Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was originally discovered as the tumor product that causes humoral hypercalcemia of malignancy. PTHrP is now known to be widely expressed in many normal fetal tissues where it may participate in the regulation of organogenesis. In this report, we document that overexpression of PTHrP in myoepithelial cells in the mammary glands of transgenic mice resulted in a form of breast hypoplasia characterized by a profound defect in branching morphogenesis of the developing mammary duct system. In addition, transgenic mice manifested a defect in lobuloalveolar development during pregnancy that seemed to be, in part, the consequence of an impaired ability to form terminal ducts in response to estrogen and progesterone stimulation. The effects of PTHrP on branching morphogenesis during breast development appeared to be the result of amino-terminal PTH-like sequences that signal through the PTH/PTHrP receptor, since overexpression of parathyroid hormone itself in the mammary glands of transgenic mice caused a similar developmental phenotype, and delivery of PTHrP (1-36) via locally implanted slow-release pellets impaired breast development in normal mice. These results suggest that PTHrP, which is a native product of mammary epithelial and myoepithelial cells may participate in normal breast development, perhaps as a locally secreted growth inhibitor.

Key words: epithelial-mesenchymal interaction, organogenesis, calciotropic hormones, myoepithelial cells, PTH/PTHrP receptor, transgenic mice

SUMMARY

Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development

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1993) and two studies have detected biochemical responses to PTHrP specifically in myoepithelial cells (Seitz et al., 1993; Ferrari et al., 1993). Thus, it appears that the mammary gland produces PTHrP during several developmental stages and that myoepithelial cells have the capacity both to produce and to respond to PTHrP.

There have been several proposals as to the function of PTHrP in mammalian physiology (reviewed in Broadus and Stewart, 1994). These include suggestions that PTHrP might contribute to either maternal or neonatal calcium homeostasis during lactation or that the peptide might regulate mammary blood flow and/or myoepithelial cell tone during lactation. In addition, given the expression of PTHrP in the non-lactating mammary gland as well as the growing recognition of PTHrP’s developmental effects in other tissues, it has been suggested that PTHrP might function in the regulation of growth and development of the mammary gland.

We recently reported that overexpression of PTHrP directed to the basal keratinocytes of transgenic mice by the keratin 14 (K14) promoter resulted in a severe disturbance in the development of hair follicles (Wysolmerski et al., 1994). In the process of consolidating breeding colonies, we noticed that the offspring of some transgenic females suffered from a high rate of perinatal mortality and that the dead pups had no milk in their stomachs. In addition, pups from the litters of other transgenic females were born frequently at an unexpectedly low birth weight and were likely to develop stunted growth and development of the mammary gland. This latter phenomenon characterized by a severe defect in branching morphogenesis.

**MATERIALS AND METHODS**

**Generation and identification of transgenic mice**

K14-PTHrP mice were generated and identified as previously reported (Wysolmerski et al., 1994). We analyzed two true-breeding lines, both having similar phenotypes. The K14-PTH transgene was constructed by replacing the cDNA fragment encoding human PTHrP (1-141) in the original K14 transgene with a 539 bp Ddel-HindlI cDNA fragment encoding the pre-pro and coding sequences for human PTH (1-84) (kindly provided by H. Kronenburg, Boston, MA; Hendy et al., 1981). Transgenic mice were generated in standard fashion and we obtained independent true-breeding lines. K14-PTH transgenic mice were identified by PCR amplification of a 171 bp sequence contained within the growth hormone portion of the transgene; genomic DNA integrity was confirmed by PCR amplification of a 171 bp sequence contained within the murine GAPDH gene. Growth hormone primers were 5'CTACGGGCTGCTCTACTGCTTCAGG and 3'GGCAGCAGTGAGTGCCAATTCAGGA. GAPDH primers were 5'CGTGAGGTCCTGCTCAGGTCTCCAG and 3'GATGGCATGAGCAGCTCAGG.

**Analysis of transgene expression**

Total cellular RNA was prepared from breast tissue or cells using a modification of the guanidinium thiocyanate/cesium chloride method and was analyzed by RNase protection assay as previously described (Daifotis et al., 1992). The cRNA probes protected the following sequences: (1) a 307 bp band corresponding to a PvuII-SaeI cDNA fragment of the human PTHrP gene, (2) a 229 bp band corresponding to a Ddel-SacI cDNA fragment of the human PTH gene (cDNA kindly provided by H. Kronenburg, Boston, MA), (3) a 220 bp band corresponding to a Sau3a-Sau3a cDNA fragment of the mouse cyclophilin gene (cDNA courtesy of J. Sutcliffe, LaJolla, CA), and (4) a 400 bp band corresponding to a PstI-PstI cDNA fragment of the mouse K14 gene (cDNA provided by J. Compton, Bethesda, MD).

Levels of immunoreactive and bioactive PTHrP 1-74 or PTH 1-84 were measured in acid-urea extracts of whole breast tissue or in conditioned media from breast cells in primary culture. Acid-urea extracts were prepared as previously described (Weir et al., 1988) and cells were cultured as described below. PTHrP 1-74 was assayed in an immunoradiometric assay with a sensitivity of 1pM (Burtis et al., 1990), and PTH 1-84 was measured with a commercial immuno-radiometric kit (Nichols Institute, San Juan Capistrano, CA) with a sensitivity of 0.7 pM. Bioactive PTHrP and PTH levels were measured in the rat osteosarcoma cell (ROS) assay (sensitivity 50 pM) (Weir et al., 1988). This method uses a PTH-sensitive ROS cell line (172/8) to measure the generation of cAMP in response to binding to PTH receptors. PTH and PTHrP concentrations reported for the ROS assay represent equivalency units derived from a standard curve employing human PTH 1-34. Results from immunoassays and the ROS assay were normalized for cell number (conditioned media) or for total protein content (tissue extracts). Total protein concentrations were determined using a commercial kit (Bio-Rad, Hercules, CA).

**Mammary cell culture**

8- to 10-week-old transgenic and normal virgin mice were killed and their 4th inguinal mammary glands were removed. Tissue was minced with sterile scissors and digested in DMEM/F12 containing 0.2% dispase grade II, 0.2% collagenase type III, 5% FBS, 50 μg/ml gentamycin, 100 units/ml nystatin, and 2.5 μg/ml amphotericin B for 10-12 hours at 37°C. Cells were then pelleted, washed twice with media (see below) and filtered through nitex mesh with a pore size of 530 μm, after which they were plated on plastic and fed with DMEM supplemented with 5% FBS, 1% glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 units/ml nystatin, 2.5 μg/ml amphotericin B, and 5 μg/ml each of insulin, hydrocortisone and prolactin. Cells were cultured at 37°C in 5% CO2, and media changed every 2-3 days. 24 hour conditioned media for assay were collected from confluent 75 cm2 flasks.

**Histological analysis**

Breast tissue was prepared for whole-mount analysis in the following fashion. Mice of appropriate ages were killed and their 4th inguinal mammary glands were removed in toto. The tissue was fixed on microscope slides by immersion in acid ethanol for 1 hour, washed in 70% ethanol and distilled water and then incubated in carmine aluminum stain (0.2% carmine, 0.5% aluminum potassium sulfate) for 10-12 hours at room temperature. After staining, the slides were dehydrated through graded ethanol and sequentially defatted in acetone and toluene before being mounted under glass coverslips with Permount (Fisher Scientific, Fair Lawn, NJ). For light microscopy, mammary tissue was fixed in 4% paraformaldehyde for 10-12 hours at 4°C, embedded in paraffin, and sectioned at 5 μm for H&E staining.

**Immunohistochemistry**

For PTHrP staining, animals were perfused with 4% paraformaldehyde in PBS for 10 minutes via intracardiac puncture. Following this, the 4th inguinal mammary glands were dissected, cut into 4-5 mm pieces and fixed in 4% paraformaldehyde for 10-12 hours at 4°C. For K14 and α-actin staining, the 4th gland was dissected fresh and fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) for 10-12 hours at 4°C. All tissue was embedded in paraffin and immunohistochemistry was performed on 5 μm sections. Sections were incubated with primary antibodies overnight at 4°C and staining was developed using the avidin-biotin peroxidase technique, employing commercially available reagents (Vectastain ABC kit,
Vector Laboratories, Burlingame, CA and ImmunoPure Metal Enhanced DAB, Pierce, Rockford, IL). PTHrP antibodies were raised in sheep and were affinity-purified for anti-PTHrP 1-36. The K14 antisera was raised in rabbits and was the generous gift of Dr Dennis Roop (Houston, TX). Antiserum to α-actin was a monoclonal antibody (Clone 1A4) purchased from Sigma (St Louis, MO).

**Slow-release pellets**

All pellets were cholesterol-based and were purchased from Innovative Research of America (Toledo, OH). Estrogen and progesterone pellets were surgically inserted subcutaneously in the region of the interscapular skin fold. They were designed to release 1.2 mg of progesterone and 24 μg of 17-β-estradiol per day for 21 days. PTHrP or placebo pellets were inserted into the 4th inguinal mammary gland fat pad, just in advance of the growing duct system of 4-week-old normal mice. Each PTHrP pellet contained 50 μg of PTHrP 1-36 and was designed to release approximately 2.4 μg of PTHrP into the local microenvironment daily for 21 days. Placebo pellets consisted of the pellet matrix without PTHrP.

**RESULTS**

The K14-PTHrP transgene is expressed in breast tissue

The observation of fostering difficulties in the K14-PTHrP mice suggested to us that, in addition to the epidermis, the K14-PTHrP transgene also might be expressed in mammary tissue. In fact, keratin 14 has been reported to be expressed in myoepithelial cells in the murine mammary gland (Smith et al., 1990). We first tested for expression of the K14-PTHrP transgene in total cellular RNA prepared from whole mammary glands taken from female transgenic mice and their non-transgenic littermates. By RNase protection analysis, the transgene was indeed expressed in the breast tissue of transgenic mice (Fig. 1). Furthermore, the levels of transgene expression mirrored those of the native murine K14 gene during several stages of breast development (see Fig. 1), demonstrating that the 2.1 kb portion of the human K14 promoter contained within the transgene faithfully recapitulated the natural pattern of K14 gene expression in the mammary gland.

We next tested for PTHrP protein production in breast tissue taken from transgenic and control mice, using immunohistochemistry. Fig. 2A demonstrates the staining pattern seen with an antibody to K14, which revealed that K14 expression was limited to the peripherally located myoepithelial cells. An identical staining pattern was seen using an antibody to α-actin (not shown), confirming that the cells staining for K14 are myoepithelial in origin. Fig. 2B and D compare breast tissue from a transgenic mouse and its littermate, stained with an antibody to PTHrP. In the normal mouse, both luminal epithelial cells and myoepithelial cells stained weakly for PTHrP (Fig. 2D). However, in the transgenic gland (Fig. 2B) there was a clear augmentation of staining in a peripheral pattern similar to that seen with the K14 antisera (compare Fig. 2A and B). On close inspection, PTHrP immunoreactivity appeared to be located in both myoepithelial cells and the basement membrane of the transgenic ducts. There also appeared to be some patchy accumulation of PTHrP peripherally in the basement membrane of the normal duct (see Fig. 2D). These results are consistent with the expected overexpression of transgene-derived PTHrP in myoepithelial cells of the K14-PTHrP mice.

In order to quantitate the level of PTHrP expression in the mammary glands of the transgenic mice, we prepared acid-urea extracts of breast tissue harvested from virgin mice killed between the ages of 8 and 12 weeks. In addition, we grew mammary epithelial cells in primary culture and measured PTHrP secretion into 24-hour conditioned media (although these were mixed cultures, as assessed by K14 staining, the proportion of myoepithelial cells was the same in cultures derived from transgenic versus normal glands (not shown)). The acid-urea extracts prepared from breast tissue interfered with the immunoassay, but we could detect bioactive PTHrP using a PTH-sensitive rat osteosarcoma cell (ROS) assay. Using this technique, we found that extracts of transgenic breast tissue contained 100 fmol of PTHrP per mg total protein, while normal breast tissue was below the detection limit of this assay. Likewise, conditioned media taken from transgenic breast cells in culture contained a mean of 363 pM of PTHrP 1-74 by immunoradiometric assay (range: 247-480 pM) and 1430 pM of PTHrP in the ROS assay (range: 1104-1759 pM). Normal breast cells in culture produced PTHrP at levels below the detection limits of both assays.

**Overexpression of PTHrP interferes with ductular and lobuloalveolar development**

The growth and development of ductular and alveolar structures in the mouse mammary gland occurs in three distinct stages: during embryogenesis, during sexual maturation and during pregnancy/lactation (Daniel and Silberstein, 1987). With this in mind, we examined mammary glands removed from either transgenic mice or their age-matched littermates at time points representing: (a) preadolescence (18-21 days), (b) adolescence (6-8 weeks), (c) sexual maturity (10-12 weeks), (d) pregnancy and (e) lactation.
Fig. 2. Immunohistochemical localization of K14 and PTHrP in breast tissue from K14-PTHrP transgenic mice and their littermates. (A) Section of breast tissue taken from a 6-week-old virgin transgenic mouse stained with K14 antisera at a 1:500 dilution. Staining was limited to the peripherally located myoepithelial cells. (B) Section of breast tissue taken from a 6-week-old virgin transgenic stained with PTHrP antisera at a 1:10 dilution. Note that the heaviest staining is located at the periphery of the duct, in myoepithelial cells and in the basement membrane. (C) Section of breast tissue taken from a 6-week-old virgin transgenic, stained with a 1:10 dilution of PTHrP antisera that had previously been preincubated with 10^{-6} M PTHrP (1-36). Note that competition with PTHrP completely abolished the staining pattern seen in panel B. (D) Section of breast tissue taken from a 6-week-old normal littermate mouse and stained with antisera to PTHrP at a 1:10 dilution. Although luminal epithelial cells in normal ducts (D) and transgenic ducts (B) stain for PTHrP with similar intensity, there is clear overexpression of PTHrP in myoepithelial cells in transgenic (B) as compared to normal (D) ducts. Scale bar represents 18 m̅ for each panel.

Fig. 3 illustrates the severe defect in branching morphogenesis seen in the K14-PTHrP mice during sexual maturation. The mammary duct system at 18-21 days of age in transgenic mice was similar in both size and branching to that seen in their littermates (not shown). However, by 6 weeks of age there was a dramatic difference in both the degree of growth and branching of the ducts in the transgenic gland (compare Fig. 3A and B). At this age, the duct system in the normal littermates had consistently neared the borders of the mammary fat pad and had a highly branched organization (Fig 3A). In contrast, in the transgenic animals the overall growth of the duct system into the fat pad appeared to be delayed, and the transgenic gland exhibited a much simpler, sparser duct structure than the control gland, with a dramatic reduction in the degree of side branching and many fewer small tertiary and quaternary ducts. This reduction in side branching was even more striking at higher magnification, as seen in Fig. 3C and D. Although by 10-12 weeks of age the transgenic duct system...
had grown nearly to the borders of the fat pad, the defect in side branching persisted (compare Fig. 3E and F).

The K14-PTHrP mice also demonstrated alterations in lobuloalveolar development during pregnancy. As shown in Fig. 4A, by day 17 of pregnancy, the normal mammary gland has developed a characteristically dense network of well-formed alveoli. In contrast, the transgenic gland appeared to have many fewer, less well-developed alveoli (see Fig. 4B). On histological examination of H&E-stained sections, the individual alveoli in the transgenic gland appeared normal in their overall architecture (Fig. 4D), but there appeared to be fewer clusters of alveoli, and the individual acini were smaller and less well expanded as compared to the normal gland (Fig. 4C). These differences persisted into lactation, at which point the expansion of alveoli due to the secretory state obliterated the ability to discern lobular architecture in whole mounts prepared from mammary glands of normal mice (Fig. 4E). In contrast, in the transgenic glands, there were reduced numbers of alveoli, allowing one to define individual lobules on whole mounts (see Fig. 4F). As was the case during pregnancy, histological review of conventional H&E sections demonstrated reduced numbers, but normal-appearing individual secretory alveoli in the transgenic glands (not shown).

Overexpression of PTHrP inhibits terminal duct development in early pregnancy

Given the profound defect in side-branching during ductular proliferation in the K14-PTHrP mice, we asked if the changes in lobuloalveolar development that we noted during late pregnancy were a consequence of an impairment in terminal duct development (a necessary prelude to lobuloalveolar development) that occurred during early pregnancy. We approached this question in two ways. First, we examined the mammary glands of transgenic mice and their littermates in the first week of pregnancy. Second, we administered systemic estrogen and progesterone to virgin mice in order to stimulate terminal duct proliferation. As expected, both of these analyses revealed a significant defect in the induction of terminal ducts in the transgenic animals. Fig. 5A and B compares whole mounts taken from a transgenic mouse (Fig. 5B) or control (Fig. 5A) on day 7 of pregnancy. As can be seen, the transgenic glands showed reduced numbers of small terminal ducts as compared to the normal duct system. Similarly, in virgin mice exposed to 7 days of systemic estrogen and progesterone the normal duct system (Fig. 5C) demonstrated a robust growth of terminal ducts. In contrast, the transgenic ducts were nearly devoid of terminal ducts (see Fig. 5D) and thus appeared to be almost completely resistant to the effects of the estrogen and progesterone.

Overexpression of parathyroid hormone in breast tissue causes defects in branching morphogenesis during mammary gland development

PTH and PTHrP share a short stretch of amino-terminal homology that allows each protein to bind and to activate a common PTH/PTHrP receptor (see Fig. 6) (Broadus and Stewart, 1994). Although PTHrP may exert many of its physiological effects through the actions of this receptor, there is also evidence that PTHrP may be processed to give rise to several biologically active peptides that would presumably signal through other than the conventional PTH/PTHrP receptor (Broadus and Stewart, 1994). In order to test whether amino-terminal PTHrP, acting through the PTH/PTHrP receptor, was responsible for the mammary gland phenotype in our K14-PTHrP mice, we performed a second transgene experiment in which PTH itself was overexpressed using the keratin-14 promoter.

As detailed in Materials and Methods, we inserted a cDNA

Fig. 4. Whole-mount analysis of 4th inguinal mammary glands taken from K14-PTHrP transgenic mice (B,D,F) and their normal littermates (A,C,E) during pregnancy and lactation. (A) A normal mouse at day 17 of pregnancy; (B) a transgenic mouse on day 17 of pregnancy. (C,D) Conventional sections (5 μm) of breast tissue taken from a transgenic mouse (D) or a normal littermate mouse (C) at day 12 of pregnancy and stained with hematoxylin and eosin. Note that the transgenic mice (B,D) have a reduced density of alveoli that appear somewhat less expanded than those in the normal mice (A,C). (E,F) Whole mounts prepared from lactating mammary glands taken from a transgenic mouse (F) or normal control (E). Note the reduced mass of secretory alveoli in the transgenic gland. Scale bar represents 250 μm in A,B; 66 μm in C,D and 2.5 mm in E,F.
fragment encoding human pre-pro PTH (1-84) into the K14 cassette used previously and generated three true-breeding lines of K14-PTH mice. Each line expressed the transgene RNA in breast tissue (not shown), and each had a similar phenotype (see below). We analyzed breast tissue and breast cells from these mice for PTH content in a fashion similar to our analysis in the K14-PTHrP mice. Acid-urea extracts prepared from breast tissue taken from 8- to 12-week-old virgin transgenic mice contained 13 fmol per mg total protein of PTH in the ROS assay, and conditioned media from breast cells grown in primary culture contained 240 pM of bioactive PTH in the ROS assay (range: 75-396 pM) and 56 pM PTH 1-84 by immunoradiometric assay (range: 38-75). These values were some one-tenth to one-fifth those seen in the K14-PTHrP mice (see above).

The K14-PTH mice had only minor and inconsistent changes in their coat, but demonstrated a clear mammary gland phenotype. Fig. 7 compares whole mounts of breast tissue taken from 8- to 12-week-old virgin transgenic mice contained 13 fmol per mg total protein of PTH in the ROS assay, and conditioned media from breast cells grown in primary culture contained 240 pM of bioactive PTH in the ROS assay (range: 75-396 pM) and 56 pM PTH 1-84 by immunoradiometric assay (range: 38-75). These values were some one-tenth to one-fifth those seen in the K14-PTHrP mice (see above).

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Locally released PTHrP 1-36 impedes breast development in normal mice

Given the findings in the K14-PTH mice, we sought to confirm that amino-terminal PTHrP would inhibit ductular proliferation in mammary tissue by testing the effects of locally delivered PTHrP (1-36) in normal mice. We surgically implanted slow-release pellets containing PTHrP (1-36) into one 4th inguinal mammary gland of eight normal 3- to 4-week old mice, just ahead of the growing duct system. Into the same mice we placed a placebo pellet in an analogous position in the contralateral 4th mammary gland. Four mice also received slow-release pellets delivering systemic estrogen and progesterone. The mice were killed after 2 weeks of exposure to the pellets, and their 4th mammary glands were processed for whole-mount analysis in order to determine if the pellets had an effect on the continued growth of the mammary duct system.

In the mice that did not receive systemic hormones, the PTHrP pellets appeared to exert only subtle effects on duct growth over the course of the experiment (not shown), but, as shown in Fig. 8, if duct development was accelerated by the systemic delivery of estrogen and progesterone, the PTHrP pellets exerted a consistent and profound effect on the growth of the duct system. As shown, in all four mice, although there appeared to be little effect on branching, the growth of the duct system into the fat pad appeared to be slowed by pellets con-
taining PTHrP but was unhindered by the presence of placebo pellets (compare Fig. 8A and B). Thus, delivery of PTHrP (1-36) to the breast microenvironment by pellet seemed to exert an inhibitory effect on ductular proliferation in much the same fashion as did delivery of PTHrP (1-141) to the myoepithelial cells in the transgenic mice.

**DISCUSSION**

The development of the mouse mammary gland occurs in distinct stages (reviewed in Daniel and Silberstein, 1987; Topper and Freeman, 1980), each of which depends on a bidirectional interaction of the mammary epithelium with the stromal components of the mammary fat pad (Daniel and Silberstein, 1987). These interactions, in turn, are regulated by the coordinated responses of both epithelium and stroma to systemic hormones, locally derived growth factors and the extracellular matrix (Daniel and Silberstein, 1987; Topper and Freeman, 1980).

In this report, we document that overexpression of PTHrP in myoepithelial cells of the murine breast results in an impairment of mammary gland development during adolescence and early pregnancy. During sexual maturation, K14-PTHrP mice manifest a slower rate of ductal elongation, as well as a marked inhibition of side branching. There also appear to be changes in lobuloalveolar development in the transgenic mice characterized by the delayed formation of a reduced number of alveoli. It is difficult to discern whether this latter defect is simply the result of a reduced ductal mass or whether overexpression of PTHrP also impairs lobuloalveolar development per se, especially since K14 expression has been detected in developing alveolar cells in early pregnancy (Smith et al., 1990). However, our observations that the transgenic mice display an impairment in the development of terminal ducts in early pregnancy as well as in response to systemic estrogen and progesterone in virgin animals suggest that the impairment in lobuloalveolar development may follow largely from a reduction in ductular development. These defects appear to result from the actions of amino-terminal PTHrP on the PTH/PTHrP receptor, as overexpression of PTH in transgenic mice impairs ductal elongation, and branching and local delivery of PTHrP 1-36 to the mammary glands of normal mice antagonizes ductal elongation.

The results described above clearly demonstrate that overexpression of PTHrP can inhibit ductal proliferation and branching. Previous studies have suggested that mammary epithelial cells elaborate some growth inhibiting factor(s) that helps to maintain the proper spacing of ducts within the gland (Faulkin and Deome, 1960), so that it is interesting to speculate as to whether PTHrP might normally play a role as a locally active mammary growth inhibitor. The exact cellular pattern of PTHrP expression in the developing mammary gland remains unclear, so that it is difficult to discern how closely the pattern of PTHrP expression in the K14-PTHrP mice might mimic that of normal mice. However, as noted in the Introduction, PTHrP is present in the developing mammary gland and has been localized to both luminal and myoepithelial cells in the virgin mammary ducts as well as to the developing alveolar cells during pregnancy (Liapis et al., 1993; Grone et al., 1994; Rakopoulos et al., 1992). Receptors for PTHrP exist on mammary cells (Urena et al., 1993), and myoepithelial cells have been shown to respond to both PTHrP and PTH (Seitz et al., 1993; Ferrari et al., 1993). Further, preliminary experiments in our laboratory indicate that PTH/PTHrP receptor mRNA is present in both mammary epithelial cells and mammary stromal cells in culture (unpublished observations). Therefore, it would appear that we have targeted a mammary compartment that normally produces PTHrP and that has the ability to respond to PTHrP. Furthermore, the presence of PTH/PTHrP receptors on myoepithelial cells is noteworthy, as morphological studies have suggested that these cells are involved in generating side branches during ductal development, perhaps by dedifferentiating to form cap cells for the lateral bud (Williams and Daniel, 1983). It is possible that the mammary defects seen in the K14-PTHrP mice result in large part from autocrine effects of the excess PTHrP on myoepithelial cell function. Finally, although it is not known whether estrogen and/or progesterone alter PTHrP secretion by mammary epithelial cells, from the results of our pellet experiments, it appears that PTHrP can antagonize ductal elongation induced by systemic estrogen and progesterone in normal mice.

**Fig. 7.** Whole-mount analysis of mammary gland morphology in K14-PTH transgenic mice. (A,B) Whole mounts prepared from the 4th inguinal mammary glands taken from an 8-week-old K14-PTH transgenic mouse (B) and her normal littermate (A). (C,D) Higher magnification views of the same mammary glands depicted in A and B: C is from the normal gland and D is from the transgenic gland. Note that K14-PTH mice display the same defects in duct elongation and branching as the K14-PTHrP mice (compare with Fig. 3). Scale bar represents 4.3 mm in A,B and 55 µm in C,D.
Overexpression or underexpression of several other molecules in the mammary gland results in an inhibition of breast development. The most severe phenotype has been reported with the disruption of the lymphoid enhancer-binding factor 1 (LEF-1) gene by homologous recombination (vanGelder et al., 1994). LEF-1-deficient mice lack mammary glands, apparently due to an early failure of mammary bud formation. Lack of colony stimulating factor-1 in the op/op mouse has been reported to cause some impairment in ductal development but seems to exert its major effect on lobuloalveolar development (Pollard and Hennighausen, 1994). Overexpression of whey acidic protein has also been reported to interfere with lobuloalveolar development during pregnancy and lactation (Burdon et al., 1991). Finally, overexpression of TGF-β using either the MMTV promoter or the whey acidic protein promoter has been found to inhibit ductular proliferation or lobuloalveolar development, respectively (Pierce et al., 1993; Jhappan et al., 1993). In fact, the phenotype of the MMTV-TGF-β mice is remarkably similar to what we observe in the K14-PTHrP mice during sexual maturation. Like K14-PTHrP mice, the MMTV-TGF-β mice display impairments in both ductal elongation and side branching. PTHrP has been shown to have TGF-β-like effects and shares with TGF-β the ability to increase fibronectin production in several cell types (Insogna et al., 1989; Eielson et al., 1994). It has been postulated that TGF-β 3 produced by myoepithelial cells may act to inhibit side branching by modulating the production of basement membrane components by these cells in an autocrine fashion (Robinson et al., 1991). It is possible that PTHrP may affect myoepithelial cell function in a similar manner. It is clear that the basal lamina of the virgin duct is important in regulating branching morphogenesis, for its disruption through overexpression of stomelysin-1 in mammary epithelial cells leads to a dramatic increase in sidebranching and premature lobuloalveolar development (Sympton et al., 1994). It is also possible that PTHrP may be a downstream effector of TGF-β’s actions in the mammary gland, as TGF-β has been found to upregulate PTHrP production by mammary epithelial cells in culture (Ferrari et al., 1992).

The K14-PTH mice recapitulated the mammary phenotype of the K14-PTHrP mice but had only very mild abnormalities in their coat. One possibility for this discrepancy is that the skin phenotype of the K14-PTHrP mice results from the actions of a portion of the PTHrP molecule that does not encompass the amino terminus and therefore does not act through the classical PTH/PTHrP receptor. However, it is perhaps more likely that PTH expression in the epidermis of the K14-PTH mice was below a threshold necessary to interfere significantly with hair follicle development. In the skin of these mice, there appeared to be only a 1.5- to 2-fold increase in bioactive PTH (by ROS assay) relative to the native bioactive PTHrP levels in control mice, whereas in K14-PTHrP skin we achieved a 10- to 12-fold level of overexpression (unpublished observations, Wysolmerski et al., 1994). We presently do not have a ready explanation for the differences in our ability to overexpress these two proteins in skin, but ongoing experiments are attempting to address this question.

Soon after the isolation of PTHrP and the characterization of its gene, it became evident that PTHrP was widely expressed in the fetus (Broadus and Stewart, 1994). In fact, PTHrP and the PTH/PTHrP receptor have been reported to be one of the earliest peptide hormone/receptor pairs to be expressed during development (de Stolpe et al., 1993). These observations have given rise to speculation that PTHrP may have important functions in development, and our observations on mammary gland development in the K14-PTHrP mice represent the third example where overexpression or underexpression of PTHrP has led to defects in organogenesis. We previously reported that overexpression of PTHrP in the epidermis of these same mice led to a failure and/or delay in the induction of hair follicle growth (Wysolmerski et al., 1994). Karaplis et al. (1994) have demonstrated that disruption of the PTHrP gene through homologous recombination is associated with disordered chondrocyte differentiation and premature calcification in the skeleton of PTHrP-deficient mice, resulting in perinatal death. In the aggregate, these experiments provide compelling support for the participation of PTHrP in developmental events during organogenesis in at least some of the many sites in which it is expressed during fetal life. It is our hope that further study of the mechanisms leading to the mammary gland abnormalities in these transgenic mice will provide insight into the natural role of PTHrP in mammary gland development as well as into its potentially broader roles in organogenesis.

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