entire ventral dermis is transformed into mammary mesenchyme (Wysolmerski *et al.* 1998, Foley *et al.* 2001). Therefore, it appears that PTHrP instructs the mesenchymal cells around the developing mammary bud to choose a mammary-specific fate and to direct subsequent mammary morphogenesis.

In addition to its actions during embryonic mammary development, PTHrP also may participate in the

regulation of ductal branching morphogenesis during puberty. We have previously shown that overexpression of PTHrP in mammary epithelial cells, using the keratin 14 (K14) promoter, causes severe defects in ductal development during puberty and early pregnancy (Wysolmerski *et al.* 1995). Overexpression of PTHrP appears to have two main consequences, both of which become manifest only after the beginning of puberty. Firstly, the ducts grow through the mammary fat pad at a slower rate, and secondly, the main ducts give rise to many fewer secondary and tertiary branches. Furthermore, local delivery of PTHrP to the mammary gland slows the rate at which normal ducts grow (Wysolmerski *et al.* 1995). These results suggest that PTHrP might regulate the function of TEBs, a hypothesis consistent with localization studies showing that, during puberty, PTHrP is predominantly expressed by cap cells of end buds, whereas the PTH-1R is predominantly expressed on stromal cells surrounding the TEBs (Dunbar *et al.* 1998). However, given the profound influence of PTHrP on embryonic mammary development, we wondered if some aspects of the K14-PTHrP phenotype might be secondary to the embryonic actions of PTHrP. In order to examine these questions, we used a tetracycline-regulated system to control the timing of PTHrP overexpression within the mammary gland. In the present study, we demonstrate that the K14-PTHrP mammary phenotype is the result of both embryonic and pubertal overexpression of PTHrP. Defects in the rate at which the epithelial ducts grow through the fat pad are due to the overexpression of PTHrP during puberty, whereas the defects in ductal branching during puberty appear to be a consequence of prior overexpression of PTHrP during embryogenesis.

Materials and Methods

Transgenic mice

The K14-tTA transgene was generated by inserting a 2.1 kb portion of the human K14 promoter upstream of the tetracycline transactivator (*tTA*) gene (Wysolmerski *et al.* 1994, Dunbar *et al.* 1998, Foley *et al.* 2001). The Tet_O-PTHrP transgene was generated by inserting a 568 bp human PTHrP(1–141) cDNA and 2.2 kb of human growth hormone sequences downstream of a minimal CMV promoter containing seven concatamerized tetracycline-repressor binding sites. The growth hormone sequences provide RNA processing sites; they are transcribed but not translated (Wysolmerski *et al.* 1994, Dunbar *et al.* 1998, Foley *et al.* 2001). Transgenic mice were generated by pronuclear injection using standard techniques, and genotypes were analyzed by PCR (Foley *et al.* 2001). Tet_O-PTHrP mice were identified by amplification of a 171 bp sequence of the human growth hormone gene using the following primers: forward, ctacgggctgctactgcttcagg; reverse, ggcaactgagtggaactc

caagg (Dunbar *et al.* 1999). K14-tTA mice were identified by amplification of a 159 bp sequence of the *tTA* gene using the following primers: forward, aacaaccgtaaacctgcc; reverse, aaatctgccagctttccc. Tet_O-β-gal mice were the gift of Dr Lothar Hennighausen, and were genotyped as described previously (Furth *et al.* 1994). K14-tTA/Tet_O-PTHrP double transgenic mice were created by crossing K14-tTA transgenic mice with Tet_O-PTHrP transgenic mice. Tet_O-PTHrP transgene expression was suppressed by feeding double transgenic mice 150 µg/ml tetracycline hydrochloride (Roche, Indianapolis, IN, USA) in 5% sucrose water. All animal experimentation was conducted in accord with accepted standards of humane animal care.

β-Galactosidase assay

β-Galactosidase activity was measured in embryonic and adult mammary tissue according to the procedure described by Furth *et al.* (1994). For adult tissue, the no. 4 inguinal mammary glands of mice were dissected and scored every 5 mm, to allow penetration of the fixative and reaction mixture. For embryonic tissue, whole embryos were harvested and fixed, and ventral skin was dissected after fixation. The tissue was fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in phosphate buffered saline (PBS) for 1 h at room temperature and then washed twice in PBS. To assay for β-galactosidase, the tissue was incubated in a buffered solution containing 0.1% 4-chloro-5-bromo-3 indolyl β-D-galactopyranoside, 2 mM MgCl₂, 5 mM EGTA, 0.02% Nonidet P-40, 5 mM K₃Fe(CN)₆ and 5 mM K₄Fe(CN)₆.6H₂O at 30 °C for at least 1 h and up to 24 h, depending on the color reaction. After staining, the tissue was post-fixed in 4% paraformaldehyde overnight at 4 °C and embedded in paraffin. Sections 5 µm thick were cut and counterstained with eosin.

RNA isolation and RNase protection analysis

Total RNA was isolated from mammary tissue using Trizol reagent (Gibco, Gaithersville, MD, USA). RNase protection analysis was performed as described previously (Daifotis *et al.* 1992) using 2×10^5 c.p.m. of labeled antisense cRNA probes corresponding to a 153-bp Bgl II-Pvu II fragment of the human growth hormone gene and a 220-bp Sau3a-Sau3a fragment of the mouse cyclophilin gene.

Whole-mount analysis

Whole-mount analysis was performed on mammary tissue as previously described (Wysolmerski *et al.* 1995). Briefly, the no. 4 inguinal mammary glands were removed and mounted on a microscope slide. The tissue was fixed in acid ethanol for 1 h at room temperature, washed in 70% ethanol and distilled water and incubated in carmine aluminum stain (0.2% carmine, 0.5% aluminum potassium

sulfate) for 10–12 h at room temperature. After staining, the mammary glands were dehydrated through graded ethanol and defatted in acetone (6–8 h) and toluene (15 min) before being mounted under glass coverslips using Permount (Fisher Scientific, Fair Lawn, NJ, USA). For light microscopy, mammary tissue was fixed in 4% paraformaldehyde for 10–12 h at 4 °C, embedded in paraffin and sectioned at 5 µm for hematoxylin & eosin (H&E) staining. Histomorphometry was performed on whole mounts and the following parameters were measured: total duct length was measured from the nipple region to the leading edge of the most distal endbud; the percentage of fat pad penetration was calculated by dividing the total duct length by the distance from the nipple region to the most distal aspect of the fatty stroma; branches per 5 mm² records the average total number of branch points occurring within 15 random sample areas each 5 mm² in size; the number of branches off primary duct records the absolute number of branches arising from the entire length of the three or four main ducts.

Cell proliferation and cell death assays

Five-week-old mice were injected with 100 µCi [³H]thymidine (Amersham, Piscataway, NJ, USA) 45 min before the animal was killed. Hormone-treated mice were injected (i.p.) with an aqueous solution of estradiol (24 µg/day) and progesterone (1.2 mg/day) for 2 days before injection of [³H]thymidine (Wysolmerski *et al.* 1995). The mice were killed and the no. 4 inguinal mammary glands were harvested, fixed in 4% paraformaldehyde overnight, washed in 70% ethanol, embedded in paraffin and sectioned at 5 µm. The sections were then deparaffinized, rehydrated through graded ethanol to distilled water and post-fixed in 10% buffered formalin for 5 min at room temperature. After this, sections were counterstained with hematoxylin, dehydrated through graded ethanol, dipped in photographic emulsion (NTB-2, Kodak), air dried and stored at 4 °C for 7–10 days in the dark with desiccant. After being developed, the sections were again dehydrated through graded ethanol and coverslipped.

Cell death assays were performed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) analysis using the *In Situ* Cell Death kit from Boehringer-Mannheim (Roche). Briefly, sections of mammary tissue were incubated with proteinase K at a concentration of 20 µg/ml in 10 mM Tris HCl for 5 min at 37 °C. They were then washed in 1 × PBS and incubated with the terminal deoxytransferase reaction mixture at 37 °C for 45 min in the dark. After TUNEL labeling, the sections were washed with 1 × PBS and mounted for fluorescent microscopy using the Prolong Antifade reagent (Molecular Probes).

Apoptosis and cell proliferation were quantitated using photomicrographs of TEBs from TUNEL and [³H]thymidine experiments. Fifteen TEBs from a total of five mice

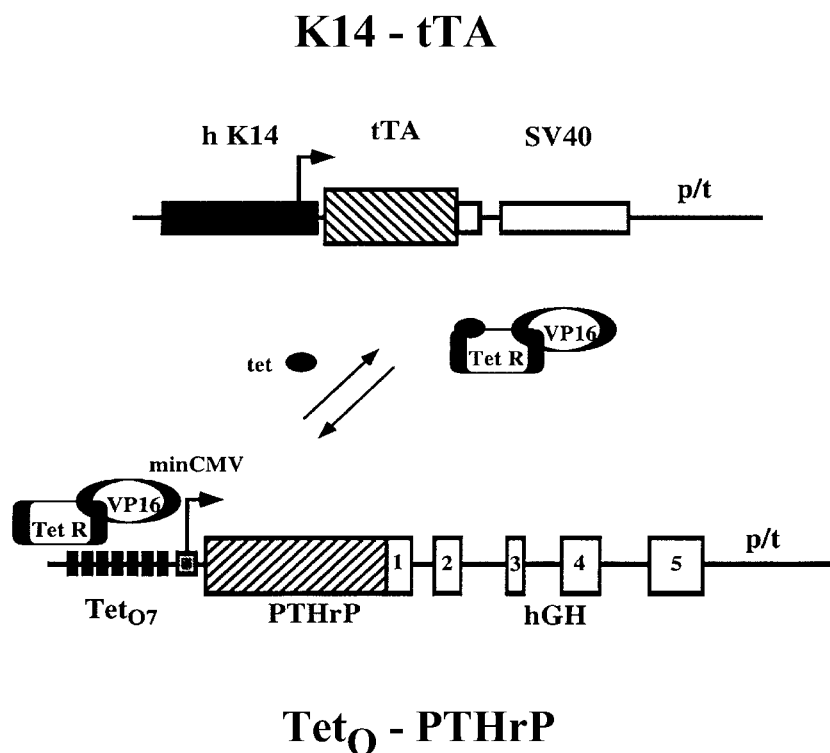
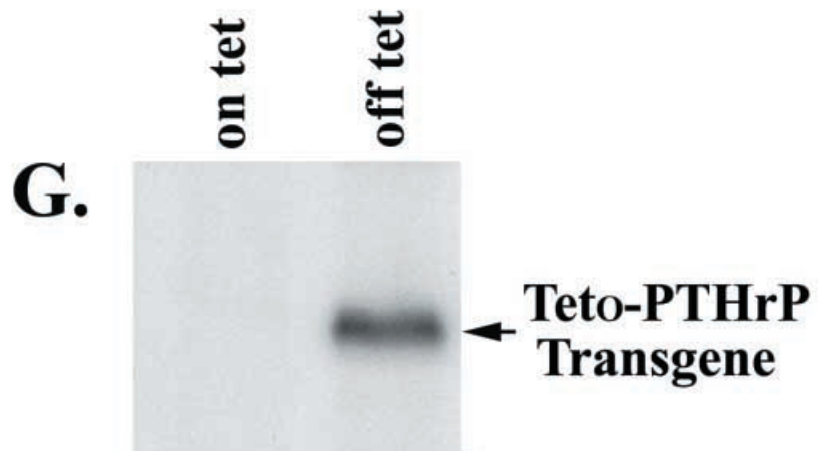
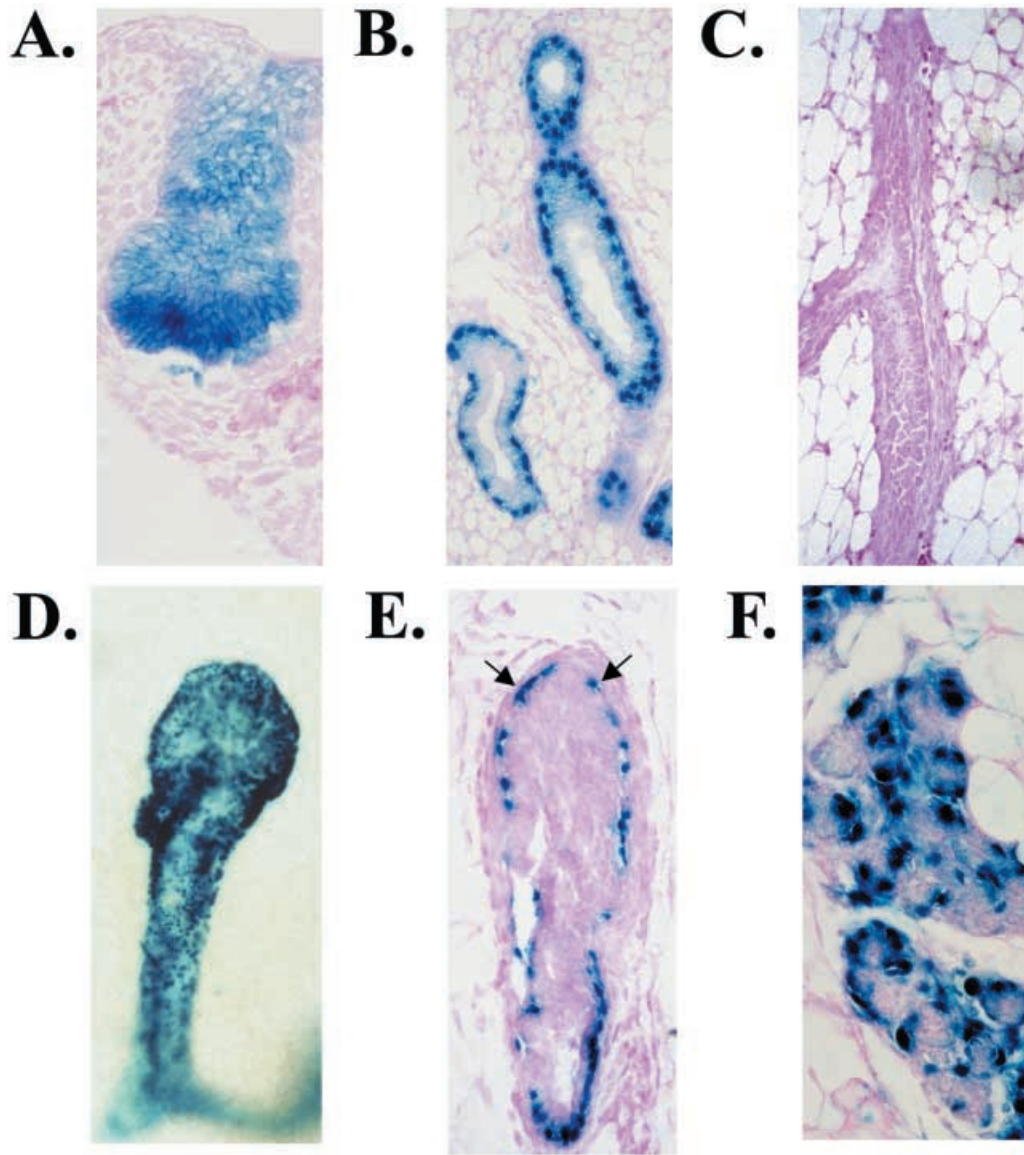


Figure 1 Regulated K14-PTHrP transgenic mouse. The K14-tTA transgene consisted of the human (h) K14 promoter driving expression of the (tet) tetracycline transactivator (tTA) cDNA, which encodes a fusion protein consisting of the DNA binding domain of the tetracycline repressor protein (Tet R) and the transcriptional activation domain of VP16. SV40 sequences are included for RNA processing. The Tet_O-PTHrP transgene consists of the human PTHrP(1–141) cDNA under the control of a minimal CMV (minCMV) promoter with seven tetracycline operon binding sites (tet_{O7}) and human growth hormone (hGH) gene sequences provided for RNA processing. These GH sequences are transcribed but not translated. In double transgenic mice, the tTA protein is produced in tissues expressing K14. This is a ‘tet-off’ system: if tetracycline is absent, the tTA protein binds to and activates transcription of the Tet_O-PTHrP transgene; however, if tetracycline is present, the tTA protein is prevented from binding and the Tet_O-PTHrP transgene remains silent. p/t, polyadenylation/termination sequences.

(three per mouse) were counted for each experimental condition. For TUNEL analysis, clusters of positively labeled nuclei occurring within a single cell diameter were considered to be fragments of a central cell and were

counted as a single apoptotic cell. For cell proliferation experiments, cells demonstrating four or more silver grains located over nuclei were counted as positive (Zeps *et al.* 1996).

Figure 2 (A–F) Mammary tissues harvested from K14-tTA/Tet_O-β-gal double transgenic mice and stained for β-galactosidase activity: A, B, D–F were raised off tetracycline and C was raised on tetracycline. (A) Section (5 μm) through a mammary bud from an embryo at embryonic day 15. (B) Similar section taken from an adult virgin mammary gland. (C) Section (5 μm) through a duct from an adult mammary gland from a mouse raised on tetracycline. (D) Terminal end bud from a whole-mount preparation of a 5-week-old mammary gland. (E) Section (5 μm) through a terminal end bud from a 5-week-old mouse. (F) Section (5 μm) through developing lobuloalveolar units from a 12-day pregnant gland. Note that β-galactosidase activity is present in all the epithelial cells of the embryonic gland (A), but only in the myoepithelial cells of the mature virgin duct (B) and the developing lobuloalveoli (F). β-Galactosidase is also present in the cap cells at the leading edge of the terminal end buds (D, and arrows in E) and the differentiating myoepithelial cells at the neck region. In the presence of tetracycline, no β-galactosidase activity was detected (C). (G) RNase protection assay on RNA prepared from mammary glands harvested from mature virgin K14-tTA/Tet_O-PTHrP mice raised either on tetracycline or off tetracycline. Note that, in the presence of tetracycline, the Tet_O-PTHrP transgene is not expressed, whereas in the absence of tetracycline, transgene-derived mRNA is easily detected.



Results

Generation and validation of K14-tTA/Tet_O-PTHrP double transgenic mice

In order to determine if the pubertal defects caused by overexpression of PTHrP were due to effects of PTHrP during mammary embryogenesis, we created a binary K14-PTHrP transgenic mouse that enabled us to regulate PTHrP production in a temporal fashion. As shown in Fig. 1, we made two transgenic mouse lines, one bearing a K14-tTA transgene and the other bearing a Tet_O-PTHrP transgene. Neither of these individual transgenic strains had defects in mammary ductal development, demonstrating that the tTA protein was not toxic to mammary epithelial or myoepithelial cells (data not shown). We next bred these two transgenic strains together to generate double transgenic (K14-tTA/Tet_O-PTHrP) mice. As we utilized a 'tet-off' system, double transgenic mice fed tetracycline should not produce excess PTHrP, but withdrawal of tetracycline should allow for PTHrP overexpression.

In order to test the ability of the K14-tTA transgene to activate target transgene expression correctly in this fashion, we first bred the K14-tTA line to a Tet_O-lacZ reporter mouse (Furth *et al.* 1994). The resulting double transgenic (K14-tTA/Tet_O-lacZ) mice would be expected to express β -galactosidase in a K14-dependent manner, only when tetracycline is absent. Figure 2 shows β -galactosidase staining of mammary glands from K14-tTA/Tet_O-lacZ embryos and adult females. As one can see, the epithelial cells of the embryonic mammary bud, in addition to those in the adult virgin mammary gland, appeared to be completely normal, again demonstrating that the presence of tTA within these cells was not toxic. As expected, when these mice were raised off tetracycline, β -galactosidase activity (as indicated by the blue color) was present in all the epithelial cells of the embryonic mammary bud and in the myoepithelial cells of the adult virgin mammary gland (Fig. 2A, B). In contrast, no β -galactosidase activity was observed in the mammary glands of double transgenic mice raised on tetracycline (Fig. 2C), demonstrating that the Tet_O-lacZ transgene is fully repressed in the presence of tetracycline. β -galactosidase activity was also detected in TEBs (Fig. 2D,E), both in the cap cells at the leading edge and the differentiating myoepithelial cells at the sides and neck, and in myoepithelial cells within the developing lobuloalveolar units during pregnancy (Fig. 2F). All of these sites have been previously shown to express K14 (Taylor-Papdimitriou & Lane 1987, Smith *et al.* 1990, Wysolmerski *et al.* 1995, 1998). Similar results were seen when the skin of the K14-tTA/Tet_O-lacZ mice was analyzed for β -galactosidase activity (Foley *et al.* 2001). These results demonstrate that the K14-tTA transgene activates target transgene expression in a tetracycline-dependent manner only at sites of K14 expression. In the

mammary gland, these include the epithelial cells of the embryonic mammary bud, the myoepithelial cells of the postnatal gland, and the cap cells of the TEB during puberty.

We next investigated whether tetracycline administration could effectively regulate PTHrP transgene expression in the mammary glands of K14-tTA/Tet_O-PTHrP double transgenic mice. Expression of the Tet_O-PTHrP transgene was assayed in total cellular RNA prepared from whole mammary glands of double transgenic mice on or off tetracycline. As can be seen in Fig. 2E, K14-tTA/Tet_O-PTHrP mice raised off tetracycline expressed the Tet_O-PTHrP transgene, but double transgenic mice raised on tetracycline did not. Furthermore, the levels of transgene expression in the K14-tTA/Tet_O-PTHrP mice raised off tetracycline were similar to those seen in the original K14-PTHrP mice (data not shown). Therefore, the binary K14-PTHrP system provides an effective 'on-off' switch to the original K14-PTHrP transgene and allows for temporal regulation of PTHrP overexpression in the mammary gland.

K14-tTA/Tet_O-PTHrP double transgenic mice recapitulate the mammary phenotype of the K14-PTHrP single transgenic mice

The mammary phenotype of K14-PTHrP transgenic mice consists of impaired ductal elongation and ductal branching during puberty. In order to determine if we could recapitulate these changes using our tetracycline-regulated system, we analyzed mammary development in 7-week-old K14-tTA/Tet_O-PTHrP female mice, raised either on tetracycline (never exposed to PTHrP overexpression) or off tetracycline (always exposed to PTHrP overexpression). As shown by whole-mount analysis in Fig. 3A, mammary glands from K14-tTA/Tet_O-PTHrP mice raised on tetracycline appeared to be completely normal. At this stage of development, the epithelial duct system had consistently reached the borders of the mammary fat pad, and it displayed a highly organized and complex branched architecture. In contrast, in double transgenic mice raised off tetracycline, both aspects of the K14-PTHrP phenotype were reproduced. As seen in Fig. 3B, the overall penetration of the duct system into the fat pad was reduced, and there was a reduction in the degree of branching. Therefore, the K14-tTA/Tet_O-PTHrP double transgenic mice reproduce the full spectrum of abnormalities caused by PTHrP overexpression, and these defects are completely prevented by feeding the mice tetracycline.

Abnormalities of ductal elongation are caused by postnatal overexpression of PTHrP and abnormalities in ductal branching are caused by prenatal overexpression of PTHrP

Although K14-driven transgene expression begins in the mammary bud as early as embryonic day 15 (Dunbar *et al.*

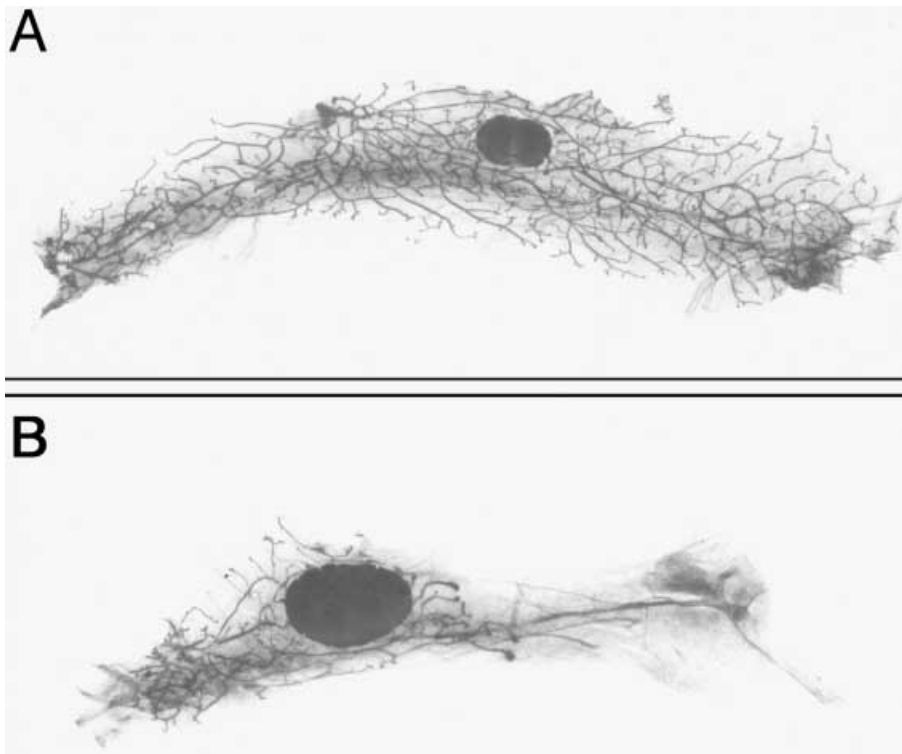


Figure 3 Whole-mount analysis of mammary glands harvested from 7-week-old K14-tTA/Tet_O-PTHrP mice raised either on tetracycline (A) or off tetracycline (B). In the presence of tetracycline, mammary ducts develop normally (A); in the absence of tetracycline, there are defects in ductal extension and branching (B).

1999), mammary abnormalities in the K14-PTHrP mice were not evident until the onset of puberty. Therefore, our working hypothesis was that overexpression of PTHrP during puberty would be responsible for the abnormal ductal proliferation and branching that occurred during this period. However, because PTHrP deficiency causes profound defects in embryonic mammary development, we examined the possibility that some of the effects of PTHrP overexpression might also be the result of PTHrP acting on the embryonic gland. In order to address this question, we utilized our tetracycline-regulated system to alter the timing of transgenic PTHrP production. K14-tTA/Tet_O-PTHrP double transgenic mice were exposed to tetracycline either from conception until birth, or from birth until they were killed, to provide for postnatal or prenatal overexpression of PTHrP respectively. Mammary gland development was analyzed towards the end of puberty, at 7 weeks of age. As shown in Fig. 4A, at this stage of development the duct system in normal mice has reached the borders of the mammary fat pad and has a highly branched organization. As compared with wild-type mice, when double transgenic mice were exposed to PTHrP overexpression only after birth, the overall growth of the epithelial ducts through the mammary fat pad was

significantly impaired and the tips of the growing ducts had not reached the distal end of the fat pad (Fig. 4B). However, as compared with either the original K14-PTHrP mice or the double transgenic mice never exposed to tetracycline, these mice exhibited minimal defects in the branching pattern of the epithelial ducts. Similar results were obtained when the mice were taken off tetracycline at the beginning of puberty (3 weeks of age, data not shown). In contrast, when double transgenic mice were exposed to PTHrP overexpression only before birth, the opposite pattern emerged: the glands from these mice demonstrated a severe reduction in branching complexity but only a minor reduction in the rate of ductal elongation (Fig. 4C).

In order to quantify the defects noted above, we performed histomorphometry in glands harvested from 10 double transgenic mice (five exposed to postnatal overexpression of PTHrP and five exposed to prenatal overexpression). The results are shown in Table 1. Ductal elongation, as assessed either by the total length of the duct system or by the percentage of the fat pad penetrated by ducts, was significantly reduced in mice exposed to postnatal, but not prenatal, transgene expression. Branching complexity, as determined by measuring the average

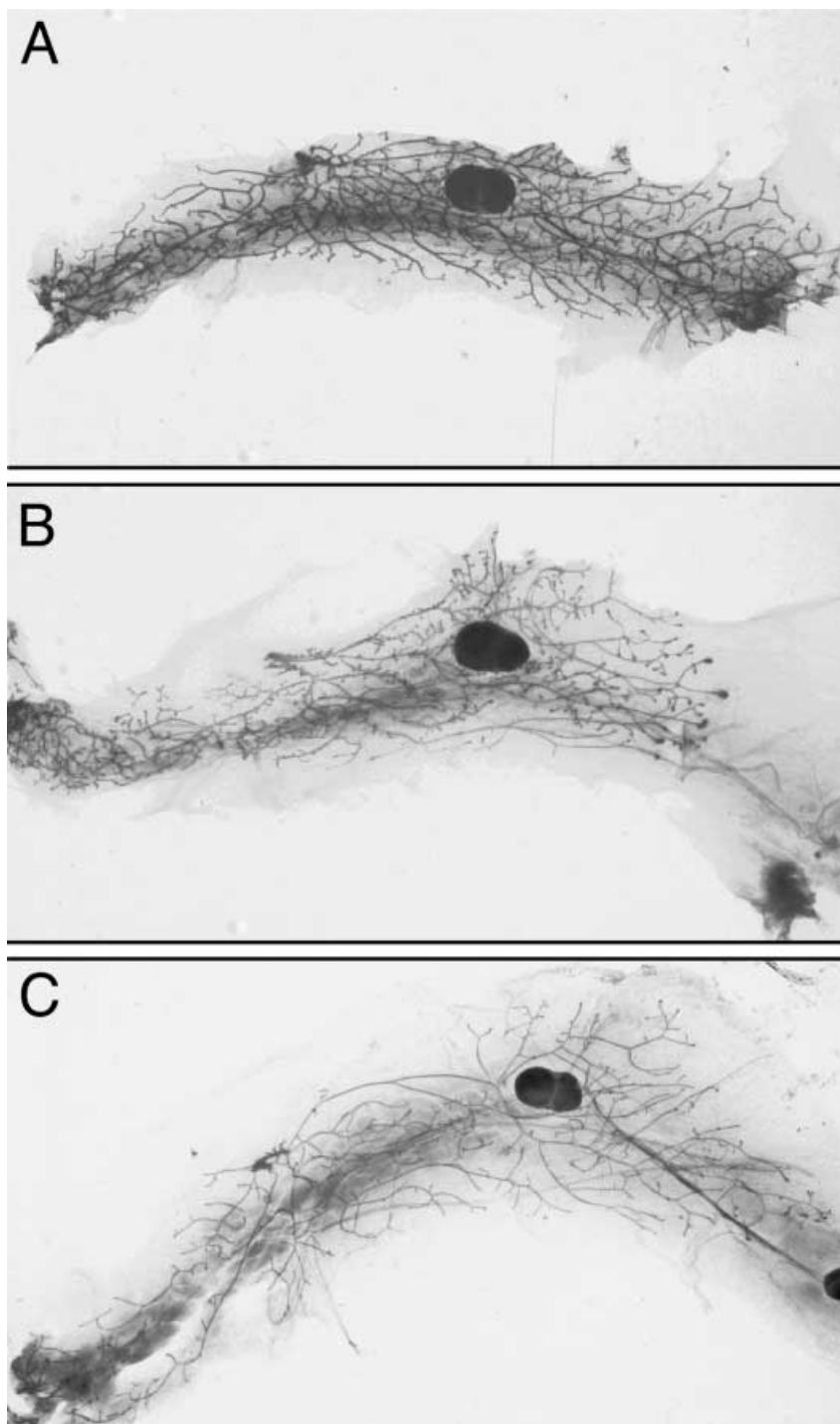


Figure 4 Whole-mount analysis of mammary glands harvested from a wild-type mouse (A), or from K14-tTA/TetO-PTHrP double transgenic mice exposed to PTHrP overexpression only after birth (B) or only before birth (C). All mice were killed at 7 weeks of age. Note that when PTHrP was expressed only after birth, there were significant defects in ductal extension, but only very mild defects in branching (B); however, when PTHrP was overexpressed only before birth, there was a significant reduction in ductal branching, but only slight impairments in duct extension. K14-tTA/TetO-PTHrP control mice that were always receiving tetracycline were also analyzed and were identical to wild-type mice.

Table 1 Histomorphometry of double transgenic (Dbl Tg) mammary glands: each measurement was performed on five separate glands, and node analysis was performed on 15 sample areas, each 5 mm² in size

	Total duct length (cm)	Fat pad penetration (%)	Branches per 5 mm² (No.)	Branches off primary duct (No.)
Wild-type	1.5 ± 0.1	91 ± 3	66 ± 6	19 ± 2
Dbl Tg				
PTHrP overexpression after birth	0.9 ± 0.1*	59 ± 5*	52 ± 3	18 ± 1
PTHrP overexpression before birth	1.3 ± 0.1	81 ± 5	24 ± 2*	13 ± 1*

Data are represented as the means ± S.E.M. **P*<0.05 compared with wild-type (Student's *t*-test).

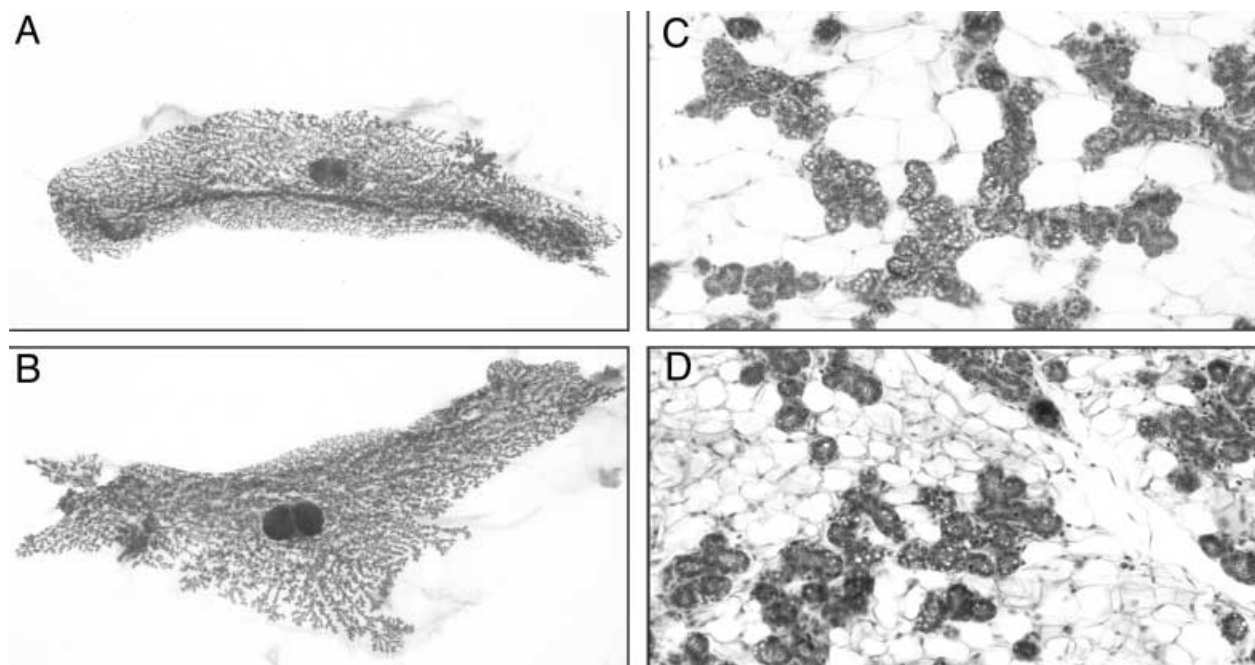


Figure 5 Lobuloalveolar development in K14-tTA/Tet_O-PTHrP double transgenic mice exposed to PTHrP overexpression after ductal development was completed. (A,C) Taken from wild-type mice at day 15 of pregnancy. (B,D) From double transgenic mice that were raised on tetracycline until 8 weeks of age and then were allowed to become pregnant 2 weeks later. These mice were also killed on day 15 of pregnancy. (A,B) Whole mounts. (C,D) Sections (5 μm) stained with H&E. Note that if PTHrP overexpression is delayed until after duct development is completed, there is no defect in lobuloalveolar development.

number of branch points per 5 mm² area or by counting the number of branches arising from each primary duct, was significantly reduced in double transgenic mice exposed to prenatal, but not postnatal, PTHrP overexpression. Taken together, these results suggest that the impairment in ductal elongation in the K14-PTHrP mice is due to the effects of PTHrP during puberty, but that the defects in ductal branching are due to the effects of PTHrP during embryonic development.

Overexpression of PTHrP during pregnancy does not affect lobuloalveolar development

In addition to alterations in ductal development during puberty, K14-PTHrP mice also demonstrate a delay in the

formation of lobuloalveolar structures, and a reduction in the total lobuloalveolar mass formed during pregnancy. Our initial characterization of this defect had suggested that it was partly a consequence of the inability to form terminal ductules during the early stages of pregnancy. However, it was unclear if overexpression of PTHrP might also directly impair lobuloalveolar differentiation. To address this question, we used the tetracycline-regulated system to overexpress PTHrP only during pregnancy. In order to allow for normal ductal development, K14-tTA/Tet_O-PTHrP double transgenic mice were raised on tetracycline until the end of puberty, when the adult branching pattern had been fully established. At that point, tetracycline was withdrawn and transgene expression was initiated. After 2 weeks, the mice were allowed to

become pregnant and lobuloalveolar development was examined at 15 days of gestation. As shown in Fig. 5, if PTHrP was overexpressed only after the completion of ductal development, there appeared to be no effect on mammary development during pregnancy (Fig. 5B,D). The glands from these mice demonstrated robust lobuloalveolar development and were indistinguishable from those of controls (Fig. 5A,C). These data suggest that overexpression of PTHrP in myoepithelial cells has no direct effect on lobuloalveolar differentiation and that the defects in pregnant K14-PTHrP mice are a consequence of the pre-existing abnormalities of ductal morphogenesis.

Impairment of ductal elongation during puberty is associated with alterations in cell turnover within terminal end buds

At the onset of puberty, the distal ends of the mammary ducts form specialized structures called terminal end buds (TEBs), which serve as sites of active cellular turnover and differentiation. Various studies have shown that the TEBs are the main locus of epithelial proliferation and apoptosis during puberty (Daniel & Silberstein 1987), and presumably the balance between these processes determines the rate at which the ducts grow through the mammary fat pad. We have previously shown that TEBs and their surrounding stroma are also the major site of *PTHrP* and *PTH-1R* gene expression during puberty (Dunbar *et al.* 1998), therefore we hypothesized that overexpression of *PTHrP* during puberty might impair ductal elongation by altering epithelial cell proliferation, epithelial cell death, or both, within the TEBs. In order to approach this question, we examined rates of cellular proliferation and apoptosis within the TEBs of K14-PTHrP transgenics and compared them with those in wild-type mice. Similarly, we studied K14-tTA/Tet_O-PTHrP double transgenic mice in which transgene expression was activated at birth, as they display defects in ductal elongation in the absence of significant branching defects. We studied mice during early puberty (4–5 weeks of age) at baseline and after 48 h of treatment with exogenous estrogen and progesterone.

We assessed TEB proliferation by measuring [³H]thymidine incorporation (Fig. 6A). At baseline, 14 ± 1.5% of epithelial cells in wild-type end buds incorporated thymidine. After hormone treatment, significantly more of the epithelial cells (23 ± 0.83%) within TEBs were labeled. Overexpression of PTHrP did not appear to affect baseline proliferation within the TEBs, as there were no differences in thymidine incorporation between wild-type mice (14 ± 1.5%) and K14-PTHrP transgenics (14 ± 1.1%) or K14-tTA/Tet_O-PTHrP double transgenics that were exposed to PTHrP overexpression after birth (13.8 ± 2.1%). However, overexpression of PTHrP did prevent the increase in proliferation that normally occurs in response to hormone treatment, as rates of proliferation after estrogen and progesterone treatment did not increase either in the

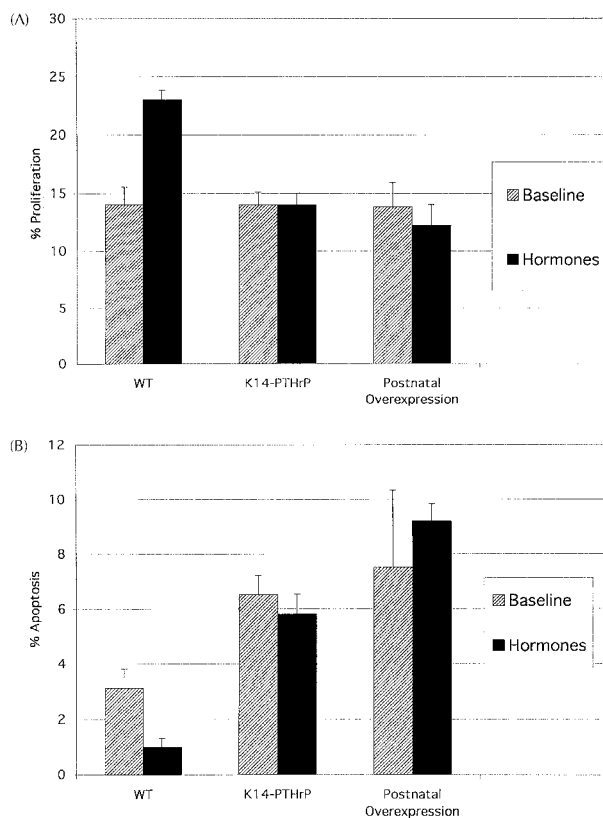


Figure 6 Effects of PTHrP overexpression on cell turnover in terminal end buds during puberty. (A) End bud cell proliferation in 5-week-old mice as determined by [³H]thymidine incorporation, showing the percentage of epithelial cells incorporating thymidine within end-buds of wild-type (WT), standard K14-PTHrP transgenics and K14-tTA/Tet_O-PTHrP double transgenic mice exposed to PTHrP overexpression only after birth (postnatal overexpression). Baseline: data for 5-week-old mice; Hormones: data for 5-week-old mice treated with exogenous estradiol and progesterone for 48 h before being injected with [³H]thymidine. Thymidine incorporation was statistically significantly greater in wild-type mice treated with hormones than in wild-type mice at baseline. Incorporation was statistically significantly lower in both transgenic groups treated with hormones than in the hormone-treated wild-type mice. (B) End bud cell apoptosis in 5-week-old mice as determined by TUNEL assay, showing the percentage of epithelial cells undergoing apoptosis within end buds of wild-type (WT), standard K14-PTHrP transgenics and K14-tTA/Tet_O-PTHrP double transgenic mice exposed to PTHrP overexpression only after birth (postnatal overexpression). Baseline: data for 5-week-old mice; Hormones: data for 5-week-old mice treated with exogenous estradiol and progesterone for 48 h before being injected with [³H]thymidine. At baseline, there was a statistically significantly greater percentage of cells undergoing apoptosis in the transgenic groups as opposed to the wild-type mice. In hormone-treated wild-type mice, there was a statistically significant decline in the percentage of apoptotic cells, but in either group of transgenic mice, hormone-treatment had no effect on cell death rates. All data are represented as the mean ± S.E.M. Statistical significance was assessed by Student's *t*-test, and significance was set as a *P* value of <0.05.

K14-PTHrP TEBs ($14 \pm 1.0\%$) or in the K14-tTA/Tet_O-PTHrP double transgenic TEBs ($12.2 \pm 1.8\%$).

We assessed TEB apoptosis by TUNEL assay (Fig. 6B). In wild-type mice, an average of $3.1 \pm 0.7\%$ of epithelial cells within TEBs were undergoing apoptosis at baseline, and this number decreased to $0.98 \pm 0.3\%$ of the cells upon hormone treatment. In contrast to the results regarding cell proliferation, overexpression of PTHrP did affect baseline rates of cell death within the TEBs. Rates of TEB apoptosis were significantly greater both in K14-PTHrP transgenic mice ($6.5 \pm 0.7\%$) and in K14-tTA/Tet_O-PTHrP double transgenic mice that had been exposed to PTHrP overexpression only after birth ($7.5 \pm 2.8\%$). In addition, in the transgenic mice, there was no reduction of apoptosis in response to hormone treatment. As has been previously documented, apoptosis occurred within the body cells of TEBs, both in wild-type and transgenic mice.

In summary, the defects in ductal elongation caused by the overexpression of PTHrP during puberty are associated with greater basal rates of epithelial cell apoptosis in TEBs, coupled with a failure to increase cell proliferation and reduce apoptosis in response to systemic estrogen and progesterone. Furthermore, these changes do not appear to be a result of prior morphological changes in the duct system, for they also occur in K14-tTA/Tet_O-PTHrP double transgenic mice that have been exposed to transgene expression only after birth.

Discussion

Previous work from our laboratory has shown that overexpression of PTHrP in mice impaired mammary duct elongation and branching during puberty (Wysolmerski *et al.* 1995). In this study we have demonstrated that the effects of PTHrP on pubertal mammary development depend on the timing of its overexpression. Using a tetracycline-regulated K14 transgene, we either transiently overexpressed PTHrP during embryonic mammary development or delayed overexpression of PTHrP until embryogenesis was complete. If PTHrP was overexpressed only before birth, the ductal branching pattern was altered during puberty, but the rate of elongation was largely unaffected. Conversely, if PTHrP was overexpressed after birth or at the commencement of puberty, ductal elongation was impaired, but branching was not. Furthermore, our results suggest that overexpression of PTHrP during puberty impairs ductal elongation by increasing the basal rate of epithelial cell apoptosis in terminal end buds and by preventing the normal changes in end bud cell turnover that occur in response to estrogen and progesterone.

Our data document that the 'tet-off' tetracycline-regulated transgenic system works well in the mammary gland. Experiments with a β -galactosidase target transgene demonstrated that the normal pattern of K14 expression was faithfully reproduced by the K14-tTA transgene. In

addition, the level of tet-transactivator generated by this transgene was not toxic to mammary epithelial cells, target transgenes were not active in the absence of tTA, and target transgene expression was fully repressed in the presence of tetracycline. On the basis of our results, we believe that this binary transgenic system should be useful for future studies examining transient overexpression of target genes in the embryonic gland or specific targeting to the myoepithelial/cap cell population at specified times during mammary development.

To our surprise, we found that the defects in ductal branching during puberty in the K14-PTHrP mice were the result of embryonic exposure to PTHrP. These results are reminiscent of experiments in which transient embryonic exposure to steroid hormones caused alterations in mammary ductal development during puberty and beyond (Hoshino 1966, Hoshino & Connolly 1967, Nagasawa *et al.* 1974, Mori *et al.* 1980). The effects of steroid hormones are believed to result, in part, from permanent alterations in the estrous cycle of exposed mice (Hoshino 1966, Hoshino & Connolly 1967, Nagasawa *et al.* 1974, Mori *et al.* 1980). It is unlikely that our results were due to systemic changes in the double transgenic mice. We have previously shown that circulating PTHrP concentrations are not increased in K14-PTHrP mice (Wysolmerski *et al.* 1995). In addition, these transgenic mice cycle normally and display normal fertility (unpublished data), ruling out any gross changes in their hypothalamic–pituitary–gonadal axis. However, at present it remains unclear how transient exposure to local overexpression of PTHrP before birth leads to a significant reduction in ductal branching complexity at the time of puberty, some 3–6 weeks later. We know that PTHrP has profound effects on the fate of the mesenchyme during fetal mammary development (Dunbar *et al.* 1999, Foley *et al.* 2001), so one possibility is that exposure to PTHrP alters the ability of either the periductal or fatty stroma of the fat pad to support correct branching. Previous recombination experiments have demonstrated that mammary ducts will branch in different configurations when combined with different stromal partners (Sakakura *et al.* 1976, Daniel *et al.* 1984). Alternatively, transient exposure to PTHrP might permanently affect the ability of the ductal epithelial cells themselves to give rise to side branches. The effects of PTHrP on ductal branching appear to be caused by amino-terminal PTHrP, acting on the type 1 PTH/PTHrP receptor, for we have found similar defects in transgenic mice that overexpressed parathyroid hormone (Wysolmerski *et al.* 1995). Because this receptor is not expressed on embryonic mammary epithelial cells (Wysolmerski *et al.* 1998, Dunbar *et al.* 1998), it is unlikely that PTHrP would affect epithelial cells in a typical autocrine fashion. Another possible mechanism may be via an intracrine pathway, for recent data have suggested that PTHrP can be imported into the nucleus and alter cellular behavior through a still undefined mechanism (Henderson *et al.* 1995, Massfelder *et al.*

1997, Aarts *et al.* 1999, Lam *et al.* 2000). Recombination and transplantation experiments examining different pairings of transgenic and wild-type epithelium and stroma will be necessary to determine if the effects of PTHrP on branching are due to a cell-autonomous or stromal effect (Weisen *et al.* 1999).

Our data suggest that, in addition to its effects during embryonic mammary development, PTHrP may also participate in the regulation of cell turnover in TEBs during puberty. We found that pubertal overexpression of PTHrP led to an increase in the basal rate of apoptosis within end buds, and it completely blocked the increases in end bud proliferation and decreases in end bud apoptosis observed in wild-type mice treated with exogenous estrogen and progesterone. A great deal of previous work has demonstrated that TEBs are the sites of active cellular turnover and differentiation during the pubertal growth of the mammary ducts (Russo & Russo 1980, Williams & Daniel 1983, Daniel & Silberstein 1987, Sapino *et al.* 1990, Humphreys *et al.* 1996). Therefore, the changes in proliferation and apoptosis that we observed most probably explain the delay in ductal extension caused by excess PTHrP production within the end buds of our transgenic mice. These results are also consistent with previous experiments in which slow-release pellets of PTHrP implanted into the mammary fat pad stalled the advance of the terminal end buds in wild-type mice treated with estrogen and progesterone (Wysolmerski *et al.* 1995). In the pubertal mammary gland, the *PTHrP* gene is most highly expressed within the cap cells of the terminal end buds, and the *PTH-1R* gene is most highly expressed in the stroma immediately surrounding these structures (Dunbar *et al.* 1998). Although epithelial cells in TEBs do not appear to express the PTH-1R, these are the cells the proliferation, apoptosis, or both, of which is affected by PTHrP overexpression. Therefore, it would appear that PTHrP most probably acts indirectly, via the stromal cells. We believe that our results raise the possibility that PTHrP might normally act to modulate the effects of steroid hormones on the stromal cells surrounding the end buds, thereby restraining ductal extension. However, confirmation of this hypothesis will require the demonstration of reciprocal changes in TEB cell turnover in the absence of PTHrP expression.

Consistent with the above hypothesis, the linear growth of the mammary ducts during puberty appears to be primarily a function of estrogen acting on the stromal cells of the mammary fat pad (Silberstein *et al.* 1994, Bocchinfuso & Korach 1997, Cunha *et al.* 1997). It is believed that estrogen modulates signaling by local growth factor networks responsible for governing cellular proliferation and apoptosis within the terminal end buds (Imagawa *et al.* 1990). Several signaling systems have been implicated as potential mediators of the effects of estrogen. These include the epidermal growth factor (EGF) family of ligands and receptors (Ankrapp *et al.* 1998, Sebastian

et al. 1998, Luetkeke *et al.* 1999, Weisen *et al.* 1999), insulin-like growth factor-1 (Kleinberg *et al.* 2000) and hepatocyte growth factor/scatter factor (Yang *et al.* 1995). In addition, transforming growth factor (TGF)- β has been shown to be a potent inhibitor of end bud proliferation and ductal growth (Daniel *et al.* 1989, Robinson *et al.* 1991, Pierce *et al.* 1993). Furthermore, overexpression of TGF- β in the mammary glands of transgenic mice produces a phenotype that is remarkably similar to that of the K14-PTHrP and K14-tTA/Tet_O-PTHrP mice (Pierce *et al.* 1993). If PTHrP does act in a negative feedback loop to restrain the proliferative effects of estrogen, one might expect that expression of PTHrP or its receptor would be modified by estrogen. Although estrogen has been reported to upregulate, downregulate or not change the concentrations of PTHrP in breast cancer cell lines (Kurebayashi & Sonoo 1997, Funk & Wei 1998, Sugimoto *et al.* 1999), we have found no evidence that hormone treatment affects levels of whole gland PTHrP or PTH-1R mRNA in early adolescent mice (unpublished results). However, it has been reported that the production of PTHrP by normal mammary epithelial cells in culture is enhanced by treatment with either EGF or TGF- β (Ferrari *et al.* 1992, 1994, Sebag *et al.* 1994, Yin *et al.* 1999). More research will be needed to determine where PTHrP signaling might fit in the hierarchy of changes induced by steroid hormones in the terminal end buds.

In our original description of the K14-PTHrP mammary phenotype, we felt that the delay in lobuloalveolar development that we observed was a function of a defect in terminal duct formation, rather than a defect in lobuloalveolar differentiation *per se* (Wysolmerski *et al.* 1995). Our observations in the K14-tTA/Tet_O-PTHrP double transgenic mice support this interpretation. If we delayed the onset of transgene expression until after the duct system was formed, PTHrP overexpression had no effect on the development of lobuloalveolar structures during pregnancy. This suggests that the defects that we observed during pregnancy in the single transgenic mice were indeed related to pre-existing abnormalities in the ductal tree. We conclude that overexpression of PTHrP, at least within myoepithelial cells, does not inhibit lobuloalveolar differentiation.

In summary, we have developed a tetracycline-regulated PTHrP transgene that has allowed us to investigate the effects of PTHrP overexpression at specific times in the life cycle of the mammary gland. Using this approach we have learned that transient overexpression of PTHrP during embryonic mammary development reduced the capacity of the growing ducts to form side branches during puberty. In addition, overexpression of PTHrP during puberty itself slows the rate at which mammary ducts grow through the fat pad. This inhibition of ductal elongation is associated with an increase in the rate of cell death in terminal end buds and resistance to

the proliferative and anti-apoptotic effects of exogenous hormones on end bud cells. Our results suggest that PTHrP may function in terminal end buds as a natural inhibitor of hormone-induced ductal growth during puberty.

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