

Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development

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SUMMARY

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product that causes humoral hypercalcemia of malignancy. PTHrP is now known to be widely expressed in normal tissues and growing evidence suggests that it is an important developmental regulatory molecule. We had previously reported that overexpression of PTHrP in the mammary glands of transgenic mice impaired branching morphogenesis during sexual maturity and early pregnancy. We now demonstrate that PTHrP plays a critical role in the epithelial-mesenchymal communications that guide the initial round of branching morphogenesis that occurs during the embryonic development of the mammary gland. We have rescued the PTHrP-knockout mice from neonatal death by transgenic expression of PTHrP targeted to chondrocytes. These rescued mice are devoid of mammary epithelial ducts. We show that disruption of the PTHrP gene leads to a failure of the initial round of branching growth that is responsible for transforming the mammary bud into the rudimentary

mammary duct system. In the absence of PTHrP, the mammary epithelial cells degenerate and disappear. The ability of PTHrP to support embryonic mammary development is a function of amino-terminal PTHrP, acting via the PTH/PTHrP receptor, for ablation of the PTH/PTHrP receptor gene recapitulates the phenotype of PTHrP gene ablation. We have localized PTHrP expression to the embryonic mammary epithelial cells and PTH/PTHrP receptor expression to the mammary mesenchyme using *in situ* hybridization histochemistry. Finally, we have rescued mammary gland development in PTHrP-null animals by transgenic expression of PTHrP in embryonic mammary epithelial cells. We conclude that PTHrP is a critical epithelial signal received by the mammary mesenchyme and involved in supporting the initiation of branching morphogenesis.

Key words: Epithelial-mesenchymal interaction, Branching morphogenesis, Ectodermal dysplasia, Genetic rescue, Keratin 14, Organogenesis, PTH/PTHrP receptor, Mammary mesenchyme

INTRODUCTION

Parathyroid hormone-related peptide (PTHrP) was initially isolated from tumors causing the paraneoplastic syndrome of humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). Its name reflects the fact that PTHrP and parathyroid hormone (PTH) are the products of genes that have diverged from a common ancestor (Broadus and Stewart, 1994). Unlike PTH, which is produced only by the parathyroid glands and circulates as a classic peptide hormone that regulates systemic calcium metabolism, PTHrP is produced by

a wide variety of fetal and adult tissues, does not circulate and exerts its actions locally (Broadus and Stewart, 1994). PTH and PTHrP retain a high degree of homology in their amino-terminal portions, and PTH and amino-terminal species of PTHrP have retained the use of a common G-protein-coupled receptor, the PTH/PTHrP receptor (Jüppner et al., 1991). PTHrP has also been shown to undergo post-translational processing to generate several other peptides, at least one of which has been demonstrated to have biological activity subserved by an as yet unidentified receptor distinct from the PTH/PTHrP receptor (Wu et al., 1996; Kovacs et al., 1996).

PTHrP has been implicated in the regulation of a variety of biological processes such as cell growth and differentiation, the regulation of pancreatic islet cell function, the regulation of smooth muscle tone and the facilitation of placental calcium transport (Philbrick et al., 1996). Although the exact physiological functions of PTHrP remain unclear in mature organisms, a series of recent experiments in transgenic mice has demonstrated that PTHrP serves important roles during fetal development. Disruption of the PTHrP and PTH/PTHrP receptor genes and overexpression of PTHrP in chondrocytes have shown that PTHrP regulates chondrocyte differentiation during endochondral bone formation (Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996; Weir et al. 1996). In the absence of PTHrP or the PTH/PTHrP receptor, chondrocytes appear to differentiate and ossify prematurely, resulting in a chondrodystrophy that leads to the neonatal death of the knockout mice. Overexpression of PTHrP in chondrocytes leads to the opposite phenotype, a profound delay in the differentiation of chondrocytes resulting in the birth of mice with a cartilaginous skeleton. In addition to effects in chondrocytes, PTHrP has been implicated as playing a role in epithelial-mesenchymal interactions during hair follicle and mammary gland development. Overexpression of PTHrP in keratinocytes (Wysolmerski et al., 1994) results in either a delay or failure of hair follicle initiation, and its overexpression in mammary myoepithelial cells (Wysolmerski et al., 1995) has been shown to impair mammary ductal development.

Shortly after its discovery, PTHrP mRNA was found to be expressed in the lactating mammary gland and PTHrP was found in high concentrations in milk (Thiede and Rodan, 1988; Budayr et al., 1989). The role of PTHrP during lactation remains obscure, but it is now clear that PTHrP is expressed at various stages during mammary gland development, and overexpression of PTHrP in myoepithelial cells has been shown to retard ductular growth and to impair side branching during sexual maturation as well as to inhibit the formation of terminal ductules during early pregnancy (Wysolmerski et al., 1995). In addition, PTHrP introduced directly into the mammary fat pads of normal mice has been shown to impair estrogen- and progesterone-induced ductular proliferation (Wysolmerski et al., 1995). Because the skeletal phenotypes of PTHrP underexpression and overexpression were exact opposites of each other, we hypothesized that PTHrP gene ablation might also lead to defects in ductular growth and or branching. However, because mammary development occurs to a great extent after birth, and because the PTHrP-knockout mice die at birth, in order to test this hypothesis, we needed to devise a strategy to rescue these mice from their neonatal demise. In this report, we describe our strategy for rescuing the PTHrP-null mice, and we demonstrate that PTHrP is essential for mammary gland development.

MATERIALS AND METHODS

Mouse strains and identification of knockout embryos.

The disrupted PTHrP allele (Karaplis et al., 1994) was progressively outbred onto a CD-1 background and mice heterozygous for this allele were mated to produce PTHrP-null embryos. The date of the appearance of a vaginal plug was considered to be day 0 of embryonic

life. Embryos were removed from the uterus and genotyped with respect to the presence or absence of neomycin gene sequences and the presence or absence of an intact PTHrP-coding region (exon IV) by PCR, utilizing the following primer sets: wild-type murine PTHrP gene – forward 5'-GCTACTGCATGACAAGGGCAAGTCC and reverse 5'-CATCACCCACAGGCTAGCGCCAAC (421 bp product), and bacterial neomycin gene – forward 5'-GGAGAGGCTATTCG-GCTATGAC and reverse 5'-CGCATTGCATCAGCCATGATGG (315 bp product). This allowed the identification of wild-type, heterozygous and homozygous PTHrP-null embryos. The disrupted PTH/PTHrP receptor allele was progressively bred onto a Black Swiss background, and homozygous-null embryos were produced and identified in like fashion (Lanske et al., 1996). The PTH/PTHrP receptor primer pair utilized for this purpose amplified a 270 bp portion of the PTH/PTHrP receptor gene and consisted of the following sequences: forward 5'-GCAGAGATTAGGAAGTCTTGGA and reverse 5'-AGCCGTCGT-CCTTGGGAAGTGT.

Col II-PTHrP/PTHrP-null mice were produced in the following fashion. The col II-PTHrP and PTHrP-null alleles were first bred onto a CD-1 background for several generations to minimize any potential exacerbating effects of their original different genetic backgrounds. Then col II-PTHrP transgenic mice were crossed to PTHrP-null heterozygotes to generate offspring carrying both the transgene and a PTHrP-null allele. These were again crossed to PTHrP-null heterozygotes to generate col II-PTHrP hemizygous, PTHrP-null homozygous mice (col II-PTHrP/PTHrP-null mice). The PTHrP-null allele was identified as outlined above. The col II-PTHrP transgene was identified in like fashion using the following primers that identified a 510 bp section of the murine procollagen II promoter/human PTHrP cDNA junction segment: forward 5'-TCTT-AGCATTCTTGGAAGAAC and reverse 5'-ATCAGATGGTGAAGG-AAG.

K14-PTHrP/PTHrP-null embryos were produced by mating K14-PTHrP transgenic hemizygotes (Wysolmerski et al., 1994) with mice heterozygous for the PTHrP-null mutation. Offspring of this cross that were both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null gene were then crossed to mice heterozygous for the PTHrP-null allele to produce mice homozygous for a disrupted PTHrP gene and hemizygous for the K14-PTHrP transgene. The K14 transgene was identified as previously described (Wysolmerski et al., 1995).

K14-PTHrP, col II-PTHrP/PTHrP-null (double rescue) mice were produced as follows. We first created K14-PTHrP hemizygous, PTHrP-null heterozygous and col II-PTHrP hemizygous, PTHrP-null heterozygous mice as described above. These mice were then crossed to one another to generate K14-PTHrP/col II-PTHrP double transgenic, PTHrP-null homozygotes (double rescue) mice. The various alleles were identified as outlined above.

Each of the various types of embryos was also sexed based on the presence or absence of a 240 bp band amplified from the SRY gene using the following primers: forward 5'-CGG-GATCCATGTCAAGCGCCCCATGAATGCATTTATG and reverse 5'-GCGGAATTCACCTTAGCCCTCCGATGAGGCTGATAT (Geise et al., 1994).

Histology/immunohistochemistry

Embryos were harvested by caesarean section and fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was then removed, and the embryonic mammary glands were identified using transmitted light and photographed under low magnification. Subsequently, the mammary glands were dissected from the ventral skin and embedded in paraffin. Serial 5 µm sections were cut and stained with hematoxylin and eosin for microscopic examination. Immunohistochemistry was performed using standard techniques. The mouse casein antibody is a rabbit polyclonal antibody (kind gift of B. Vonderhaar, NIH, Bethesda MD) and was used at a dilution of 1:200. All primary incubations were performed for 12 hours at 4°C,

and primary antibody binding was detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3' diaminobenzidine as a chromagen. Slides were counterstained using hematoxylin. Apoptosis was detected by terminal deoxynucleotidyl transferase labelling (TUNEL assay) employing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Mannheim, Germany).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed on 5 μ m paraffin sections of embryonic mammary glands as follows. Probes corresponded to a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the PTH/PTHrP receptor gene, as previously described (Weir et al., 1996). Sense and antisense riboprobes were generated from linearized fragments using an in vitro transcription kit (Promega, Madison, WI) in the presence of 35 S-UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 μ g/ml in PBS for 17 minutes at room temperature), and acetylated with 0.25% acetic anhydride in the presence of 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 minutes. Sections were then rinsed in 2 \times SSC and incubated for 30 minutes in 0.66% N-ethylmaleimide (Sigma Chemical Co., St Louis, Mo) in 2 \times SSC, rinsed again in 2 \times SSC, dehydrated in graded alcohol, treated with chloroform for 5 minutes, rehydrated and then air dried. The probes (1.5×10^7 cts/minute/ml) were then hybridized to the samples for 17 hours at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1 \times Denhardt's solution, 4 \times SSC, 250 μ g/ml tRNA, 100 μ g/ml salmon sperm DNA and 50 mM DTT. After hybridization, sections were rinsed in 1 \times SSC and washed twice in 2 \times SSC/50% formamide for 5 minutes at 52°C, rinsed in 2 \times SSC, and treated with 30 μ g/ml RNase A in 2 \times SSC at 37°C for 30 minutes. Following two rinses in 2 \times SSC, sections were again washed in 2 \times SSC/50% formamide at 52°C for 5 minutes, dehydrated through graded ethanol, air dried and dipped in a 1:1 mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development, sections were counterstained with hematoxylin and mounted for microscopic examination.

RESULTS

Col II-PTHrP rescued PTHrP-null mice lack mammary glands

Disruption of the PTHrP gene by homologous recombination resulted in defects in skeletal development including inappropriate ossification of the costal cartilage, resulting in a shield chest and respiratory failure (Karaplis et al., 1994). Most other tissues appear to have developed normally in these mice, but the neonatal death of these animals had precluded a full examination of the role of PTHrP in sites, such as the mammary gland, which develop after birth. Because overexpression of PTHrP via a procollagen II-PTHrP (col II-PTHrP) transgene produced a skeletal phenotype reciprocal to that seen in the PTHrP-knockout mice (Weir et al., 1996; Karaplis et al., 1994), we reasoned that delivery of PTHrP to chondrocytes, via this transgene, might rescue the skeletal phenotype of the PTHrP-knockout mice and allow these animals to survive beyond birth. Our goal was to produce a mouse that lacked PTHrP in all tissues except cartilage, where it would be supplied by the col II-PTHrP transgene. To this end, we bred the col II-PTHrP transgene onto a PTHrP-null background to produce col II-PTHrP/PTHrP-null mice. These mice survived to maturity but suffered from multiple

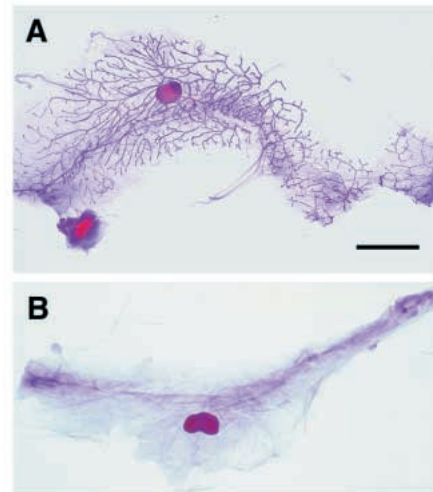


Fig. 1. Whole-mount analysis of mammary glands from col II-PTHrP/PTHrP-null mice and normal littermates. The fourth inguinal mammary glands were resected from 4-month-old normal and col II-PTHrP/PTHrP-null mice, fixed in acid ethanol and stained with carmine aluminum. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In contrast, the col II-PTHrP/PTHrP-null gland (B) is devoid of epithelial structures; only the lymph node and vasculature are present within the fat pad. These results are representative of the findings in 2 col II-PTHrP/PTHrP-null females. Scale bar represents 5 mm.

abnormalities including defects in the integument and its appendages, and failures of tooth eruption and mammary epithelial development, a phenotype reminiscent of the collection of human syndromes known as ectodermal dysplasias (Freire-Maia and Pinheiro, 1994). In this report, we detail the effects of the loss of PTHrP on mammary development.

Fig. 1 demonstrates the morphology of whole mammary glands taken from 4-month-old, female col II-PTHrP/PTHrP-null (Fig. 1B) and normal littermate (Fig. 1A) mice. As one can see, the mature virgin mammary gland (Fig. 1A) consists of a series of branched epithelial ducts filling out a specialized stromal compartment known as the mammary fat pad. In contrast, col II-PTHrP/PTHrP-null mice lacked any evidence of mammary epithelial ducts (Fig. 1B). The mammary fat pad and its vasculature appeared to form normally, but were devoid of any mammary epithelium. Furthermore, examination of the ventral epidermis failed to demonstrate any nipple structures. These data suggested that PTHrP is essential for the development of the mammary epithelial duct system and nipples.

Loss of PTHrP results in a failure of the mammary epithelial primary growth spurt

The formation of the embryonic murine mammary gland is essentially a two-step process. The first step, occurring between E10 and E12, is the formation of the mammary buds. In female mice, the mammary buds remain relatively quiescent until E16 when they begin the second step, an initial round of branching morphogenesis, which leads to the formation of a mammary duct system with approximately 15-20 branches by

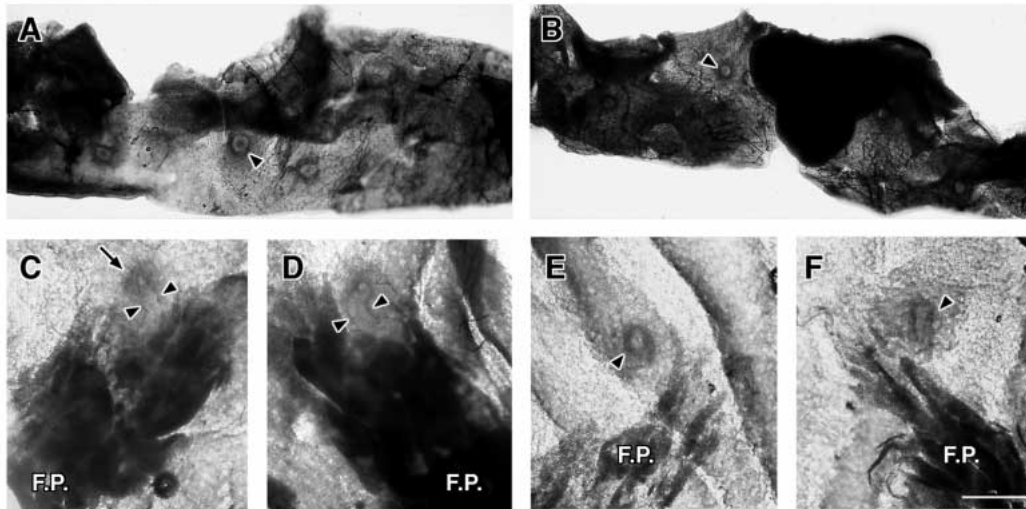


Fig. 2. Comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. The ventral epidermis was dissected from the respective embryos and photographed under low magnification using transmitted light in order to examine the gross structure of the embryonic mammary glands. (A,B) The mammary buds appear as round structures projecting upwards from the undersurface of the epidermis (arrowheads). Note that at E15, the mammary buds in the knockout embryos (B) appear similar to those in the normal embryos (A) (7 PTHrP-null female embryos analyzed, 6 wild-type embryos analyzed). In contrast, by E18 there is a dramatic difference in the appearance of the mammary structures in knockout (E,F) as compared to normal (C,D) embryos. At this point, the normal mammary structure consists of a developing nipple (dark halo, arrow in C) and an elongated primary duct (translucent tube-like structure between arrowheads in C,D) which is just beginning to form initial branches as it makes contact with the developing mammary fat pad (labelled F.P.). Note that in the PTHrP-knockout embryos (E,F), the mammary glands fail to elongate and remain bud-like (E) or slightly ectatic (F) in their appearance. There are no primary ducts that extend to the fat pads, and the developing fat pads (F.P.) themselves appear diminished in size (13 PTHrP-null female embryos examined, 6 wild-type embryos examined). Scale bar represents 160 μ m for all panels.

birth (Sakakura, 1987). The nipples of mice are formed on or about E18 as a circular invagination of the epidermis, referred to as the nipple sheath (Sakakura, 1987). Because col II-PTHrP/PTHrP-null mice lack nipples and given the timing of nipple formation at E18, we reasoned that the loss of epithelial ducts resulting from the lack of PTHrP most likely occurred during the embryonic development of the mammary gland. Therefore, we returned to the original PTHrP-knockout embryos and examined embryonic mammary gland growth at days E12-13, E15 and E18, and at birth.

Figs 2 and 3 demonstrate the gross and microscopic appearance, respectively, of the mammary rudiments from mice homozygous for a disrupted PTHrP gene as compared to their wild-type littermates. As shown in Figs 2A,B and 3A,B, the mammary buds appeared normal in PTHrP-null embryos at E15. This was also the case at E12-13 (data not shown). In contrast, there was a dramatic difference in the appearance of the PTHrP-knockout ducts as compared to those in wild-type embryos at E18. As seen in Fig. 2C,D, at this age in the normal embryos, the mammary bud has given rise to a primary duct, which has elongated to make contact with the developing mammary fat pad and has formed several initial branches. In the knockout embryos, the mammary buds failed to make this transition and appeared similar to those at E15 (Fig. 2E,F). Furthermore, the mammary fat pads, although present, appeared somewhat diminished in size in the knockout embryos.

On microscopic examination, one could see that, by E18, the normal ducts had extended into the lower dermis and had formed initial branches that could be seen amongst the

preadipocytes constituting the developing mammary fat pad (see Fig. 3C,D). At this point, the normal mammary glands also had well-developed nipple sheaths surrounding the origins of the primary ducts (Fig. 3C). In contrast, as seen in Fig. 3E, at E18 the PTHrP-knockout ducts appeared not to have undergone the primary growth spurt. Instead of extending to the fat pad and branching, epithelial ducts were uniformly found only in the upper portions of the dermis, where, typically, they were enveloped by a dense condensation of fibroconnective tissue. In addition, there was no evidence of nipple sheath development surrounding the origins of the epithelial ducts in the PTHrP-knockout embryos. When examined at higher magnification, the epithelial cells within the knockout ducts often appeared to be degenerating. As compared to normal epithelial cells (Fig. 3F), there was separation of the PTHrP-knockout epithelial cells (Fig. 3G) from the basement membrane, the cells borders were indistinct, and many nuclei appeared pyknotic. Consistent with this observation, by birth, there were only scattered remnants of degenerating mammary ducts that could be found on serial sectioning of the PTHrP-null embryos while, in wild-type embryos, the mammary ducts were firmly established within the mammary fat pad and had developed the expected branching pattern (data not shown). In summary, in the absence of PTHrP, mammary development proceeds normally through the mammary bud stage but subsequently falters as the buds fail to undergo the initial phase of branching morphogenesis and the mammary epithelial cells then degenerate.

PTHrP overexpression has been shown to delay chondrocyte differentiation and apoptosis, whereas disruption of the PTHrP

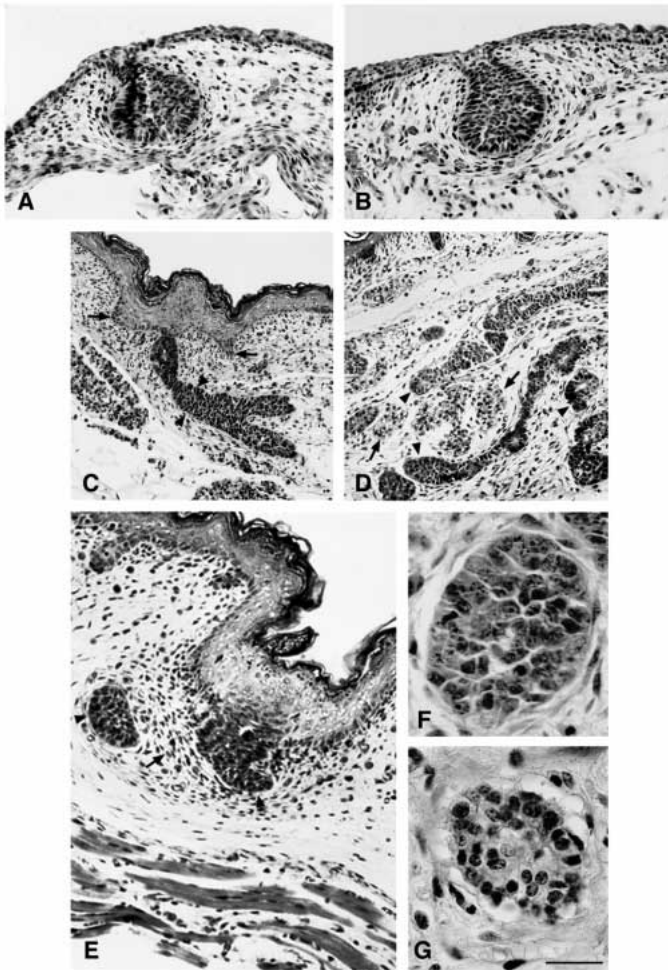


Fig. 3. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. (A,B) Photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15 (3 knockout and 3 wild-type embryos examined). At this stage, the mammary bud consists of an invagination of mammary epithelial cells surrounded by a condensation of mammary mesenchyme, and the microscopic appearance of the PTHrP-knockout buds was entirely normal. (C-G) Photomicrographs of H&E-stained sections through mammary glands dissected from PTHrP-knockout (E,G) and normal littermate (C,D,F) embryos at E18 (5 knockout and 4 wild-type embryos examined). In a normal embryo (C,D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad). In contrast, in the PTHrP-knockout embryos, (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). (F,G) High power photomicrographs of mammary epithelial ducts in cross-section taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that, in the knockout duct (G), the epithelial cells appear to be degenerating; many nuclei are pyknotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are separating from the basement membrane. Scale bar represents 16 μm in A,B; 25 μm in C,D; 17 μm in E; 5 μm in F,G.

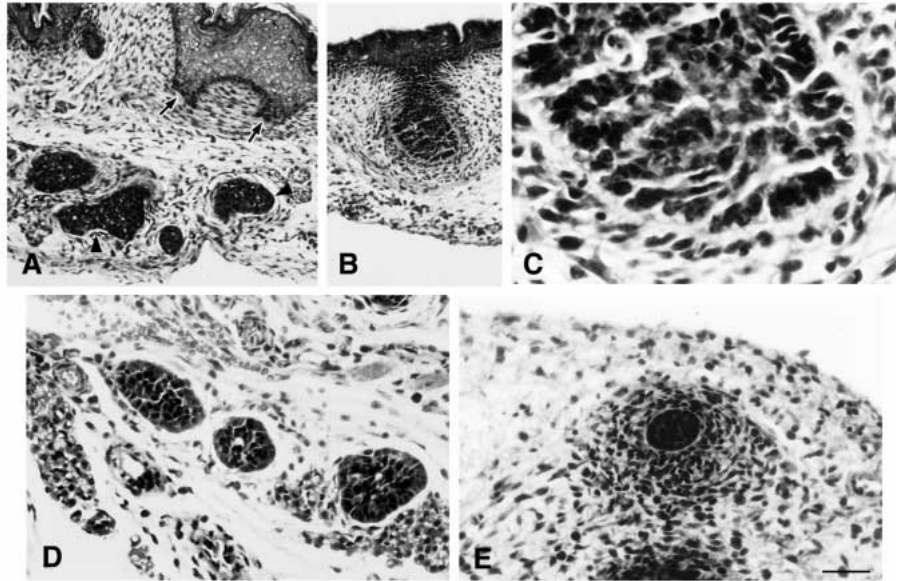
gene results in a form of growth failure associated with premature differentiation and apoptosis of chondrocytes in the growth plate of developing bones (Weir et al., 1996; Amling et al., 1997; Karaplis et al., 1994; Lee et al., 1996; Vortkamp et al., 1996). Given these findings and the apparent degeneration of the mammary epithelial cells in the PTHrP-knockout embryos, we examined these cells for evidence of apoptosis and/or inappropriate differentiation at E18 by TUNEL assay and by immunohistochemistry for β -casein. Although apoptotic cells were easily detected in normal ducts, there were no apoptotic cells within the knockout ducts (data not shown). Likewise, immunohistochemistry for β -casein revealed no evidence that the knockout mammary epithelial cells were undergoing premature cytodifferentiation; there was no staining for β -casein in either normal or knockout epithelial cells at E18 (data not shown). These data suggest that, unlike the events in cartilage, the failure of mammary development in PTHrP-knockout mice appeared neither to be associated with premature differentiation of the mammary epithelial cells nor with widespread apoptosis of these cells.

Ablation of the PTH/PTHrP receptor gene recapitulates the mammary phenotype of PTHrP-knockout mice

As mentioned in the Introduction, PTHrP gives rise to several biologically active peptides (Broadus and Stewart, 1994; Wu et al., 1996). In addition, it has been suggested that PTHrP may be targeted to the nucleus and exert biological activity via an intracrine pathway (Henderson et al., 1995). Previous experiments had implicated soluble, amino-terminal PTHrP acting via the PTH/PTHrP receptor as important in the regulation of branching morphogenesis in the mammary gland during sexual maturation and pregnancy (Wysolmerski et al., 1995). In order to determine if this was also the case during embryogenesis, we examined mammary gland development in PTH/PTHrP receptor-null embryos (Lanske et al., 1996) over the same time frame as in the PTHrP-null embryos.

Fig. 4 demonstrates the appearance of the mammary rudiment in PTH/PTHrP receptor knockout mice and control littermates. As seen in the PTHrP-knockout embryos, in the receptor-knockout mice, the primary round of branching morphogenesis failed, leading to the subsequent degeneration of the mammary epithelial ducts. Just as with the PTHrP knockouts, the mammary buds appeared to form appropriately in the receptor knockout mice (data not shown), but clear differences in the appearance of the receptor-knockout mammary rudiment as compared to normal littermates were apparent by E18. As shown in Fig. 4, by E18, the normal duct system (Fig. 4A,D) had grown to the fat pad and begun to branch, while the knockout mammary duct failed to elongate or branch and remained bud-like in its appearance (Fig. 4B). Examination at higher magnifications revealed that the mammary ducts in the receptor knockout mice (Fig. 4C,E) remained in the upper dermis, were enveloped within an abnormal condensation of stroma and appeared to be degenerating, a picture nearly identical to that seen with mammary ducts devoid of PTHrP (see Fig. 3). Furthermore, as with the absence of PTHrP, the receptor knockout embryos formed no nipple sheath (see Fig. 4B). Therefore, ablation of PTHrP or the PTH/PTHrP receptor led to the same phenotype,

Fig. 4. Histologic comparison of the embryonic mammary glands of PTH/PTHrP receptor-knockout and normal littermate embryos at E18. (A) Photomicrograph of H&E-stained sections of mammary rudiment from a normal littermate. Note the initial branches of the primary duct (arrowheads) within the lower dermis. Also, note the developing nipple sheath (arrow) (3 embryos examined). (B) Photomicrograph of H&E-stained sections of mammary rudiment from PTH/PTHrP receptor-knockout embryo (5 receptor-knockout embryos examined). Note that the mammary duct has not elongated, that the mammary rudiment remains bud-like in its appearance and that there is no nipple sheath. (C) Higher magnification of B. Note that the epithelial cells appear to be degenerating; there are many pyknotic nuclei and the cell borders are indistinct, similar to the appearance of the PTHrP-knockout epithelial cells at this time point. (D,E) H&E-stained cross-sections of epithelial ducts from normal (D) and PTH/PTHrP receptor-knockout (E) mammary glands at E18. Note the lacy, delicate appearance of the stroma surrounding the normal ducts (D) as they make contact with the mammary fat pad. In contrast, note the condensation of stroma surrounding a rare PTH/PTHrP receptor-knockout duct (E) that has attempted to grow out from the mammary bud. Scale bar represents 20 μm in A,B; 4.5 μm in C; 10.4 μm in D,E.



a failure of the initial phase of branching morphogenesis during embryonic mammary development.

Localization of PTHrP and PTH/PTHrP receptor gene expression during embryonic mammary gland development

We next determined the sites of PTHrP and PTH/PTHrP gene expression in normal Balb/c mammary rudiments from E12

through E18 by in situ hybridization. As shown in Fig. 5A-C, PTHrP mRNA expression in the developing mammary rudiment was limited to the epithelial cells, especially those cells located peripherally, adjacent to the basement membrane. PTHrP mRNA was also detected in keratinocytes within the epidermis as well as within developing hair follicles, although it appeared that the highest levels of expression were within the mammary epithelial structures. Expression of the PTHrP

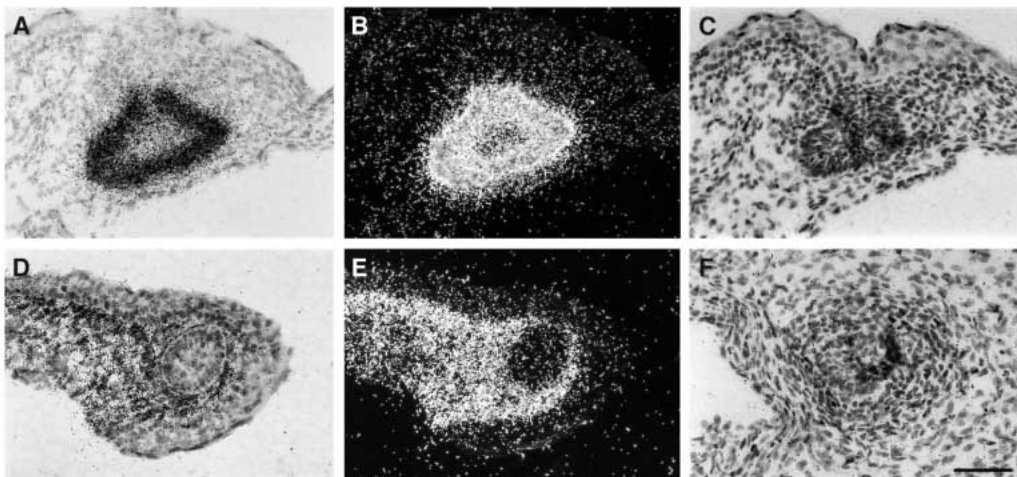


Fig. 5. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. (A-C) In situ hybridization for PTHrP mRNA in normal mammary rudiments at E16. (A,B) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. (C) Bright-field image of a similar section hybridized to sense probe as a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare A and C). (D-F) In situ hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. (D,E) Bright-field and dark-field images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. (F) Bright-field image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to D. Scale bar represents 15 μm for all panels.

gene did not appear to be induced at any specific point during the time period that we examined (E12-18). Rather, PTHrP mRNA was continuously expressed at high levels in mammary epithelial cells in the mammary bud as well as in the growing ducts during the initial phase of branching morphogenesis.

In contrast to the epithelial expression pattern seen for PTHrP, expression of the PTH/PTHrP receptor was limited to the mesenchyme. As seen in Fig. 5D-F, PTH/PTHrP receptor mRNA was expressed throughout the embryonic dermis, including the dense mammary mesenchyme. At E12-13, the expression of the receptor mRNA appeared to be fairly uniform throughout the dermal mesenchyme (data not shown), but, from E15 onward, there appeared to be more intense hybridization of the receptor antisense probe in the upper, more cellular dermis (Fig. 5E). At E18, at a point at which the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in the stromal cells surrounding the growing mammary ducts as they became surrounded by the developing fatty stroma (data not shown). As with PTHrP gene expression, the PTH/PTHrP receptor gene was expressed throughout the time frame examined, and there was not a specific point at which its expression appeared to be induced. Therefore, within the embryonic mammary gland, PTHrP and the PTH/PTHrP receptor appear to represent an epithelial/mesenchymal signalling unit in which PTHrP is produced by mammary epithelial cells and interacts with its receptor on mammary mesenchymal cells.

Transgenic expression of PTHrP rescues the mammary glands of PTHrP-knockout mice

We hypothesized that the failure of mammary development seen in the PTHrP and PTH/PTHrP receptor-knockout embryos was due to the loss of PTHrP-mediated paracrine signalling between the mammary epithelium and mammary mesenchyme. This working hypothesis suggested that reintroducing PTHrP into the local microenvironment of the mammary bud might prevent the failure of mammary development in these mice. Keratin-14 expression is known to be induced in embryonic skin beginning at E15-16 (Kopan and Fuchs, 1989), about the time of the primary growth spurt of the mammary rudiment. Furthermore, the keratin-14 gene had been shown to be expressed in epithelial cells in the adult mammary gland (Smith et al., 1990; Wysolmerski et al., 1995). Therefore, we examined K14 expression in the embryonic mammary gland and found that it was expressed uniformly in embryonic mammary epithelial cells beginning on or about E15 (data not shown). Since we had shown that the K14-PTHrP transgene faithfully reproduced the native pattern of K14 expression in the mature mammary gland (Wysolmerski et al., 1995), we used this transgene as a vehicle to reintroduce PTHrP into the mammary environment of the PTHrP-null mice. We took a two-tiered approach. First, in order to ascertain if replacement of PTHrP into mammary epithelial cells rescued embryonic mammary development, we bred the K14-PTHrP transgene onto a homozygous PTHrP-null background to produce K14-PTHrP/PTHrP-null mice. These mice were devoid of PTHrP in all tissues except for those expressing the K14 gene. Second, in order to examine the effects of PTHrP replacement on the subsequent development of the mammary duct system within adolescent mice, we bred

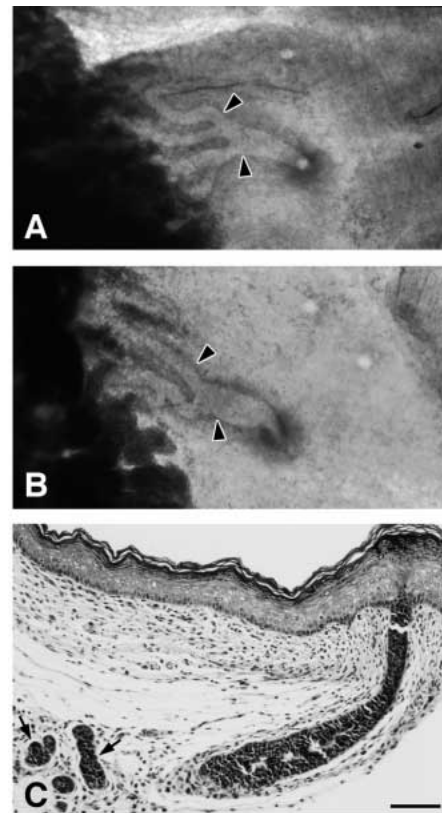


Fig. 6. Mammary development in K14-PTHrP/PTHrP-null mice. (A,B) Photographs of the whole mammary glands of a normal (A) and K14-PTHrP/PTHrP-null neonate (representative of 2 K14-PTHrP/PTHrP-null animals analyzed). The ventral skin was dissected and the mammary glands were viewed under low magnification using transmitted light. In both cases, the mammary gland consists of a primary duct (between arrowheads) that forms several branches before entering the mammary fat pad (dark area to the left). (Contrast B with the lack of a primary duct in the original PTHrP-null embryos as shown in Fig. 1E,F) (C) Photomicrograph of an H&E-stained section through the mammary gland of a K14-PTHrP/PTHrP-null neonate. Note that the primary duct extends from the epidermis through the dermis and forms its initial branches (arrows), as occurs in normal mammary development. However, despite the rescue of mammary epithelial development, note the lack of a nipple sheath. Scale bar represents 67 μ m in A,B; 17 μ m in C.

both the col II-PTHrP and K14-PTHrP transgenes onto a homozygous PTHrP-null background to produce col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. These double-rescue mice lacked PTHrP in all tissues except for chondrocytes and sites of K14 expression.

As expected, the K14-PTHrP/PTHrP-null mice died at birth due to the skeletal abnormalities resulting from the lack of chondrocyte PTHrP expression but, as opposed to the original PTHrP-knockout mice, these mice had mammary glands. As described in the previous sections, by birth, the epithelial duct system in the PTHrP-knockout embryos had completely degenerated. In contrast, as seen in Fig. 6, at birth, the K14-PTHrP/PTHrP-null mice had a well-formed primary duct that extended into the mammary fat pad and formed the expected initial branches. Interestingly, grossly, the primary ducts in the

K14-PTHrP/PTHrP-null neonates often appeared somewhat dilated as compared to normals. On H&E section, one could see that the epithelial duct system in the K14-rescued mice had extended below the upper dermis and, although the primary ducts again often appeared somewhat dilated histologically, they formed normal-appearing secondary ducts within the fatty stroma of the mammary fat pad (see Fig. 6C). Of note, despite the near normal appearance of the ductal tree, there remained no nipple sheath, as was also the case in the PTHrP-null embryos (compare Figs 3E and 6C). Therefore, expression of PTHrP in the embryonic mammary cells of PTHrP-null embryos under the control of the K14 promoter allowed the mammary bud to undergo the primary growth spurt but did not rescue nipple sheath formation.

The double-rescue mice lived to maturity in similar fashion to the col II-PTHrP/PTHrP-null mice. Although the double-rescue mice also lacked nipples, they had a mammary duct system. Fig. 7A demonstrates the fourth and fifth inguinal mammary glands taken from a mature double-rescue female. As can be seen, in these mice the reintroduction of PTHrP via the K14 transgene resulted in the successful completion of the initial round of branching morphogenesis and the appropriate extension of the mammary duct system into the mammary fat pad (Fig. 7A,B). However, the resultant duct system appeared to be that of a sexually immature animal. We had observed that female col II-PTHrP/PTHrP-null mice suffer from a form of hypothalamic hypogonadism (unpublished observations), and we hypothesized that this might have impaired the development of the mammary glands in the double-rescue mice. To address this issue, adult, double-rescue females were treated with subcutaneous estrogen and progesterone for 2 weeks. As shown in Fig. 7C,D, ductal growth in the mammary glands of hormonally treated double-rescue females progressed to the borders of the mammary fat pad and was appropriately branched. Therefore, replacement of PTHrP expression in the developing mammary gland via the K14 promoter was sufficient to support the early morphogenesis of the ductal epithelium and to allow for its subsequent growth and ramification.

DISCUSSION

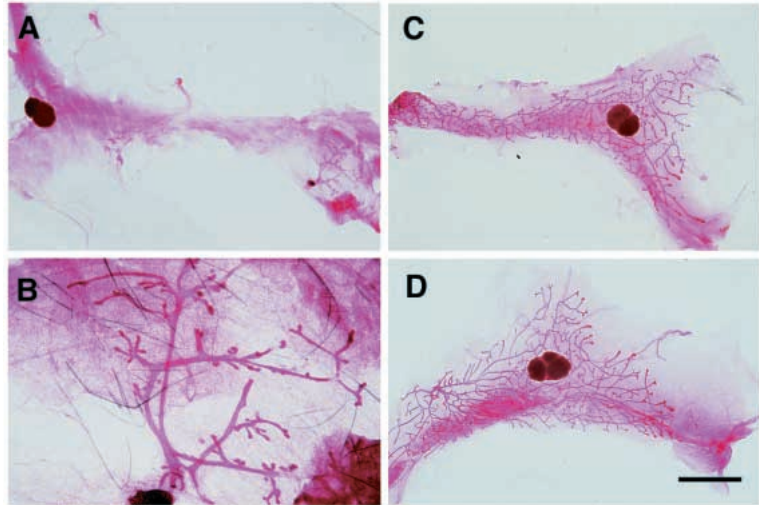
This report records a series of observations that clearly demonstrate that amino-terminal PTHrP is required for the development of the mammary epithelial duct system in mice. First, col II-PTHrP/PTHrP-null mice (devoid of PTHrP in all tissues except for cartilage) lack all mammary epithelial ducts. Second, in PTHrP-knockout embryos, we found a primary failure of branching morphogenesis during embryonic mammary gland development. Third, deletion of the PTH/PTHrP receptor recapitulated the failure of mammary development seen in the PTHrP-knockout embryos. Finally, reintroduction of PTHrP into mammary epithelial cells via the K14-PTHrP transgene rescued the failure of embryonic mammary development seen in the absence of PTHrP and allowed the subsequent development of the mature mammary duct system within the mammary fat pad.

The formation of the embryonic mammary gland occurs in two steps: first, the formation of the mammary bud and, second, the initiation of branching morphogenesis that leads to

the formation of the immature ductal tree (Sakakura, 1987). In PTHrP-knockout embryos, the mammary buds formed appropriately, but they failed to undergo the transition successfully into the initial round of branching growth that leads to the typical immature ductal tree. In the absence of PTHrP, the mammary epithelial structures failed to elongate and/or penetrate into the developing fat pad, remaining in the upper dermis and becoming surrounded by a dense condensation of fibroconnective tissue. The mammary epithelial cells subsequently degenerated; the nipple sheath failed to form, and, by birth, all traces of the mammary epithelial duct system disappeared, explaining the lack of mammary structures in the mature col II-PTHrP/PTHrP-null mice. The exact nature of the epithelial cell degeneration in the PTHrP-knockout embryos remains unclear. PTHrP has been shown to regulate chondrocyte differentiation and apoptosis in the developing growth plate (Weir et al., 1996; Amling et al., 1997; Lee et al., 1996; Vortkamp et al., 1996). However, the loss of the mammary epithelial cells in PTHrP-null embryos did not appear to be associated with either their premature differentiation (as measured by β -casein expression) or apoptosis. Histologically, the stromal condensation around the degenerating ducts in the PTHrP-null mice is reminiscent of the androgen-mediated stromal reaction that leads to the deterioration of the mammary rudiment in male embryos (Sakakura, 1987; Kratochwil and Schwartz, 1976). Despite this similarity, in female knockout embryos, the mammary buds appeared normal through E15, a point at which the mammary buds in normal male littermates are actively degenerating. This asynchrony makes it unlikely that modulation of PTHrP secretion and/or PTH/PTHrP receptor signalling is a central feature of the response of the mammary bud to fetal androgens. However, it remains a possibility that alterations in PTHrP signalling might play some role in the deterioration of the mammary epithelial cells in normal male embryos and we are currently pursuing a series of experiments to test this possibility.

The formation of the embryonic mammary gland is a classic example of inductive development involving epithelial-mesenchymal interactions (Sakakura, 1991; Cunha, 1994). Both the formation of the mammary bud and the initial round of branching morphogenesis appear to be critically dependent on a series of reciprocal and sequential signals exchanged between the mammary epithelium and the dense mammary mesenchyme (Thesleff et al., 1995; Cunha, 1994; Cunha et al., 1995; van Genderen et al., 1994; Weil et al., 1995; Yang et al., 1995). Several experiments have suggested that the presumptive mammary epithelium plays an important role in promoting the condensation and formation of the dense mammary mesenchyme (van Genderen et al., 1994; Kratochwil et al., 1996; Thesleff et al., 1995). However, once formed, the mammary mesenchyme appears to direct the formation of the mammary epithelial duct structure as well as to contribute to mammary epithelial cell cytodifferentiation. For example, heterotypic recombination experiments have demonstrated that mesenchymal cells from the fetal mammary gland can induce nonmammary epithelial cells to form mammary ducts and to make milk proteins (Cunha et al., 1995) and can even induce the formation of mammary bud-like structures from the epidermis of non-mammalian species (Propper, 1973; Propper and Gomot, 1973). Likewise, recent studies have demonstrated

Fig. 7. Mammary development in col II-PTHrP, K14-PTHrP/PTHrP-null (double rescue) mice. (A) Whole-mount analysis of the 4th and 5th inguinal mammary glands taken from a mature double-rescue female mouse (representative of 3 double-rescue mice examined). As one can see, epithelial ducts are present and have penetrated into the fat pad, but the duct appears sexually immature. (B) Higher magnification of 5th inguinal gland shown in A. (C,D) Whole-mount analysis of a mature normal gland (D) and an estrogen- and progesterone-treated double rescue gland (representative of 2 hormonally treated double-rescue mice). As can be seen after 2 weeks treatment of daily 17 β -estradiol (1mg/day) and progesterone (1 ng/day), the double rescue epithelial ducts (C) have grown to fill out the fat pad and appear similar to normals (D). Scale bar represents 5 mm in A and B, and 1 mm in C and D.



that signals derived from mesenchymal cells are important in regulating the overall rate of ductular proliferation as well as the pattern of branching that occurs during the process of branching morphogenesis (Yang et al., 1995; Witty et al., 1995; Phippard et al., 1996). We have demonstrated that, during embryonic mammary development, PTHrP gene expression is limited to the mammary epithelium while PTH/PTHrP receptor gene expression is restricted to the mesenchyme. In the context of the phenotype discussed above, these findings suggest that PTHrP acts as an epithelial message that must be received by the mammary mesenchyme in order for it to support branching growth.

Although mammary development does not appear to be abnormal in the PTHrP knockout embryos until E15-16, we have found that the PTHrP and the PTH/PTHrP receptor genes appear to be expressed in the mammary bud from its formation, at E12, onward. Furthermore, our K14 transgene crossing experiment suggests that PTHrP is largely dispensable before E15. K14 expression does not appear before this point, and therefore the mammary epithelium in the K14-PTHrP/PTHrP-null mice does not produce PTHrP before E15. Despite this delay in PTHrP secretion, as compared to normal mice, K14-PTHrP/PTHrP-null mice successfully initiate branching growth of the mammary ducts. This would imply that the critical period of PTHrP signalling for initiating branching morphogenesis is just before the primary growth spurt at E15-16. However, since nipple sheath development was not rescued in the K14-PTHrP/PTHrP-null mice and since the primary duct did not appear to be completely normal, PTHrP most likely also exerts earlier effects on the mesenchyme. Future study of the effects of PTHrP on mammary mesenchymal cells should help to clarify the details of the temporal requirements for PTHrP signalling during embryonic mammary development.

In summary, we have found that, during embryonic mammary gland development, PTHrP is a necessary participant in the epithelial-mesenchymal interactions leading to the formation of the rudimentary epithelial duct system. Specifically, PTHrP is produced by the mammary epithelium and appears to act on the mesenchyme, allowing it to support the initiation of branching morphogenesis. We have previously reported that the overexpression of PTHrP in mammary

myoepithelial cells had dramatic effects on the process of branching morphogenesis during sexual maturation and pregnancy (Wysolmerski et al., 1995), indicating that PTHrP likely plays an important role in the regulation of this process throughout mammary development. There is also growing evidence of the participation of PTHrP in the reciprocal epithelial-mesenchymal interactions that govern epithelial development in sites other than the mammary gland. For example, the pattern of epithelial PTHrP expression and mesenchymal PTH/PTHrP receptor expression seen in the developing mammary gland has been noted in other developing organs (Lee et al., 1995). In addition, col II-PTHrP/PTHrP-null mice have defects in other ectodermally derived organs (skin, teeth and sebaceous glands) that are dependent on epithelial-mesenchymal interactions for their development (unpublished observations). We anticipate that PTHrP will be found to participate in the regulation of mesenchymal cell function during the development of a number of epithelial organs, and it is our hope that further study of the effects of PTHrP during embryonic mammary development will provide a framework for the general understanding of PTHrP's role in regulating mesenchymal function during organogenesis.

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