AD_____

Award Number: DAMD17-96-1-6198

TITLE: The Role of PTHrP in Mammary Gland Development and
        Tumorigenesis

PRINCIPAL INVESTIGATOR: John J. Wysolmerski, M.D.

CONTRACTING ORGANIZATION: Yale University
                          New Haven, Connecticut 06520-8047

REPORT DATE: September 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20010716 077

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Final (1 Sep 96 – 31 Aug 00)

4. TITLE AND SUBTITLE
The Role of PTHrP in Mammary Gland Development and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-96-1-6198

6. AUTHOR(S)
John J. Wysolmerski, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, Connecticut 06520-8047

E-MAIL:
john.wysolmerski@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color graphics.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

PTHrP was discovered as the tumor product responsible for the syndrome of humoral hypercalcemia of malignancy. It is know known to be an important developmental regulatory molecule in several sites, including the mammary gland. Over the four years of this grant, we have used a variety of animal models to investigate the functions of PTHrP during mammary development. We have discovered that PTHrP is necessary for mammary gland formation in the embryo. Our data suggest that PTHrP signaling is involved regulating an intricate series of cell fate decisions that govern the proper differentiation of the mammary epithelium and mammary mesenchyme as well as the proper morphogenesis of the mammary ducts and nipple. We have also studied the effects of PTHrP overexpression during the pubertal development of the mammary gland. Here, it appears that PTHrP regulates epithelial cell proliferation and apoptosis within terminal endbuds, and antagonizes the effects of estrogen and progesterone on these processes. Finally, overexpression of PTHrP appears to increase the incidence and shorten the latency of mammary tumor formation when mice are treated with a chemical carcinogen.

14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 77
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Introduction

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product responsible for causing the clinical syndrome of humoral hypercalcemia of malignancy (HHM) (1). In this syndrome, PTHrP is released into the circulation by malignant cells, resulting in a typical constellation of biochemical abnormalities resembling hyperparathyroidism. The similarity of HHM and hyperparathyroidism is now understood on a molecular basis. The parathyroid hormone (PTH) and PTHrP genes arose by duplication from a common ancestral gene and continue to share a high degree of homology in their amino-terminal ends, a feature that allows them to signal through the use of a common receptor (termed the PTH/PTHrP type I receptor or PTHR1) (1,2). In the setting of malignancy, PTHrP, which normally acts as a local autocrine or paracrine factor, is secreted into the circulation by tumor cells and interacts with PTHR1's in bone and kidney, mimicking the actions of PTH (1).

As noted in the preceding paragraph, PTHrP normally acts as a local autocrine and/or paracrine factor. It is expressed in a great number of tissues, where it appears to play a role in the regulation of cellular proliferation and differentiation during development (3). One of these sites is the mammary gland. PTHrP mRNA has been shown to be expressed in the embryonic mammary epithelium, as well as by mammary epithelial cells during pregnancy and lactation (3,4). In addition, PTHrP has been shown to be secreted by mammary myoepithelial cells in culture (5,6). The PTH/PTHrP receptor is found on mammary stromal cells and on mammary myoepithelial cells (4-6). Therefore, the mammary gland contains the elements of both autocrine and paracrine signaling loops for PTHrP. That these loops are important to the physiology of mammary development is evident by the results of the overexpression of PTHrP in mammary myoepithelial cells (7). As reviewed in the original proposal, we used the human keratin-14 (K14) promoter to target PTHrP overexpression to myoepithelial cells. This resulted in a severe impairment of branching morphogenesis and mammary ductal proliferation during sexual maturation and pregnancy (7). These results led us to hypothesize that PTHrP acts as a local growth inhibitor, contributing to the regulation of ductal proliferation and morphogenesis during mammary development.

The intent of the current grant was to test the hypothesis that PTHrP serves as a locally-produced mammary growth inhibitor by examining the effects of PTHrP on branching morphogenesis and on mammary epithelial cell proliferation and transformation. In order to test this hypothesis we proposed a series of four technical objectives that encompassed a mixture of experiments *in vitro* and in transgenic animals. As summarized in previous reports, over the last four years, we have made great strides in understanding PTHrP's function during embryonic mammary development. In addition, we have also begun to unravel its effects during the pubertal development of the gland as well as its effects on mammary tumor formation and metastasis. We will present these findings below, organized by technical objective. Published data that has been presented in previous annual reports will be sumarized briefly. New and unpublished data from the final year of the proposal will be presented in more depth.

BODY

Technical Objective 1. – Effects of the loss of PTHrP on mammary gland development

As has been detailed in the original proposal and in previous annual reports, we utilized a transgenic approach to rescue the PTHrP knockout mice from a neonatal death caused by skeletal dysplasia (4). When we did this we discovered that PTHrP is necessary for the formation of mammary glands during embryonic development. In the absence of either PTHrP or its receptor, PTHR1, the epithelial mammary bud forms but subsequent morphogenesis fails. The expected androgen-mediated destruction of the mammary bud in male embryos does not take place (8). In females, the primary growth spurt does not occur, and the mammary epithelial cells disappear by birth, leaving the embryos without mammary glands or nipples. In addition to these observations, we also documented the pattern of PTHrP and PTHR1 expression during embryonic mammary development. The PTHrP gene becomes highly expressed by mammary epithelial cells on the second half of E11, just after the mammary bud has begun to take shape (4,8). It is not expressed within the developing epidermis at this time. The PTHR1 gene is expressed in the mesenchyme surrounding the mammary bud and beneath the developing epidermis (4,8). These patterns of expression suggested to us that PTHrP acted as a critical signal from epithelium to mesenchyme that regulated the morphogenetic function of the mammary mesenchyme. Experiments over the last year have now clearly shown that PTHrP signaling regulates a series of ventral cell fate decisions that are necessary for mammary gland and nipple development. The information summarized above is presented in greater detail in the appended published manuscripts (Wysolmerski et al 1998, Development; Dunbar et al 1999; Development). Our most recent work pertaining to the function of PTHrP during embryonic mammary development is described in the following paragraphs and the appended preprint (Foley et al, Development, in press).

In last year's annual report, we presented data that suggested that PTHrP was responsible for inducing the differentiation of the primary mammary mesenchyme from the presumptive dermal mesenchyme found beneath the embryonic skin, and we presented the model shown in Fig. 1. This model suggests that it is the presence of PTHrP in the mammary epithelial cells that leads the surrounding mesenchyme to choose a mammary fate. This in turn has consequences for the future morphogenesis of the mammary ducts. As presented last year, we validated this model by showing that when PTHrP is misexpressed in the basal keratinocytes of K14-PTHrP transgenic embryos, the dermis on the ventral surface of the mice is transformed into mammary mesenchyme (8). This past year, we performed experiments that demonstrate that PTHrP participates in a series of cell fate decisions that are critical to the maintenance and morphogenesis of the mammary epithelial cells, the differentiation of the mammary mesenchyme and the differentiation of the epidermis comprising the nipple.

We took advantage of having both loss of function (PTHrP and PTHR1 KO's) and gain of function (K14-PTHrP transgenics) systems to study the consequences of

5

alterations in PTHrP signaling on embryonic mammary development. Using a series of differentiation markers, we showed that in the absence of PTHrP signaling, no mammary mesenchyme is formed and the epithelial cells of the mammary bud differentiate down an epidermal pathway. In the presence of diffuse epidermal overexpression of PTHrP, the ventral dermis is transformed into mammary mesenchyme and the epidermis becomes nipple skin. These cell fate decisions require embryonic exposure to PTHrP, for when overexpression of PTHrP is delayed until after birth (using a binary tetracycline-regulated K14-tTA/TetO-PTHrP transgene) the ventral epidermis develops normally. These changes are also a consequence of PTHrP's effects on the mesenchyme, for when the K14-PTHrP transgene is bred onto a PTHR1 KO background, the ventral epidermis also develops normally. These data are incorporated into a refined model shown in Fig. 2 that summarizes our current understanding of the role of PTHrP during embryonic mammary development (see appended preprint – Foley et al).

In addition to the above experiments, we have begun to address the potential signaling pathways that may explains PTHrP's effects on the mammary mesenchyme. Thus far, we have demonstrated that the changes in cell fate seen in response to PTHrP signaling correlate nicely with changes in the expression of LEF1 and β-catenin, two critical molecules in the wnt-signaling cascade (9). Normally, LEF1 expression is induced within the mammary mesenchyme and suppressed in the keratinocytes destined to become the nipple skin. In the absence of PTHrP signaling, there is a loss of mammary mesenchyme LEF1 expression, and an inappropriate retention of LEF1 expression in the presumptive nipple epidermis. In the presence of ectopic PTHrP expression in K14-PTHrP transgenics, LEF1 is inappropriately expressed in what should be the dermis and inappropriately lost in the ventral keratinocytes, reproducing the pattern normally seen in the nipple region. Likewise, we found that β-catenin is normally expressed within the mammary mesenchyme, but not in the dermis. In the absence of PTHrP signaling β-catenin expression is lost in the mammary mesenchyme and in the presence of epidermal PTHrP expression, β-catenin is inappropriately induced in the ventral dermis. These data suggest that the PTHrP and wnt signaling cascades may interact during the formation of the embryonic mammary gland and nipple, a possibility that will be the focus of future efforts. These findings were recently accepted for publication in Development (see Foley et al, appended preprint).

We completed the original experiments described within this technical objective and performed many others. In fact, because the original experiments showed that PTHrP signaling was absolutely necessary for the formation of the murine mammary gland, we devoted most of our energies over the past four years to this aspect of the project. This has provided us with a solid understanding of PTHrP's role during embryonic mammary development and has extended the knowledge base regarding this phase of mammary development. In fact we have recently demonstrated that PTHrP-signaling is also required for breast development in human fetuses (10).

6

Technical Objective 2. - The effects of PTHrP on the branching morphogenesis of mammary epithelial cells and Technical Objective 3 – Examination of the effects of PTHrP on hormonally-induced proliferation in mammary epithelial cells.

These objectives arose from our original hypothesis that PTHrP regulates epithelial-stromal interactions that support the proper morphogenesis of the mammary epithelial duct system during the pubertal development of the mammary gland as well as during embryogenesis. In addition, we had hypothesized that PTHrP acted as a local antagonist of hormonally-stimulated proliferation of the mammary ducts during puberty. As summarized in the original proposal and in last year's report, there are two lines of evidence that would support these concept. First, transgenic mice overexpressing PTHrP have an impairment in branching morphogenesis of the epithelial ducts that manifests itself at the time of puberty (7). In addition, the transgenic ducts do not grow through the mammary fat pad as rapidly as they should during puberty (7). Second, during the pubertal growth of the mammary gland, PTHrP is expressed in the epithelial cells of the end-buds and the PTH/PTHrP receptor is expressed in the stromal cells surrounding the end buds (11). End buds are specialized structures at the tips of the growing ducts where active cell proliferation, differentiation and forward growth of the duct system actually occurs (12). We had originally envisioned a series of experiments to examine the effects of PTHrP on the branching morphogenesis and proliferation of mammary epithelial cells in culture. However, we were never successful in establishing culture systems that could reproduce what we had observed *in vivo* in the K14-PTHrP transgenic mice. Therefore, in the past year we have tried to answer these questions using the original K14-PTHrP transgenic mice and newer tetracycline-regulated K14-PTHrP trnasgenic mice.

In last year's annual report we introduced the tetracycline-regulated PTHrP transgenics. However, we will again describe them briefly in this report. As outlined in Figure 3, we used a "tet-off" system so that the addition of tetracycline into the water supply of the mice shuts off transgene expression. We created two lines of transgenic mice; K14-tTA mice expressed the tetracycline transactivator under the control of the K14promoter, and ptet-PTHrP mice expressed the PTHrP gene under the control of a minimum promoter with seven tetracycline-responsive elements. Next, we bred these mice together to generate double transgenics, which overexpressed PTHrP at sights of K14 expression as before, but now with transgene expression dependent on the presence or absence of tetracycline in the animals' water. In other words, the transgene gained an on-off switch. The ability to turn transgene expression on or off enabled us to examine the consequences of PTHrP overexpression at different points in the development of the mammary gland. Before using these mice in our experiments, however, we first validated the fidelity of the K14-tTA transgene by breeding these mice to a βGal responder strain. As expected, these mice expressed βGal only in sites of K14 expression and only in the absence of tetracycline (see Fig 4).

We next used this system to examine the effects of PTHrP overexpression at different times during mammary development. . Because the K14-PTHrP mammary phenotype becomes manifest during puberty, we had expected that concurrent exposure to PTHrP was necessary for this phenotype to emerge. However, we were surprised to

learn that the original K14-PTHrP transgenic mammary phenotype could be separated into two different phenotypes and only one of these was caused by PTHrP overexpression during puberty. In our original K14-PTHrP transgenic mice, the mammary gland appeared relatively normal until the onset of puberty. During puberty, the transgenic ducts demonstrate impairments in both ductal elongation and branching. The transgenic endbuds grow through the mammary fat pad at a slower rate than normal and the duct system develops many fewer side branches, resulting in a simpler and sparser gland. By altering the timing of PTHrP overexpression we have learned that the impairment in ductal branching is a result of the effects of embryonic overexpression of PTHrP and the impairment of ductal elongation is a result of pubertal overexpression of PTHrP. As shown in Table 1, histomorphometric studies in the tetracycline-regulated transgenics have documented that if PTHrP is overexpressed before birth, but not after birth, branching is impaired, but ductal elongation is normal. In contrast, if PTHrP is overexpressed only after birth or during puberty, ductal elongation is impaired but branching is normal. These latter results agree with our prior observations that placement of PTHrP pellets in advance of the growing duct system led to an inhibition of ductal elongation but not branching (7).

As noted above, at the onset of puberty, the distal ends of the pre-existing mammary ducts form specialized structures called terminal end buds (TEBs), which serve as sites of active cellular turnover and differentiation (12). Various studies have shown that the TEBs are the main locus of epithelial proliferation and apoptosis during puberty and presumably the balance between these processes determines the rate at which the ducts grow through the mammary fat pad (13,14). We have previously shown that TEB's are also the major site of PTHrP and PTHR1 gene expression during puberty (11). Therefore we hypothesized that overexpression of PTHrP during puberty might impair ductal elongation by altering epithelial cell proliferation and/or epithelial cell death 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 to wild-type mice. We similarly studied K14-tTA/Tet$_0$-PTHrP double transgenics in whom transgene expression was activated at birth, since 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 hours of treatment with exogenous estrogen and progesterone.

We assessed TEB proliferation by measuring ^{3}H-thymidine incorporation. As shown in Fig. 5, at baseline, 14 +/- 1.5% of epithelial cells in wild-type endbuds incorporated thymidine. After hormone treatment, significantly more of the wild-type epithelial cells (23+/-0.83%) within TEBs were dividing . 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$_0$-PTHrP double transgenics that were exposed to PTHrP overexpression after birth (13.85+/-2.13%). 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

8

not increase in either the K14-PTHrP TEB's (14+/-1.0%) or in the K14-tTA/Tet$_o$-PTHrP double transgenic TEBs (12.19+/-1.8%).

We assessed TEB apoptosis by TUNEL assay. As shown in Fig. 6, in wild-type mice, an average of 3.11+/-0.7% of epithelial cells within TEBs were undergoing apoptosis at baseline, and this number decreased to 0.98+/-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 higher both in K14-PTHrP transgenic mice (6.5+/-0.7%) and in K14-tTA/Tet$_o$-PTHrP double transgenic mice that had been exposed to PTHrP overexpression only after birth (7.5+/-2.83%). In addition, in the transgenic mice there was no reduction of apoptosis in response to hormone treatment.

In summary, the defects in ductal elongation caused by the overexpression of PTHrP during puberty are associated with higher 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 minimal branching defects due to the initiation of transgene expression only after birth.

Although we were not able to perform the cell culture experiments originally described in technical objectives 2 and 3, we feel that these data in vivo establish that PTHrP can act locally at TEBs to inhibit hormonally-regulated cell proliferation and apoptosis. Furthermore, since PTHrP and its receptor are normally expressed within the TEBs of the pubertal gland, it is likely that PTHrP normally participates in the regulation of these processes. The challenge for the future will be to begin to sort out the mechanisms by which PTHrP antagonizes the effects of systemic hormones on endbud proliferation and apoptosis. We are currently preparing a manuscript describing our experiments with the tetracycline-regulated transgene.

Technical Objective 4 – The effects of PTHrP on mammary tumor formation

Because PTHrP appears to inhibit the hormonally-stimulated proliferation of mammary epithelial cells in TEB's during normal development, we had hypothesized that overexpression of PTHrP might inhibit the formation of hormone-dependent mammary tumors in mice. To investigate this possibility, as outlined in the original proposal, we crossed our K14-PTHrP transgenic mice to GR mice (15), and then examined the incidence of tumor formation after multiple pregnancies. We analyzed 11 GR/K14-PTHrP transgenic mice and 13 plain GR mice. After 3-5 pregnancies 9/11 (82%) GR/K14-PTHrP mice developed 19 tumors and 10/13 (77%) developed 24 tumors. Thus it appeared that overexpression of PTHrP did not inhibit tumor formation. In order to confirm that these tumors actually did overexpress PTHrP we analyzed tumors harvested from either GR/K14-PTHrP or plain GR mice for the presence of transgene mRNA by RNase protection analysis. As expected, the tumors from the GR/K14-PTHrP mice clearly did express the transgene, and cultured cells from GR/K14-PTHrP tumors

secreted large amounts of PTHrP. Therefore, although the tumors arising from GR/K14-PTHrP mice both expressed the transgene and overproduced PTHrP, it appeared that this did not inhibit tumor formation in the GR mouse tumor model.

In the time that has passed since the original proposal was written, it has been proposed that PTHrP may be important to the process of forming bone metastases from breast cancer. In order to test this hypothesis as well as to examine our original hypothesis that PTHrP would inhibit tumor formation in another model, we treated a cohort of our transgenic animals and controls with DMBA to examine the effects of PTHrP overexpression in a chemical-induced tumor model (16). Our protocol was as follows. Nineteen female K14-PTHrP transgenic mice and 22 female control littermates between the ages of 7 and 9 weeks were treated with 7,12-dimethyl-antracene (DMBA). The DMBA was administered by gavage at doses of 1mg weekly for 4 consecutive weeks. The mice were then housed with males and were allowed to proceed through multiple rounds of pregnancy over the course of 1 year. Each mouse was palpated weekly to detect the presence of mammary tumors. Once established, the tumors were allowed to reach a size of 2 – 2.5 cm, after which the mice were sacrificed. At sacrifice, blood was drawn for the measurement of circulating calcium and PTHrP levels and tumor tissue was harvested for histology and analysis of transgene expression. A necropsy was performed and all the visceral organs were harvested for histologic examination to detect metastases from the primary tumor. After the viscera were removed, radiographs of the entire skeleton were obtained using a Hewlett-Packard Faxitron. Finally, the appendicular and axial skeletons of each mouse were decalcified and processed for histologic examination. The experiment was terminated at 1 year folowing DMBA treatment.

As shown in Fig. 7, surprisingly, in this model the overall incidence of tumors in the K14-PTHrP transgenic mice appeared to be higher (84% at 1 year) than in the controls (59% at one year). In addition, tumors appeared to develop more rapidly in the K14-PTHrP transgenic cohort. The time to 50% tumor incidence was 27 weeks for the K14 transgenic mice and 32 weeks for the control mice. However, using the log rank test, the differences between these curves just missed statistical significance (p = 0.08). The tumors themselves, were primarily either adenocarcinomas or adenoacanthomas and transgenic and control tumors showed no differences in histological type. All the tumors expressed the K14-PTHrP transgene and the transgenic mice had elevated levels of circulating PTHrP and were hypercalcemic as compared to controls. However, none of the transgenic mice developed bone metastases as assessed by whole body radiography and histological examination of the axial and appendicular skeleton. None of the control mice with tumors developed bone metastases although both transgenic and control mice developed soft tissue metastases at comparable rates.

In summary, in neither the GR nor the DMBA system did PTHrP inhibit the formation of hormonally-induced mammary tumors. In fact, PTHrP overexpression appeared to augment the formation of mammary tumors in response to DMBA. This is in contrast to our observations during normal development, where PTHrP overexpression clearly antagonizes the effects of estrogen and progesterone. Although these experiments

10

did not turn out as expected, we were sucdessful in completing them. We are in the process of writing up our data from the DMBA experiments for publication.

KEY RESEARCH ACCOMPLISHMENTS – ENTIRE GRANTING PERIOD

1. Recognition that PTHrP and the PTHR1 are necessary for the formation of mammary glands.
2. Elucidation of the epithelial expression of PTHrP and the mesenchymal expression of the PTHR1 during embryonic mammary development.
3. Recognition of PTHrP as the epithelial signal responsible for the induction of androgen receptor expression in the mammary mesenchyme.
4. Elucidation of the effects of PTHrP on mesenchymal cell fate decisions during embryonic mammary development.
5. Recognition that PTHrP signaling in the mammary mesenchyme is responsible for the location and formation of the nipples.
6. Recognition of the correlation between PTHrP signaling and the expression patterns of LEF-1 and β-catenin in the mammary mesenchyme and the implication that PTHrP signaling interacts with the Wnt signaling cascade during mammary mesenchyme differentiation.
7. Creation and validation of tetracycline-regulated system for overexpression of genes in mammary epithelial cells in transgenic mice.
8. Use of above tetracycline-regulated system to separate the effects of PTHrP overexpression into developmental and concurrent effects.
9. Demonstration of the patterning defects in adolescent mammary gland development in K14-PTHrP transgenic mice to be the result of embryonic exposure to PTHrP.
10. Demonstration of the defects in ductal elongation in K14-PTHrP transgenic mice to be due to concurrent exposure to PTHrP during adolescence.
11. Demonstration that PTHrP overexpression inhibits ductal elongation by increasing the basal rate of apoptosis in terminal endbuds and by inhibiting the effects of estrogen and progesterone on endbud proliferation and apoptosis.

REPORTABLE OUTCOMES – ENTIRE GRANTING PERIOD

Manuscripts:

1. **Wysolmerski JJ**, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, Broadus AE. Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development*, **1998**, 125: 1285-1294.

2. Dunbar ME, Young P, Zhang JP, McCaughern-Carucci J, Lanske B, Orloff J, Karaplis A, Cunha G, **Wysolmerski JJ**. Stromal cells are critical targets in

the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein (PTHrP). *Developmental Biology,* **1998**, 203: 75-89.

3. Foley J, Longely BJ, **Wysolmerski JJ**, Dreyer BE, Broadus AE, Philbrick WM. Regulation of epidermal differentiation by PTHrP: evidence from PTHrP-null and PTHrP-overexpressing mice. *J Investigative Dermatology,* **1998**, 111:1122-1128.

4. **Wysolmerski JJ** and Stewart AF. The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. *Annual Rev. Physiol,* **1998**, 60: 431-460.

5. Dunbar ME and **Wysolmerski JJ**. The role of parathyroid hormone-related protein (PTHrP) in mammary development, lactation and breast cancer. Chapter for CD ROM text on mammary development, Henninghausen, L. (ed.) see Biology of the Mammary Gland web site: http://mammary.nih.gov/index.html.

6. Dunbar ME and **Wysolmerski JJ**. Parathyroid hormone-related protein: a developmental regulatory molecule necessary for mammary gland development. *J Mammary Gland Biol Neoplasia,* **1999**, 4:21-34.

7. Dunbar ME, Dann PR, Robinson GW, Hennighausen L, Zhang JP, **Wysolmerski JJ**. Parathyroid hormone-related protein is necessary for sexual dimorphism during embryonic mammary development. *Development,* **1999**, 126:3485-3493.

8. Foley J, Dann P, Hong J, Cosgrove J, Dreyer B, Rimm D, Dunbar ME, Philbrick W, **Wysolmerski JJ**. Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic mammary development. *Development,* in press.

9. **Wysolmerski JJ**, Stewart AF. Martin TJ. Physiological actions of PTH and PTHrP V: mammary, reproductive, epidermal and pancreatic tissue. in The Parathyroids: Basic and Clinical Concepts, 2nd Edition, JP Bilizekian, R Marcus, MA Levine, eds. Academic Press, in press.

10. Dunbar ME and **Wysolmerski JJ**. Mammary ductal and alveolar development, lessons learned from genetically manipulated mice. *Microscopy Research and Technique,* in press.

Abstracts/Presentations

1. **Wysolmerski, JJ,** Dunbar, M, Karaplis, AC, Broadus, AE, Philbrick, WM. PTHrP is necessary for mammary epithelial development. Chosen for an oral presentation at 1996 meeting of the American Society of Bone and Mineral Research.

2, Dunbar, ME, Philbrick, WM, Broadus, AE, **Wysolmerski, JJ.** PTHrP modulates the branching morphogenesis of mammary epithelial cells in vitro. Poster presentation at 1996 meeting of the American Society of Bone and Mineral Research.

3. **Wysolmerski JJ,** Lanske B, Kronenberg HM, Broadus AE, Philbrick WM. Disruption of the PTH/PTHrP receptor gene leads to a failure of mammary gland development. Chosen for an oral presentation at 1997 Endocrine Society Meetings.

4. "The role of PTHrP in mammary gland development" Gordon Research Conference on Mammary Gland Biology, 6/97. Plymouth State College, Plymouth, New Hampshire

5. Dunbar M, McCaughern-Carucci J, Zhang JP, **Wysolmerski JJ.** The analysis of PTHrP and PTH/PTHrP receptor expression in the murine mammary gland. Poster presentation at 1997 Gordon Research Conference on mammary gland biology.

6. "PTHrP is necessary for mammary epithelial development" Conference on Breast Development, Physiology and Cancer, 6/97, National Institutes of Health, Bethesda, Maryland

7. **Wysolmerski JJ,** Dunbar ME, Karaplis A, Lanske B, Kronenberg H, Broadus AE, Philbrick WM. Parathyroid Hormone-related Protein is Necessary for Mammry Epithelial Development. Selected for Oral Platform Presentation at 1997 Department of Defense Breast Cancer Research Program Meeting.

8. Dunbar M, Dann P, Robinson T, **Wysolmerski J.** PTHrP Signaling is Necessary for Sexual Dimorphism During Fetal Mammary Development. Selected for oral presentation, 1998 Joint Meeting of the American Society for Bone and Mineral Research and the International Bone and Mineral Society.

9. Dunbar ME, Young P, Zhang JP, McCaughern-Carucci J, Lanske B, Orloff J, Karaplis A, Cunha G, **Wysolmerski JJ.** Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein (PTHrP). Poster presentation at 1998 Joint Meeting of the American Society for Bone and Mineral Research and the International Bone and Mineral Society.

10. "PTHrP regulates fetal mammary development by modulating mesenchymal cell fate decisions", Gordon Research Conference on Mammary Gland Biology Henneker, New Hampshire, 6/9/99.

11. Dunbar, M, Dann P, Dreyer B, Broadus AE, Philbrick WM, **Wysolmerski JJ**. Transient early overexpression of PTHrP leads to subsequent defects in mammary development. Selected for oral presentation at the 1999 meeting of the Endocrine Society, San Diego, CA

12. "PTHrP regulates mammary ductal morphogenesis", San Diego, CA., Basic Symposium: Signaling Molecules in Breast Cancer, Endocrine Society Annual Scientific Meetings, 6/14/99.

13. Dann P, Dunbar M, **Wysolmerski J.** PTHrP regulates LEF-1 and beta-catenin expression during embryonic mammary development. Selected for oral presentation at the 2000 meeting of the American Society of Bone and Mineral Research, Toronto, Canada.

14. Perkins A, Dann P, Zelezny E, Guise T, Stewart A, Dunbar M, **Wysolmerski J.** Overexpression of PTHrP causes hypercalcemia, but not bone metastases in a murine model of mammary tumorigenesis. Selected for poster presentation at the 2000 meeting of the American Society of Bone and Mineral Research, Toronto, Canada.

Funding secured based on this work:

1. Agency – NIH
Number – 1 RO1 DK55501-01
Title - PTHrP in Embryonic Mammary Gland Development
Duration: 9/30/99 – 8/31/2004

2. Agency – American Cancer Society
Number – RPG-00_037-01-CNE
Title – Role of PTHrP in Breast Cancer Metastases to Bone
Duration: 1/1/2000 – 12/31/2004

CONCLUSIONS

Over the past four years, with the help of this grant, we have made remarkable strides in understanding the functions of PTHrP in the mammary gland. As often happens, we had some surprises along the way and we have come to appreciate that PTHrP's role in the breast is more complicated, but more interesting, than our original predictions. We have spent the bulk of our time and effort studying the effects of PTHrP

on embryonic mammary development. This whole area arose out of the experiments originally described in Technical Objective 1 that demonstrated that PTHrP and its receptor are required for the formation of a mammary epithelial duct system. As outlined in the Body of this report, we now appreciate that PTHrP regulates an intricate set of ventral cell fate decisions in the embryo that results in the morphogenesis of the mammary epithelial ducts and the nipples. As a result of these studies, PTHrP has joined a handful of molecules recognized to be crucial to breast development. In addition, we have established that PTHrP signaling is necessary for sexual dimorphism during murine mammary development and that PTHrP is the epithelial factor that induces androgen receptor expression in the mammary mesenchyme. Our future goals are to identify the signaling pathways upstream and downstream of PTHrP. We would like to know what turns PTHrP expression on in the mammary bud and what are the molecular consequences of PTHrP signaling within the differentiating mammary mesenchyme. With respect to the latter goal, we are now pursuing some promising preliminary data suggesting that PTHrP signaling interacts with the Wnt signaling cascade.

Our work has demonstrated that the survival, proliferation and morphogenesis of the mammary epithelial cells during embryogenesis clearly depends on clues from the mammary mesenchyme. In breast cancer, it appears that the malignant epithelial cells become somewhat independent of stromal regulation. In addition, it appears that the stroma may also contributes to the oncogenic process. Our hope is that a deeper understanding of PTHrP's role in epithelial-mesenchymal interactions during embryogenesis will help to shed light on tumor-stromal interactions in breast cancer.

In addition to our efforts directed at studying the functions of PTHrP during embryonic mammary development, we have also pursued our original observation that PTHrP affects mammary development during puberty. As reviewed in the Body of this report, we created a new strain of tetracycline-regulated K14-PTHrP transgenic mice to examine the effects of PTHrP on ductal branching and elongation during puberty. This system worked very well and, using it, we were able to show that excess amounts of PTHrP during embryogenesis were able to change the branching pattern of the epithelial ducts during puberty while excess amounts of PTHrP during puberty inhibited ductal elongation. We were surprised that transient exposure to PTHrP during embryogenesis was able to affect morphogenetic events that occurred much later during puberty. We do not yet understand how this happens and we are beginning to study this by using mammary gland transplantation techniques to determine if the effects of PTHrP are on the mammary fat pad or on the epithelial cells themselves. With regard to ductal elongation, it is clear that the reduction in the growth rate of the ducts is the result of higher rates of cell death and lower rates of proliferation in terminal endbuds. In addition, overexpression of PTHrP causes the endbuds to become resistant to the effects of exogenous hormones. We are excited by these findings for they confirm our original observations that suggested that PTHrP might act as a local growth inhibitor that antagonized the effects of estrogen and progesterone. Obviously, the effects of PTHrP in this regard were not enough to prevent the formation of hormonally-induced mammary tumors in our DMBA experiments. Nonetheless, learning more about the mechanisms by

which PTHrP antagonizes the effects of estrogen and progesterone on endbuds should be relevant to the biology of hormone-responsive breast cancer.

REFERENCES

1. Wysolmerski JJ, Broadus AE. Hypercalcemia of malignancy: the central role of parathyroid hormone-related protein. Annu Rev Med 45:189-200, 1994.

2. Jüppner H, Abou-Samra B-B, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF, Hock J, Potts JT, Kronenberg HM, Segre GV. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science 254:1024-1026, 1991.

3. Philbrick WM, Wysolmerski JJ, Galbraith S, Holt E, Orloff JJ, Yang KH, Vasavada RC, Weir EC, Broadus AE, Stewart AF. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev 76:127-173, 1996.

4. Wysolmerski JJ, Philbrick WM, Dunbar ME, Lanske B, Kronenberg H, Karaplis A, Broadus AE. Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development*, **125**: 1285-1294, 1998.

5. Ferrari SL, Rizzoli R, Chaponnier C, Gubbiani G, Bonjour JP. Parathyroid hormone-related protein increases cAMP production in mammary epithelial cells. Am J Physiol 264:E471-E475, 1993.

6. Seitz PK, Cooper KM, Ives KL, Ishizuka J, Townsend CM, Rajsraman S, Cooper CW. Parathyroid hormone-related peptide production and action in a myoepithelial cell line derived from normal human breast. Endocrinology 133:1116-1124, 1993.

7. Wysolmerski JJ, McCaughern-Carucci JF, Daifotis AG, Broadus AE, Philbrick WM. Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. Development 121:3539-3547, 1995.

8. Dunbar ME, Dann PR, Robinson GW, Hennighausen L, Zhang JP, Wysolmerski JJ. Parathyroid hormone-related protein signaling is necessary fo sexual dimorphism during embryonic mammary development. Development 126:3485-3493, 1999.

9. Eastman Q, Grosschedl, R. Regulation of Lef/TCF transcription factors by Wnt and other signals. Current Opinion Cell Biol 11:233-240, 1999.

10. Wysolmerski JJ, Cormier S, Philbrick WM, Dann P, Zhang JP, Roume J, Delezoide AL, Silve C. Absence of functional type I PTH/PTHrP receptors in humans is

associated with abnormal breast and tooth development. J Clin Endocrinol Metab, in press.

11. Dunbar ME, Young P, Zhang JP, McCaughern-Carucci J, Lanske B, Orloff J, Karaplis A, Cunha G, Wysolmerski JJ. Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein (PTHrP). Developmental Biology 203: 75-89, 1998.

12. Daniel CW, Silberstein GB. Postnatal development of the rodent mammary gland. In The Mammary Gland:Development, Regulation and Function (ed. by MC Neville and CW Daniel) pp 3-36, Plenum Publishing, New York 1985.

13. Daniel CW, Silberstein, Strickland P. Direct actions of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. Cancer Res. 47:6052-6057, 1987.

14. Humphreys RC, krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, Reed JC, Rosen JM. Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development 122: 4013-4022, 1996.

15. Muhlhock O. A note on a new inbred mouse strain GRIA. Eur J Cancer 1:123, 1965.

16. Bonser, G.M. Evolution of mammary cancer induced in virgin female IF mice with minimal doses of locally acting methylcholanthrene. J Pathol Bacteriol 68: 531-546, 1954.

Personnel:

John J. Wysolmerski

William Philbrick

Pamela Dann

Christopher Brown

Jiang-Ping Zhang

Jayme McCaughern-Carucci

UENTRAL MESENCHYME

PTHrP

No PTHrP

MAMMARY MESENCHYME

DERMIS
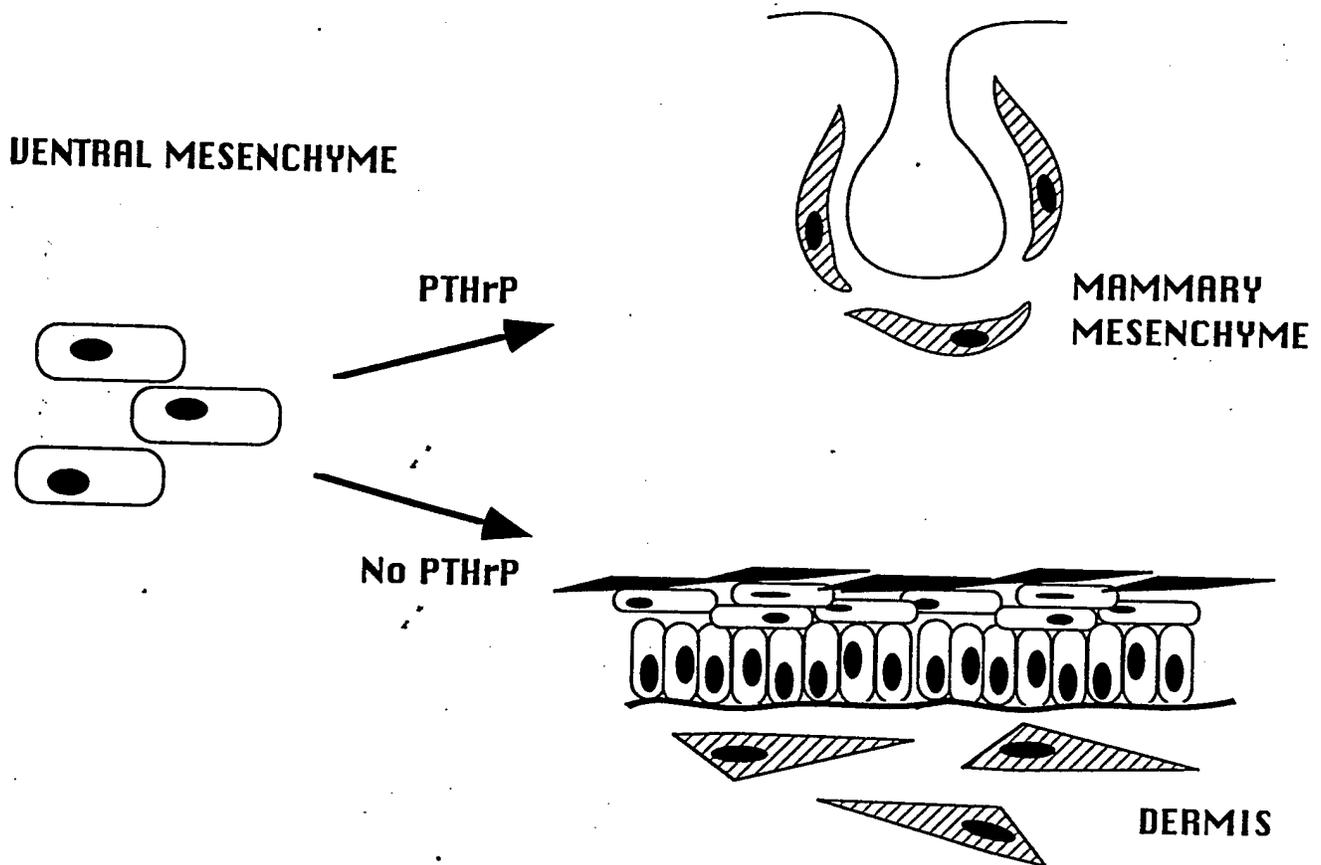
Figure 1. A model for the regulation of mesenchymal cell differentiation by PTHrP on the ventral surface of the embryo. By virtue of PPR1 expression, all of the ventral mesenchymal cells are potentially responsive to PTHrP, but during early mammary development, PTHrP expression is specific to the mammary epithelial bud. PTHrP therefore acts as a dominant signal, contributing to cell fate decisions leading the ventral mesenchyme cells surrounding the mammary epithelial bud to acquire a dense mammary mesenchyme phenotype and the ability to support mammary morphogenesis. In the absence of PTHrP production by the epidermal keratinocytes, the ventral mesenchyme acquires a dermal phenotype.

A   WILD TYPE   **B**   PTHrP and PTHR KO   **C**   K14 - PTHrP Transgenics

Localized PTHrP Signaling

Mammary Mesenchyme Differentiation

Mammary and Nipple Morphogenesis

No PTHrP Signaling

Failure of Mammary Mesenchyme Differentiation

No Nipple or Mammary Morphogenesis

Difffuse PTHrP Signaling

Inappropriate Mammary Mesenchyme Differentiation

Diffuse Ventral Nipple Formation

Figure 2. Model for the regulation of cell fate by PTHrP-signaling during mammary gland and nipple development. The different colors represent different cell fates: red circles represent mammary epithelial cells, yellow squares represent mammary mesenchyme, green ovals represent dermal mesenchyme, blue ovals represent typical epidermal cells and purple squares represent nipple cells. (A) Normally, the mammary epithelial cells express PTHrP after the bud starts to form. PTHrP signals to the dermal mesenchyme near the developing bud and, as a result, these cells become mammary mesenchyme. The mammary mesenchyme maintains the mammary fate of the epithelial cells, triggers their morphogenesis and induces the overlying epidermis to become the nipple. (B) In the absence of PTHrP signaling no mammary mesenchyme is formed. Therefore, the mammary epithelial cells revert to an epidermal fate, no morphogenesis occurs and the nipple does not form. (C) In the presence of diffuse PTHrP signaling, the entire ventral dermis becomes mammary mesenchyme and the ventral epidermis becomes nipple sheath.

Tet₀ - PTHrP
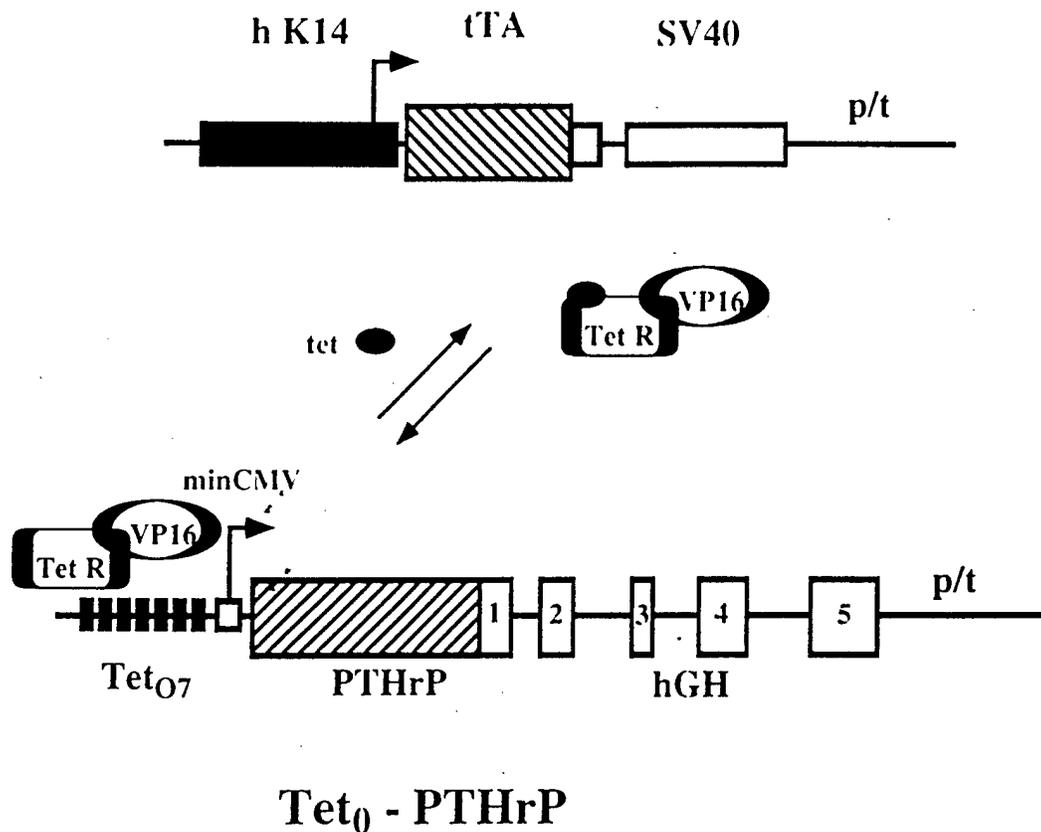
Figure 3. Tetracycline-regulated PTHrP production using a binary tet-off system. In order to introduce tetracycline regulation into our original K14-PTHrP transgenic model, we created two transgenes. The first, as shown on top, consists of the K14 promoter driving expression of the tet-transactivator protein (tTA), which contains elements of the powerful VP16 transcriptional activator. The second transgene, shown on the bottom, consists of the PTHrP coding region placed downstream of a minimal CMV promoter containing 7 tetracycline operon sequences. The minimal CMV promoter has negligible activity itself, but in the presence of tTA, which binds to the tet-operon sequences, the VP16 domain activates transcription of the PTHrP sequences. However, in the presence of tetracycline, tTA changes conformation and can no longer bind to the tet-operon sequences. Therefore, in this system tetracycline acts as an on-off switch. In animals carrying both transgenes, PTHrP is made in cells which normally express K14 in the absence of tetracycline. When tetracycline is added to the water supply of these mice it turns off expression of the transgene and no PTHrP is produced. Hence, this system now adds temporal regulation of PTHrP production to the pre-existing spatial control of PTHrP production afforded by the K14 promoter.
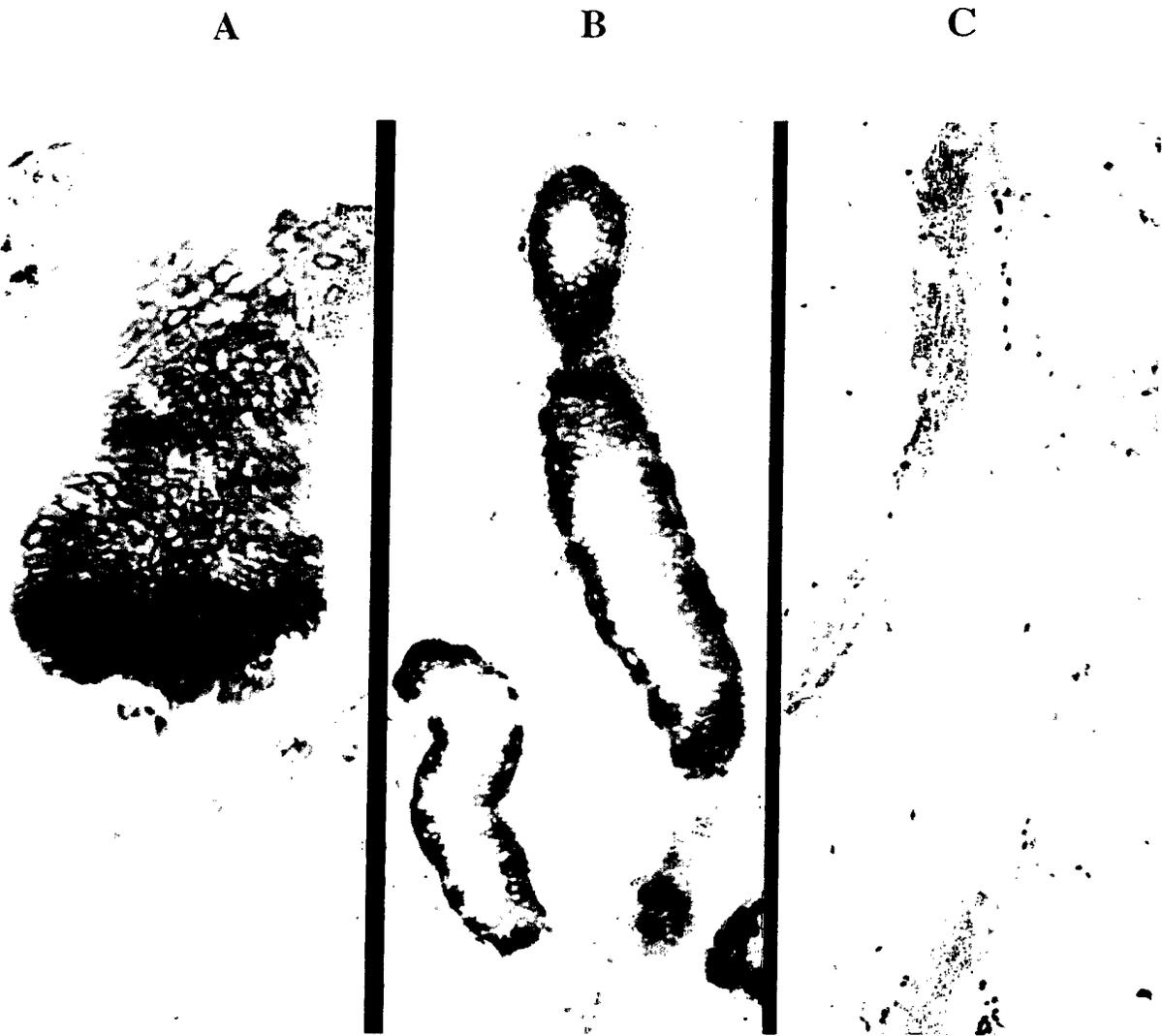
Figure 4. Expression patterns of the K14-tTA transgene in K14-tTA/pTet-
β–galactosidase (β-gal) double transgenic mice. Mammary tissue from double transgenic
mice was harvested and stained with X-gal. Cells that express the K14-tTA transgene
would be expected to turn blue with this procedure, by virtue of them containing β-gal.
In the presence of tetracycline, the expression of β-gal, and hence the blue color, should
be blocked. (A) The mammary rudiment of a double-transgenic embryo at E15, stained
with X-gal. (B) Mammary ducts, stained with X-gal, from an adult double-transgenic
mouse. (C) Mammary ducts, stained with X-gal, from an adult double transgenic mouse
receiving tetracycline. As one can see, the K14-tTA transgene is expressed within all the
mammary epithelial cells before birth (A), and in myoepithelial cells after birth (B).
These findings are identical to the expression patterns of the native K14 gene. The
addition of tetracycline to the water supply of the mice completely and uniformly
suppresses K14-tTA transgene expression in mammary epithelial cells (C).
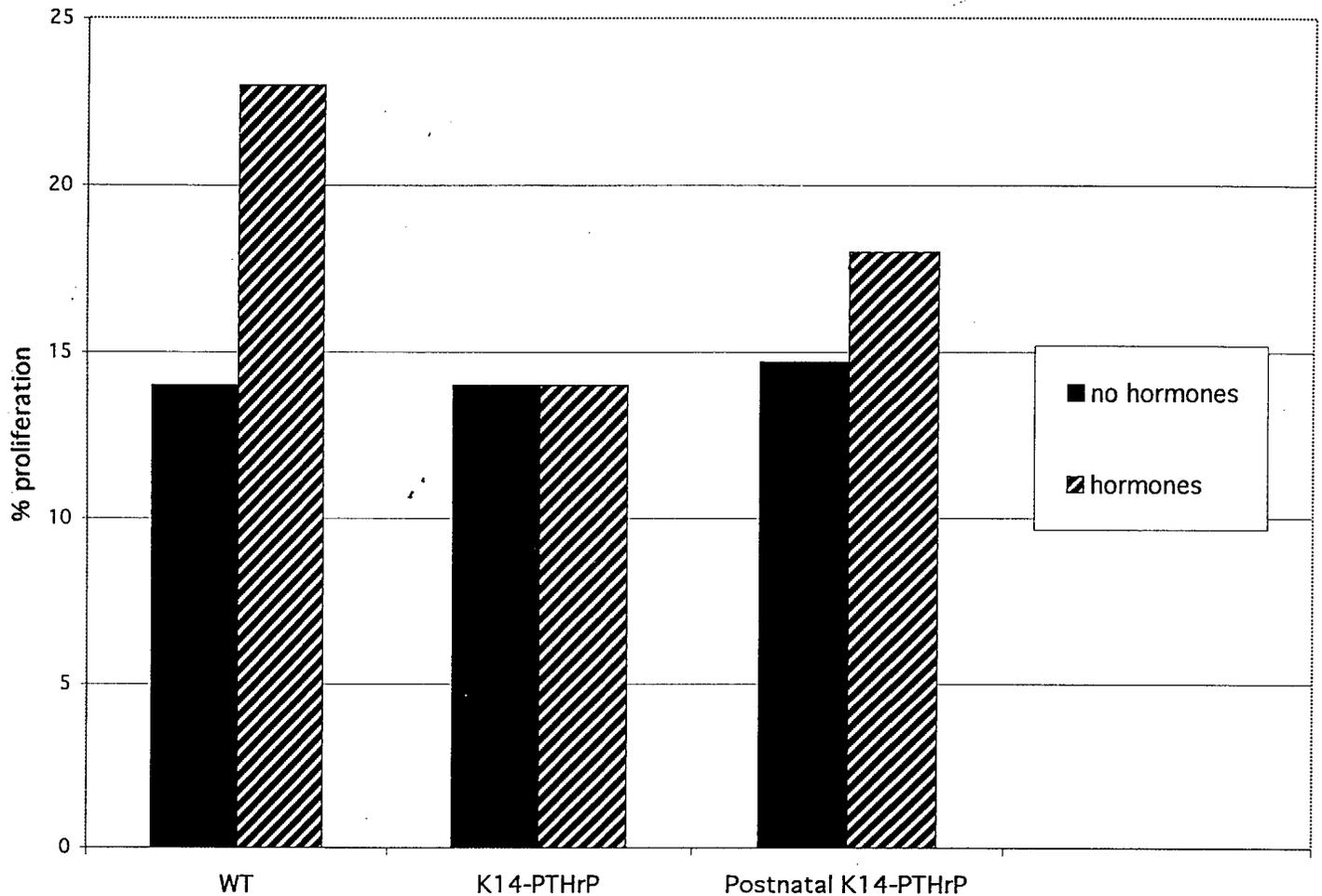
Figure 5. Terminal endbud proliferation in wild-type, K14-PTHrP transgenic and tetracycline-regulated K14-PTHrP mice at baseline and in response to exogenous hormones. Shown are the percentage of epithelial cells within terminal endbuds that incorporated 3H-thymidine. The solid bars represent mice at baseline and the striped bars represent mice that have received estrogen and progesterone for 48 hrs. In wild type mice (WT) the percentage of dividing cells increases in response to hormone injections. Continuous overexpression of PTHrP in the K14-PTHrP transgenic mice does not affect the baseline level of cell proliferation, but it blocks the increase seen in response to hormones. Likewise, when PTHrP is overexpressed only after birth, (postnatal K14-PTHrP), the basal level of proliferation is not diffeent from wild-type and there is no significant increase in cell proliferation after hormone exposure.
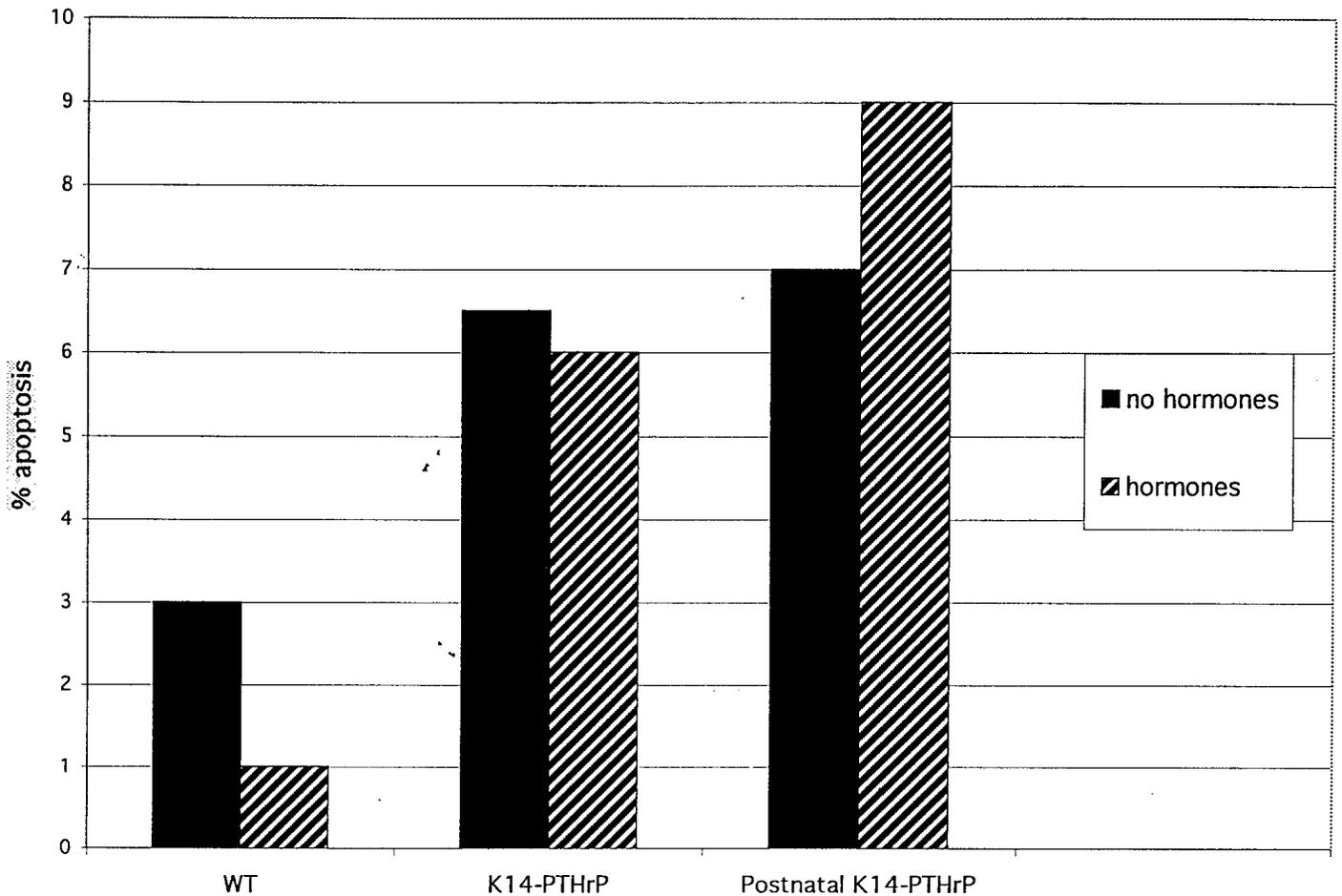
Figure 6. Terminal endbud apoptosis in wild-type, K14-PTHrP transgenic and tetracycline-regulated K14-PTHrP mice at baseline and in response to exogenous hormones. Shown are the percentage of endbud epithelial cells that are undergoing apoptosis as assayed by TUNEL staining. The solid bars represent mice at baseline and the striped bars represent mice that have received estrogen and progesterone for 48 hrs. In wild-type mice (WT) the percentage of apoptotic cells is reduced in response to exogenous hormone injection. In response to continuous overexpression of PTHrP (K14-PTHrP), the basal rate of apoptosis is higher and it does not decline in response to hormones. Likewise, in tetracycline-regulated K14-PTHrP mice, if PTHrP is overexpressed only after birth (postnatal K14-PTHrP), apoptosis rates are elevated and are unresponsive to hormone injections.
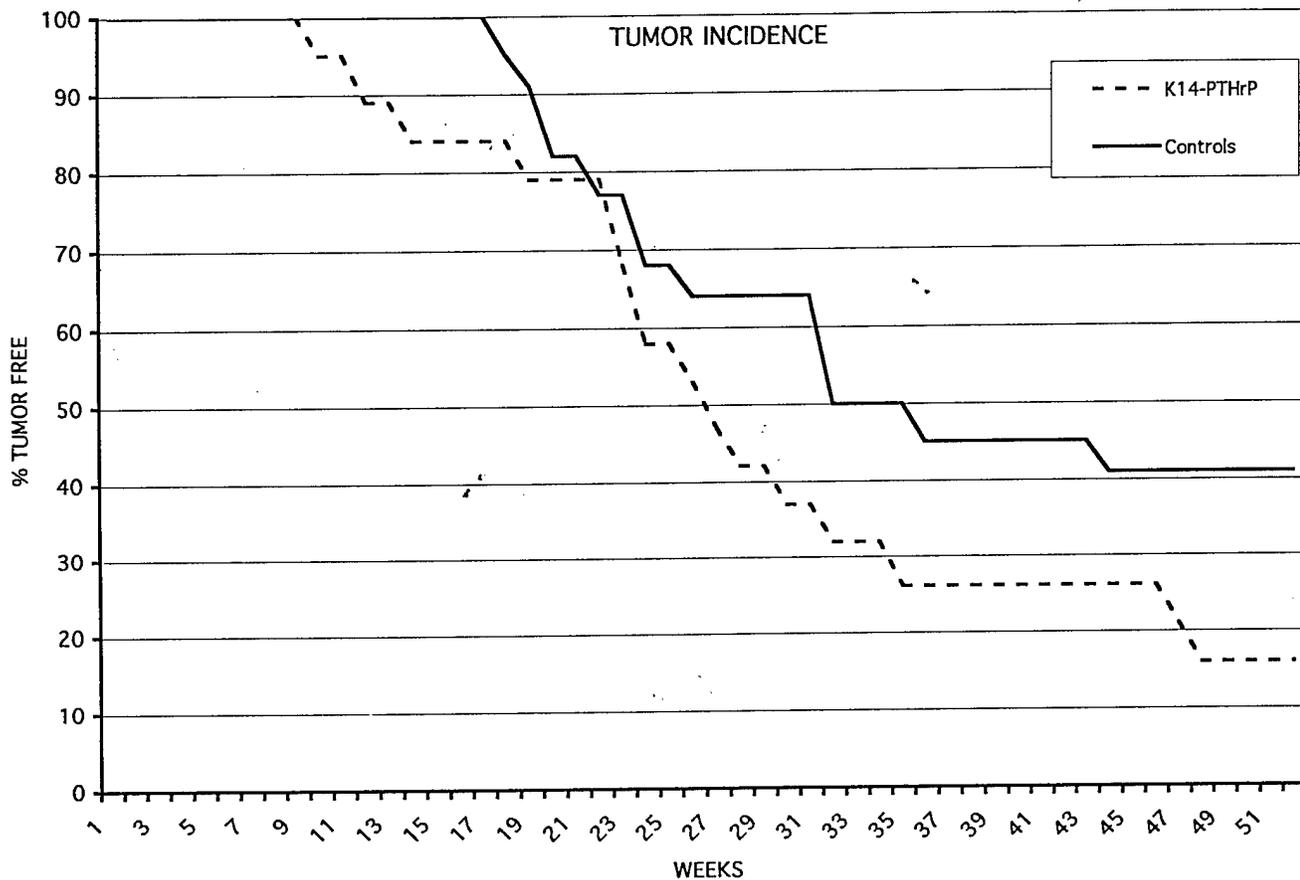
Figure 7. Mammary tumor incidence and latency in K14-PTHrP transgenic and wild-type controls after treatment with DMBA. The above curves plot the percentage of mice remaining tumor-free (y axis) against the time from treatment with DMBA (x axis, in weeks). As one can see, the overall incidence of tumors in the K14-PTHrP transgenic mice appeared to be higher (84% at 1 year) than in the controls (59% at one year). In addition, tumors appeared to develop more rapidly in the K14-PTHrP transgenic cohort. The time to 50% tumor incidence was 27 weeks for the K14 transgenic mice and 32 weeks for the control mice. However, using the log rank test, the differences between these curves just missed statistical significance (p = 0.08).

24

Appendix 1

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

John J. Wysolmerski[1,*,†], William M. Philbrick[1,*], Maureen E. Dunbar[1], Beate Lanske[2], Henry Kronenberg[2], Andrew Karaplis[3] and Arthur E. Broadus[1,4]

[1]Division of Endocrinology and Metabolism, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA
[2]Endocrine Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
[3]Division of Endocrinology and Metabolism, Department of Internal Medicine, Lady Davis Institute for Medical Research, McGill University, Montreal, Quebec, Canada
[4]Department of Molecular and Cellular Physiology, Yale University School of Medicine, 333 Cedar Street, FMP 102, Box 208020, New Haven, CT, USA
*These two individuals contributed equally to this work
†Author for correspondence (e-mail: John-Wysolmerski@Yale.edu)

Accepted 21 January; published on WWW 26 February 1998

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′-CATCACCCACAGGCTAGCGCCAACT (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-CCTTGGGAACTGT.

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-AGCATTCTTGGAGAAC 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′-GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT (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 deoxytransferase 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 μm 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 μg/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× SSC and incubated for 30 minutes in 0.66% N-ethylmaleimide (Sigma Chemical Co., St Louis, Mo) in 2× SSC, rinsed again in 2× 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× Denhardt's solution, 4× SSC, 250 μg/ml tRNA, 100 μg/ml salmon sperm DNA and 50 mM DTT. After hybridization, sections were rinsed in 1× SSC and washed twice in 2× SSC/50% formamide for 5 minutes at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 minutes. Following two rinses in 2× SSC, sections were again washed in 2× 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
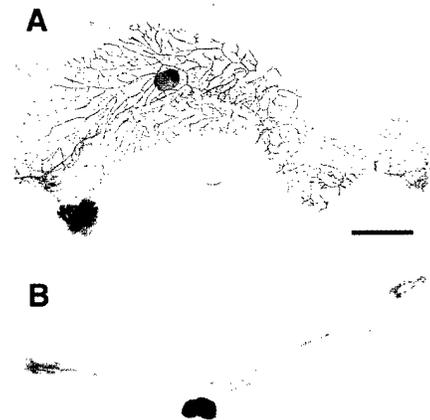

Fig. 1. Whole-mount analysis of mammary glands from col II-PTHrP/PTHrP-null mice and normal littermates. The fourth inguinal mammary glands were resected from 4-month-old normal and col II-PTHrP/PTHrP-null mice, fixed in acid ethanol and stained with carmine aluminum. The normal gland (A) is characterized 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
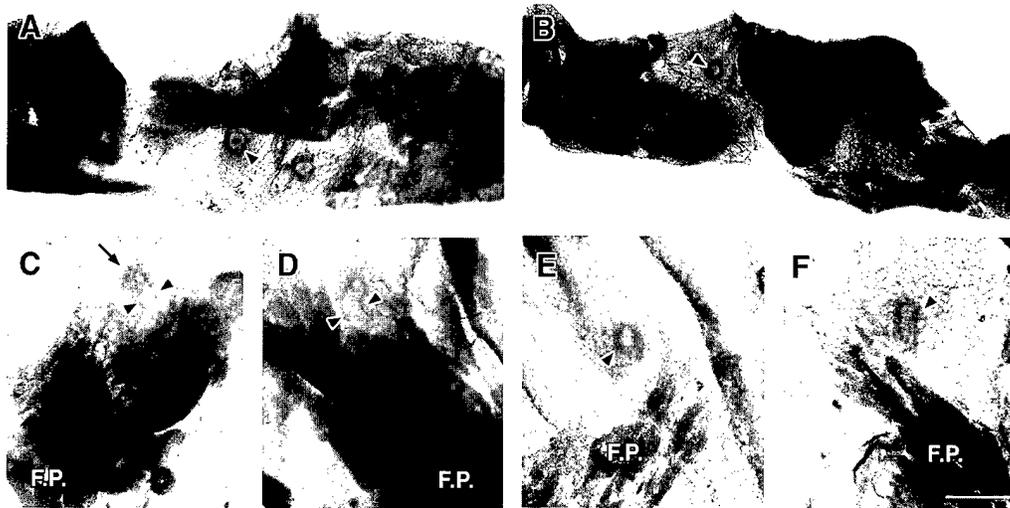
Fig. 2. Comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. The ventral epidermis was dissected from the respective embryos and photographed under low magnification using transmitted light in order to examine the gross structure of the embryonic 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 μm 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
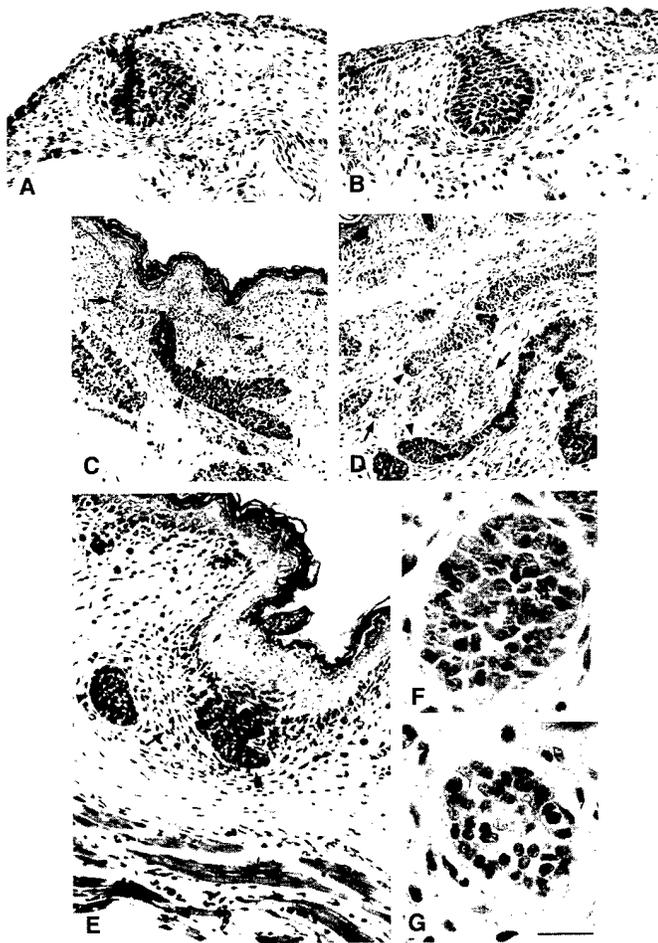
Fig. 3. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. (A,B) Photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15 (3 knockout and 3 wild-type embryos 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
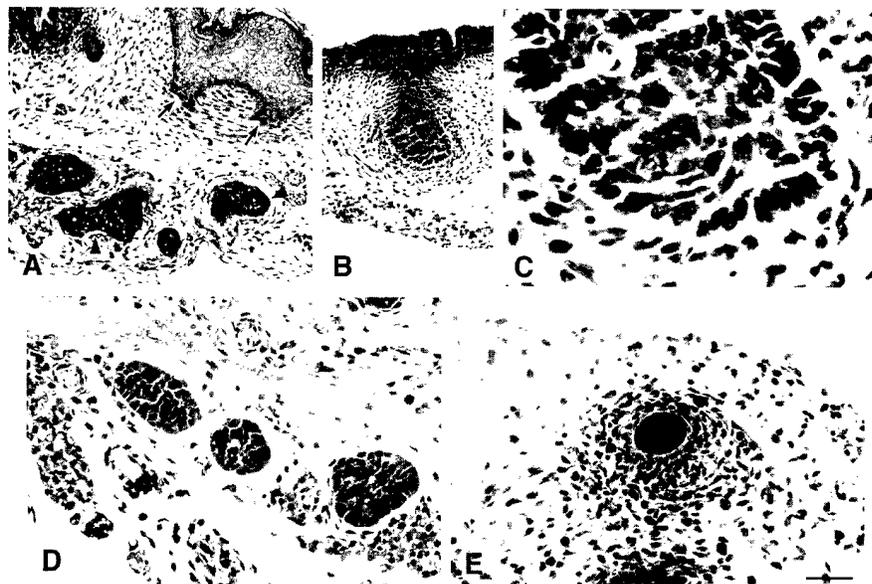
(D) and PTH/PTHrP receptor-knockout (E) mammary glands at E18. Note the lacy, delicate appearance of the stroma surrounding the normal
ducts (D) as they make contact with the mammary fat pad. In contrast, note the condensation of stroma surrounding a rare PTH/PTHrP receptor-
knockout duct (E) that has 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
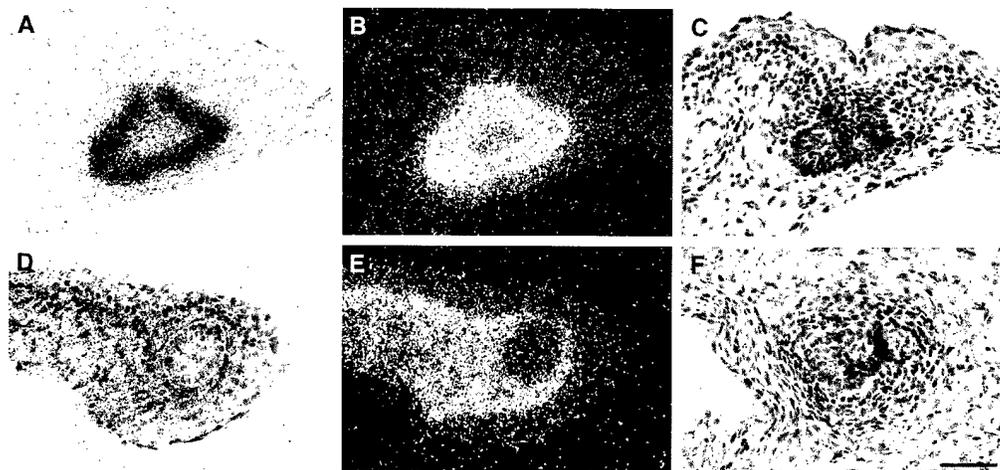
Fig. 5. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. (A-C) In situ hybridization for
PTHrP mRNA in normal mammary rudiments at E16. (A,B) Bright-field and dark-field images, respectively, of the same section hybridized
with antisense probe. (C) Bright-field 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
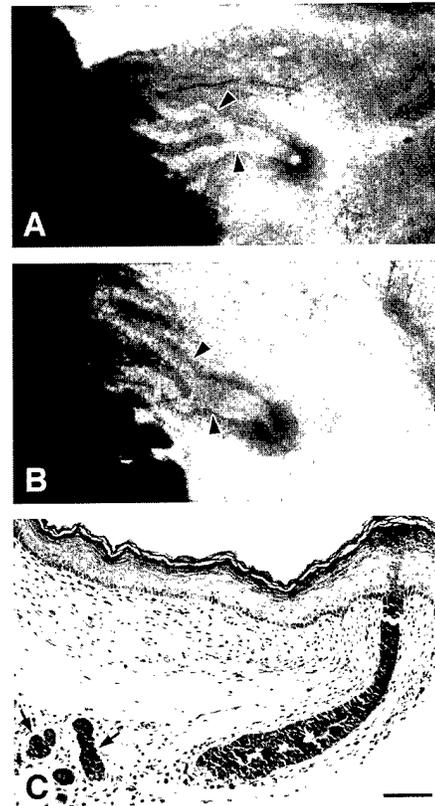

Fig. 6. Mammary development in K14-PTHrP/PTHrP-null mice. (A,B) Photographs of the whole mammary glands of a normal (A) and K14-PTHrP/PTHrP-null neonate (representative of 2 K14-PTHrP/PTHrP-null animals analyzed). The ventral skin was dissected and the mammary glands were viewed under low magnification 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 µm in A,B; 17 µm 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 (1 mg/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.
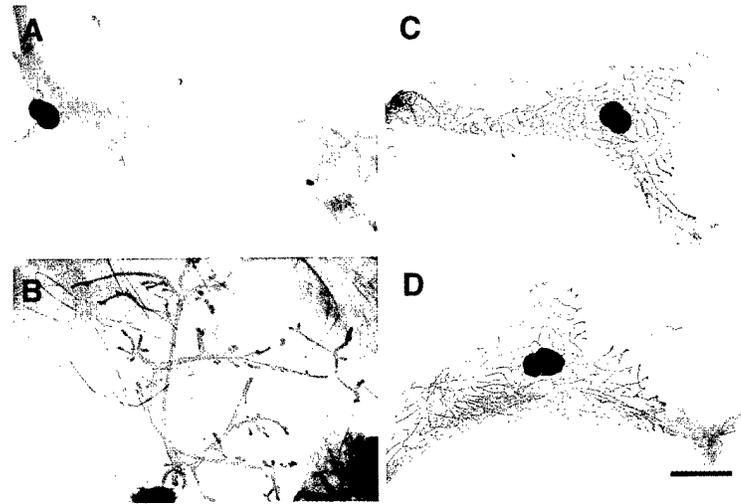
that signals derived from mesenchymal cells are important in
regulating the overall rate of ductular proliferation as well as
the pattern of branching that occurs during the process of
branching morphogenesis (Yang et al., 1995; Witty et al., 1995;
Phippard et al., 1996). We have demonstrated that, during
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.

REFERENCES

**Amling, M. Neff, L. Tanaka, S., Inoue, D., Kuide, K. Weir, E., Philbrick,
W. M., Broadus, A. E. and Baron, R.** (1997). Bcl-2 lies downstream of
parathyroid hormone-related peptide in a signaling pathway that regulates
chondrocyte maturation during skeletal development. *J. Cell Biol.* **136**, 205-
213.
Broadus, A. E. and Stewart, A. F. (1994). Parathyroid hormone-related
protein: structure, processing and physiologic actions. In *The Parathyroids*
(ed. J. P. Bilizekian, M. A. Levine and R. Marcus). New York: Raven Press.
**Budayr, A. A., Halloran, B. R., King, J., Diep, D., Nissenson, R. A. and
Strewler, G. J.** (1989). High levels of a parathyroid hormone-related protein
in milk. *Proc. Natl. Acad. Sci. USA* **86**, 7183-7185.

Cunha, G. R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74**, 1030-1044.

Cunha, G. R., Young, P., Christov, K., Guzman, R. Nandi, S., Talamantes, F. and Thordarson, G. (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* **152**, 195-204.

Freire-Maia, N., and Pinheiro, M. (1994). Ectodermal dysplasias: a clinical classification and causal review. *Am. J. Med. Genet.* **53**, 153-162.

Geise, K., Pagel, J. and Grosschedl, R. (1994). Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors. *Proc. Natl. Acad. Sci. USA* **91**, 3368-3372.

Henderson, J. E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B. M. K., Goltzman, D. and Karaplis, A. C. (1995). Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Molec. Cell. Biol.* **15**, 4064-4075.

Jüppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Hock, J., Potts, J. T., Kronenberg, H. M. and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254**, 1024-1026.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

Kopan, R. and Fuchs, E. (1989). A new look at an old problem: keratins as tools to investigate determination, morphogenesis and differentiation in skin. *Genes Dev.* **3**, 1-15.

Kovacs, C. S., Lanske, B., Hunzelman, J. L., Guo, J., Karaplis, A. C. and Kronenberg, H. M. (1996) Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proc. Natl. Acad. Sci. USA* **93**, 15233-15238.

Kratochwil, K. and Schwartz, P. (1976). Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue of testosterone. *Proc. Natl. Acad. Sci. USA* **73**, 4041-4044.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J. and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**, 1382-1394.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Jüppner, H., Segre, G. V. and Kronenberg, H. M. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science.* **273**, 663-666.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**, 453-463.

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., Kronenberg, H. M. and Segre, G. V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology* **137**, 5109-5118.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E. and Stewart, A.F. (1996) Defining the physiologic roles of parathyroid hormone-related protein in normal physiology. *Physiological Reviews* **76**, 127-173.

Phippard, D. B., Weber-hall, S. J., Sharpe, P. T., Naylor, M. S., Jayatalake, H., Maas, R., Woo, I., Roberts-Clarke, D., Francis-West, P. H., Liu, Y. H., Maxson, R., Hill, R. E. and Dale, T. C. (1996). Regulation of Msx-1,

Msx-2, BMP-2 and BMP-4 during foetal and postnatal mammary gland development. *Development* **122**, 2729-2737.

Propper, A. (1973) Evolution en culture in vitro de l'epithelium mammaire d'embryon de lapin associe au mesoderme d'osieau. *C. R. Acad. Sci. Paris* **277**, 2409-2412.

Propper, A. and Gomot, L. (1973). Control of chick epidermis differentiation by rabbit mammary mesenchyme. *Experientia* **29**, 1543-1544.

Sakakura, T. (1987). Mammary embryogenesis. In *The Mammary Gland: Development, Regulation and Function.* (ed. M. C. Neville and C. W. Daniel) pp. 37-66. New York: Plenum Press.

Sakakura, T. (1991). New aspects of stroma-parenchyma relations in mammary gland differentiation. *Int. Rev. Cytology* **125**, 165-199.

Smith, G. H., Mehrel, T. and Roop, D. R. (1990). Differential keratin gene expression in developing, differentiating, and neoplastic mouse mammary epithelium. *Cell Growth Differ.* **1**, 161-170.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39**, 35-50.

Thiede, M. A. and Rodan, G.A. (1988). Expression of a calcium mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science* **242**, 278-280.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R-G., Parslow, T. G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8**. 2691-2703.

Vortkamp, A., Lee, K., Lanske, B., Segre G. V., Kronenberg H. M. and Tabin, C. J. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Weil, M., Itin, A. and Keshet, E. (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121**, 2419-2428.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. E. and Broadus, A. E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondrol bone formation. *Proc. Natl. Acad. Sci. USA* **93**, 10240-10245.

Witty, J. P., Wright, J. H. and Matresian, L. M. (1995). Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell* **6**, 1287-1303.

Wu, T., Vasavada, R., Yang, K., Massfelder, T., Ganz, M., Abbas, S. K., Care, A. D. and Stewart, A. F. (1996) Structural and physiologic characterization of the mid-region secretory form of PTHrP. *J. Biol. Chem.* **271**, 24371-24381.

Wysolmerski, J. J. and Broadus, A. E. (1994) Hypercalcemia of malignancy: the central role of parathyroid hormone related protein. *Annu. Rev. Med.* **45**, 189-200.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M. and Philbrick, W. M. (1994). Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl. Acad. Sci. USA* **91**, 1133-1137.

Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E. and Philbrick, W. M. (1995). Overexpression of parathyroid hormone related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary development. *Development* **121**, 3539-3547.

Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartman, G., Weidner, K. M., Birchmeier, C. and Birchmeier, W. (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131**, 215-226.

Appendix 2

Parathyroid hormone-related protein signaling is necessary for sexual dimorphism during embryonic mammary development

Maureen E. Dunbar[1], Pamela R. Dann[1], Gertraud W. Robinson[2], Lothar Hennighausen[2], Jian-Ping Zhang[1] and John J. Wysolmerski[1,*]

[1]Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520-8020, USA
[2]Laboratory of Genetics and Physiology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0822, USA
*Author for correspondence (e-mail: John.Wysolmerski@Yale.edu)

SUMMARY

Male mice lack mammary glands due to the interaction of circulating androgens with local epithelial-mesenchymal signaling in the developing mammary bud. Mammary epithelial cells induce androgen receptor (AR) within the mammary mesenchyme and, in response to androgens, the mesenchyme condenses around the epithelial bud, destroying it. We show that this process involves apoptosis and that, in the absence of parathyroid hormone-related protein (PTHrP) or its receptor, the PTH/PTHrP receptor (PPR1), it fails due to a lack of mesenchymal AR expression. In addition, the expression of tenascin C, another marker of the mammary mesenchyme, is also dependent on PTHrP. PTHrP expression is initiated on E11 and, within the ventral epidermis, is restricted to the forming mammary epithelial bud. In contrast, PPR1 expression is not limited to the mammary bud, but is found generally within the subepidermal mesenchyme. Finally, transgenic overexpression of PTHrP within the basal epidermis induces AR and tenasin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP can induce the ventral mesenchyme to express mammary mesenchyme markers. We propose that PTHrP expression specifically within the developing epithelial bud acts as a dominant signal participating in cell fate decisions leading to a specialized mammary mesenchyme.

Key words: Androgen receptor, Tenascin C, Epithelial-mesenchymal interaction, Apoptosis, PTH/PTHrP receptor, Mouse

INTRODUCTION

The development of many epithelial organs depends on a series of sequential and reciprocal interactions between epithelial cells and adjacent mesenchymal or stromal cells (Thesleff et al., 1995; Birchmeier and Birchmeier, 1993). The mammary gland is an example of an organ where these epithelial-mesenchymal interactions play a critical role, especially during embryonic development (Sakakura, 1987; Cunha, 1994; Robinson et al., 1999). In mice, mammary development commences with the formation of 5 pairs of epithelial buds located on the ventral surface of the embryo. Each bud begins as a localized thickening of the epidermis first noted on embryonic day 10 (E10, appearance of the vaginal plug = E0), and between E10 and E12-13 this initial placode invaginates into the underlying mesenchyme and the mammary epithelial cells organize themselves into a characteristic "light-bulb" shape (Sakakura, 1987; Robinson et al., 1999). Initially, the mammary mesenchyme is indistinguishable from the ventral dermal mesenchyme, but by the time the mammary epithelial bud is fully formed, it is invested by several layers of mesenchymal cells that are morphologically and functionally distinct from the surrounding dermal mesenchyme (Sakakura, 1987). Recombination experiments have documented that the

mammary epithelium and mesenchyme contribute to the formation of each other during mammary bud development and, within the mature mammary bud (through E14-15), each compartment retains the capacity to induce fully the formation of the other (Propper and Gomot, 1967; Heuberger et al., 1982; Cunha et al., 1995).

One of the best-studied aspects of epithelial-mesenchymal interaction during murine mammary development is the androgen-mediated destruction of the mammary bud in males. In male embryos, beginning on E14, the mammary mesenchyme condenses around the neck of the epithelial bud and disrupts the stalk connecting the mammary bud to the overlying epidermis (Turner and Gomez, 1933; Sakakura, 1987). In most strains of mice, the mammary epithelial remnant subsequently degenerates and no nipple is formed, explaining the lack of nipples and mammary glands in adult males (Sakakura, 1987). However, the degree to which the epithelial remnant is destroyed is variable and, in rats, while the stalk is destroyed, there is little degeneration of the remaining epithelium. Several studies have shown that this process occurs as a result of the secretion of androgens by the fetal testes, which act directly on the mammary mesenchyme to trigger its condensation (Raynaud and Frilley, 1947; Raynaud, 1949; Hoshino, 1965; Neuman et al., 1970;

Kratochwil, 1977; Kratochwil and Schwartz, 1977). It has also been demonstrated that the epithelium is a necessary participant in this process, which instructs the mesenchyme to express androgen receptors (Heuberger et al., 1982). Although it is clear that the destruction of the mammary buds by androgens is dependent on a bi-directional flow of information between epithelium and mesenchyme, the identity of the signals exchanged is not known.

One molecule that has recently been implicated in epithelial-mesenchymal interactions at several sites during development is parathyroid hormone-related protein (PTHrP; Wysolmerski and Stewart, 1998). PTHrP was originally discovered as the cause of hypercalcemia in patients with a variety of cancers and it derives its name from a common ancestry shared with parathyroid hormone (PTH; Philbrick et al., 1996). PTHrP also shares the same family of G-protein-coupled receptors with PTH. The prototype of this family is the Type I PTH/PTHrP receptor (PPR1), which appears to subserve the majority of the known functions of PTHrP (Jüppner et al., 1991: Philbrick et al., 1996). During development, PTHrP has been shown to be produced by many developing epithelial structures, while the PPR1 is expressed on adjacent mesenchymal cells, suggesting a role for PTHrP in epithelial-to-mesenchymal signaling, a notion recently confirmed by several PTHrP transgenic and knockout mouse models (Lee et al., 1995; Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994, 1995, 1998).

We have recently shown that PTHrP is necessary for mammary gland development. In the absence of PTHrP or its receptor, there is a failure of the initiation of ductal branching morphogenesis and nipple formation during embryonic mammary development (Wysolmerski et al., 1998). In PTHrP or PPR-1 knockout embryos the mammary bud initially forms normally, but it fails to undergo the primary growth spurt, and the mammary epithelial cells degenerate and disappear before birth (Wysolmerski et al., 1998). Overexpression of PTHrP within the mammary gland also affects branching morphogenesis, leading to an impairment of hormonally stimulated ductal proliferation and side-branching during puberty and early pregnancy (Wysolmerski et al., 1995). Both during embryonic development and during puberty, PTHrP is produced by epithelial cells, while the PPR1 resides on mesenchymal cells during embryonic development and fat pad and periductal stromal cells during puberty (Wysolmerski et al., 1998; Dunbar et al., 1998). Given the patterns of expression of PTHrP and the PPR1 during the early stages of mammary development, and given the requirement for epithelial-mesenchymal interaction in the androgen-mediated destruction of the mammary bud, we initiated a study of PTHrP's possible involvement in this process.

In this report, we document that PTHrP and the PPR1 are necessary for the normal sexual dimorphism seen during murine mammary development. PTHrP is expressed specifically within the epithelial cells of the mammary bud concurrent with its formation, and we identify it to be an epithelial signal responsible for inducing androgen receptor and tenascin C expression within the mammary mesenchyme. Ectopic expression of PTHrP within the fetal epidermis results in the expression of mammary mesenchyme markers in the fetal dermis. These findings suggest that PTHrP participates in regulating the mesenchymal cell fate decisions that result in the formation of a specialized mammary mesenchyme.

MATERIALS AND METHODS

Mouse strains
The disrupted PTHrP and PPR1 alleles were progressively bred onto a CD-1 background to improve litter size and embryo survival, and mice heterozygous for these alleles were mated to produce homozygous PTHrP- and PPR1-null embryos (appearance of vaginal plug=day 0). Wild-type littermates were used as normal controls. Embryos were removed from the uterus and genotyped using the polymerase chain reaction as described previously (Wysolmerski et al., 1998). Keratin 14 (K14) is expressed in specific subsets of epithelial cells, including fetal mammary epithelial cells and basal keratinocytes of the skin, and we have previously documented that the K14 promoter can successfully target PTHrP transgene expression to these cells (Wysolmerski et al., 1998). In the present study, K14-PTHrP embryos were identified as reported previously, and K14-PTHrP/PTHrP-null embryos were produced by mating mice both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null allele with mice heterozygous for the disrupted PTHrP allele (Wysolmerski et al., 1998). All embryos were sexed both by visual inspection of the gonads and by amplification of a 240 bp fragment of the SRY gene by PCR (Wysolmerski et al., 1998).

Histology/immunohistochemistry
After harvesting, embryos were fixed in 4% paraformaldehyde at 4°C for 12 hours. The ventral skin was removed and the intact mammary glands were dissected and embedded in paraffin. Appropriate sections were identified by serial sectioning and hematoxylin and eosin staining, and immunohistochemistry was performed using standard techniques. The androgen receptor antibody is a rabbit polyclonal and was the kind gift of Dr Gail Prins (The University of Illinois at Chicago, Chicago, Illinois). Primary incubations with the androgen receptor antibody were performed at 4°C for 12 hours at a concentration of 0.5 or 1.0 µg/ml and were preceded by boiling of the sections for 30 minutes in 0.01 M citrate buffer pH 6.0. Competition experiments were performed with AR21, which consists of the first 21 amino acids of the androgen receptor and contains the antibody epitopes, and with peptide AR462, which consists of amino acids 462-478 and does not contain the epitopes (peptides courtesy of Dr Prins). The tenascin C antibody is also a rabbit polyclonal antiserum and was the kind gift of Drs Toshimichi Yoshida and Teruyo Sakakura (Mie University, Tsu, Japan). Primary incubations were performed at a concentration of 2.5 or 5.0 µg/ml at room temperature for 1 hour and were preceded by a 10 minute incubation in 0.1% trypsin in 0.1% (w/v) calcium chloride pH 7.8. Primary antibodies were detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and either 3, 3′ diaminobenzidine or TrueBlue™ peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as chromagens. Apoptosis was detected by terminal deoxytransferase labeling (TUNEL assay) utilizing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Indianapolis, IN).

In situ hybridization
In situ hybridization on paraffin sections was performed as previously described (Dunbar et al., 1998; Wysolmerski et al., 1998). Probes were generated from a 349 bp genomic fragment of the mouse PTHrP gene and a 238 bp cDNA fragment of the type I PTH/PTHrP receptor gene (Dunbar et al., 1998; Wysolmerski et al., 1998). Whole-mount in situ hybridization was performed using a protocol kindly provided by Dr Trevor Dale (Phippard et al., 1996). In brief, embryos were harvested, fixed for 2 hours in 4% paraformaldehyde at room temperature, treated with proteinase K (20 µg/ml) for 10-15 minutes

at room temperature and postfixed in 4% paraformaldehyde/0.1% glutaraldehyde for 20 minutes at room temperature. The embryos were then hybridized with digoxigenin-labeled riboprobes for PTHrP and PPR1 generated from the templates described above using the Genius kit (Boehringer Mannheim, Indianapolis, IN). The hybridization buffer consisted of 50% formamide, 1.3× SSC, 5 mM EDTA, 0.2% Tween 20, 0.5% CHAPS and 50 μg/ml yeast RNA and the hybridization was at 70°C overnight. Samples were then washed twice in hybridization buffer for 30 minutes at 70°C, once in 1:1 hybridization buffer:TBST at 70°C for 20 minutes and twice in TBST at room temperature for 30 minutes. Following these washes, the embryos were incubated in blocking solution consisting of 10% sheep serum and 1 mg/ml BSA in TBST for 3 hours at room temperature and then were incubated with anti-digoxigenin antiserum (Genius kit, Boehringer Mannheim, Indianapolis, IN) overnight at 4°C. The color reaction was performed according to the manufacturer's protocol and signals developed between 1 and 2 hours.

RESULTS

PTHrP and the PTH/PTHrP receptor are necessary for sexual dimorphism during murine mammary development

In order to ascertain if PTHrP signaling contributed to the androgen-mediated destruction of the male mammary bud, we examined the gross appearance of the mammary buds in PTHrP- and PPR 1-knockout mice. We performed this analysis on male embryos at E15, a time point at which the destruction of the mammary buds should normally be well advanced (Sakakura, 1987). First, we examined 48 male embryos resulting from crosses between heterozygous PTHrP-null parents. In all 10 wild-type embryos, the mammary buds were either completely absent or consisted of very small remnants. In stark contrast, in each homozygous PTHrP-knockout embryo, all mammary buds were present, well preserved and indistinguishable from those observed in female embryos. There was little evidence of haplotype insufficiency, since only one of 28 heterozygous PTHrP-knockout embryos failed to demonstrate the expected destruction of the mammary buds. We next examined 10 male PPR 1-knockout embryos and found that they uniformly also had the abnormal persistence of mammary buds at E15.

The histological findings in these embryos are shown in Fig. 1. Fig. 1A shows the typical appearance of a wild-type female mammary bud at E15. In contrast, at E15, the wild-type male bud is actively being destroyed (Fig. 1B). There is extensive mesenchymal condensation above the epithelial remnant in the region where the bud stalk appears to be degenerating. The stalk has been interrupted and the epithelial remnant, which is misshapen and degenerating (see TUNEL data below), is no longer connected to the epidermis. However, in PTHrP- and PPR 1-knockout males (Fig. 1C,D), the mammary buds appear similar to those seen in female embryos. In these embryos, there is no mesenchymal cell condensation, and the mammary mesenchyme continues to consist of several layers of cells arrayed concentrically around the epithelial bud. In addition, the epithelial stalk is intact, and the mammary epithelial cells maintain their connection with the epidermis. The mutant male buds persist until E16-E17, at which point they fail to undergo the initial round of branching morphogenesis and instead degenerate, findings identical to that previously described for
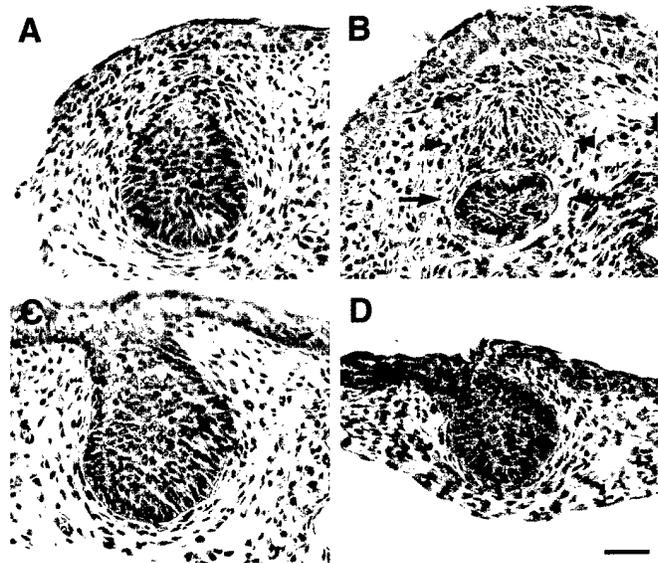

Fig. 1. Failure of the androgen-mediated destruction of the mammary buds in PTHrP- and PPR1-knockout embryos. Hematoxylin- and eosin-stained sections through E15 mammary buds taken from a wild-type female embryo (A), a wild-type male embryo (B), a PTHrP-knockout male embryo (C) and a PPR1-knockout male embryo (D). In the normal male embryo (B), the mesenchymal cells have condensed around the degenerating epithelial stalk (between arrowheads) and the epithelial remnant is misshapen and disconnected from the epidermis (arrows). In contrast, note the lack of mesenchymal condensation and the well preserved epithelial stalks in C and D. Scale bar, 110 μm.

female PTHrP and PPR-1 knockout mammary rudiments (data not shown) (Wysolmerski et al., 1998).

The destruction of the mammary bud in male embryos is an example of programmed cell death (Fig. 2). In the wild-type male bud at E15 (Fig. 2A), there is widespread TUNEL staining in the region of the degenerating epithelial stalk. This appears to involve both the epithelial cells of the stalk and the mesenchymal cells within the androgen-induced condensation. In addition, there is evidence of apoptosis occurring within the epithelial remnant that lies beneath the epidermis. In contrast, in PTHrP-knockout males (Fig. 2B), there is no apoptosis. Similar results were obtained in PPR-1 knockout embryos and, in both strains of knockout mice, the lack of TUNEL-staining was identical to the results obtained with wild-type female embryos (results not shown). Therefore, in the absence of PTHrP or the Type 1 PTH/PTHrP receptor, the pattern of sexual dimorphism normally observed during early mammary development is abolished.

PTHrP and PPR1 are necessary for androgen receptor and tenascin C expression in the dense mammary mesenchyme

The androgen-mediated destruction of the mammary bud is dependent on the presence of functional androgen receptors within the dense mammary mesenchyme, and the expression of these receptors is induced by signals from the mammary epithelium (Heuberger et al., 1982; Sakakura, 1987). The absence of an androgen response in the PTHrP- and PPR1-knockout buds combined with the epithelial expression of
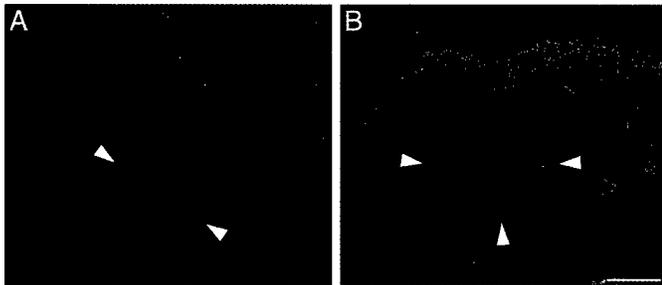
Fig. 2. Programmed cell death in male mammary buds at E15.
(A) Results of a TUNEL assay performed on a section through a
wild-type male mammary bud. Apoptotic nuclei stain bright green
whereas normal nuclei stain pale green. Note the multitude of
apoptotic nuclei in the region of the stalk and mesenchymal
condensation. Note that there is apoptosis also occurring in the
epithelial remnant (between arrowheads). (B) Results of a TUNEL
assay performed on a section through a PTHrP-knockout male
mammary bud. Note that the mammary bud (outlined by arrowheads)
is well preserved and that there is no apoptosis, as demonstrated by
the lack of bright green nuclei, in either the epithelial bud or in the
mammary mesenchyme. Scale bar, 80 μm.

PTHrP and the mesenchymal expression of the PPR1 (Dunbar
et al., 1998; Wysolmerski et al., 1998), led us to posit that
PTHrP might be the epithelial signal responsible for the
induction of androgen receptor expression within the dense
mammary mesenchyme. To investigate this possibility, we
examined androgen receptor expression in the mammary buds
of wild-type female embryos and male and female PTHrP- and
PPR1-knockout embryos at E15 by immunohistochemistry
(Fig. 3A-C). In the wild-type bud (Fig. 3B), one can appreciate
the intense nuclear staining for androgen receptor in the cells
comprising the dense mammary mesenchyme. There is no
staining in the general dermal mesenchyme. This pattern of
androgen receptor localization is identical to that seen in
previous studies using [³H]testosterone autoradiography
(Heuberger et al., 1982). In contrast, this staining pattern is

absent in the PTHrP- (Fig. 3A) or PPR1-knockout (Fig. 3C)
buds. In these glands, there are only occasional nuclei that stain
weakly for androgen receptor within the mesenchymal cells
closest to the epithelial basement membrane. The absence of
androgen receptor staining appears to be specific for the
mammary mesenchyme, for androgen receptor staining is
normal within the testes of Col II-PTHrP/PTHrP-null (Col II-
rescued) mice that lack PTHrP in all tissues except the skeleton
(data not shown) (Majdic et al., 1995; Philbrick et al., 1998;
Wysolmerski et al., 1998). Furthermore, the development of the
Wolffian ducts and the descent of the fetal testes are normal in
the absence of either PTHrP or the PPR1, demonstrating an
intact androgen response in these tissues (Gilbert, 1994;
Grumbach and Conte, 1992).

Androgen receptor expression is one of the characteristics
of the mammary mesenchyme that sets it apart from the
dermal mesenchyme (Sakakura, 1987), so that the absence
of androgen receptor expression within the mammary
mesenchyme of PTHrP- and PPR1-knockout embryos
suggested that there might be more fundamental defects in
the differentiation of these cells. The other classic marker
of the specialized mammary mesenchyme is tenascin C
(Sakakura, 1987). Therefore, we next examined PTHrP- and
PPR1-knockout mammary buds for the expression of this
extracellular matrix protein by immunohistochemistry (Fig.
3D-F). The results were similar to those seen with respect to
androgen receptor expression. The wild-type epithelial bud
(Fig. 3E) was surrounded by a halo of tenascin C within the
extracellular matrix of the dense mammary mesenchyme, but
not within the general dermal matrix. In contrast, there was
no tenascin C expression surrounding the PTHrP- (Fig. 3D)
and PPR1- knockout buds (Fig. 3F), suggesting that, in the
absence of PTHrP or PPR1, the dense mammary mesenchyme
does not differentiate properly. As with androgen receptor
expression, there did not appear to be a generalized defect in
tenascin C expression, as there was ample tenascin staining
in the developing bones of knockout embryos (data not
shown) (Erickson and Bourdon, 1989).

Fig. 3. Androgen receptor and tenascin C staining of E15 mammary buds.
(A-C) Sections stained for androgen receptor and (D-F) represent sections stained for tenascin C. (A,D) Sections through male PTHrP-knockout mammary buds. (B,E) Sections through female wild-type buds. (C,F) Sections through male PPR1-knockout mammary buds. Again, note the well-preserved mammary buds in the male knockouts. There is prominent nuclear staining for androgen receptor in the dense mammary mesenchyme of the wild-type bud in B, but little or no androgen receptor staining in the PTHrP-knockout bud in A and the PPR-1-knockout bud in C. Likewise, there is prominent staining for tenascin C in the extracellular matrix surrounding the wild-type bud (E), but an absence of similar staining in the knockouts (D,F). Scale bar, 120 μm.
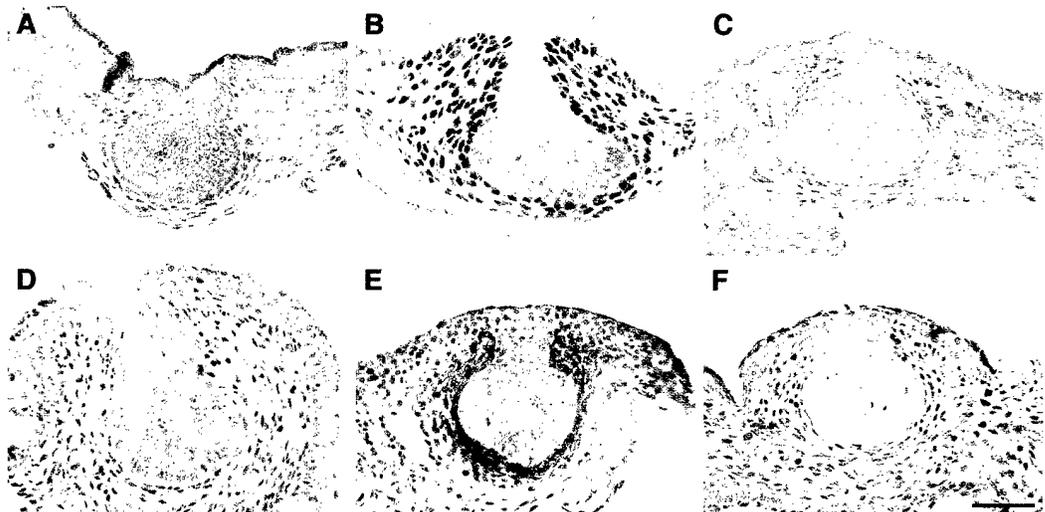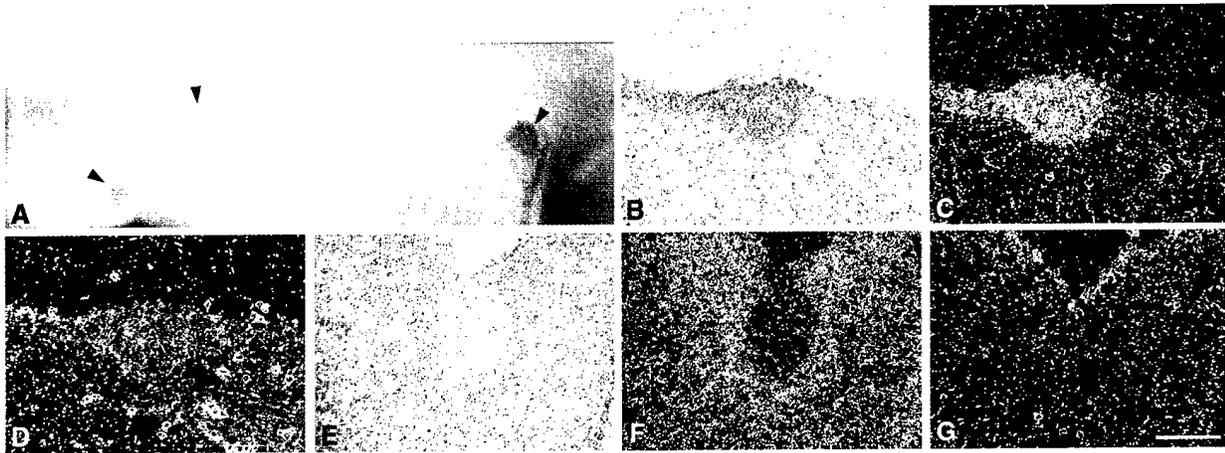
Fig. 4. Initiation of PTHrP expression in developing mammary buds. (A) A representative photograph of whole-mount in situ hybridization experiments using PTHrP antisense probe on a late E11 embryo. The photograph shows a magnified view of the ventral-lateral surface of the embryo. PTHrP expression is not seen within the general epidermis, but is limited to the mammary buds (arrowheads). (B-D) In situ hybridization for PTHrP on sections through mammary buds taken from E12 embryos. (B,C) Bright-field and dark-field views of the same section, which was hybridized to a PTHrP antisense probes. (D) Section hybridized to a PTHrP sense probe. Note that PTHrP is expressed within the epithelial cells of the mammary bud. (E-G) In situ hybridization for the PPR1 on sections through mammary buds taken from E12 embryos. (E,F) Bright-field and dark-field views of the same section, which was hybridized to a PPR1 antisense probes. (G) Section hybridized to a PPR1 sense probe. Note that the PPR1 is expressed throughout the ventral dermal mesenchyme as well as within the dense mammary mesenchyme. Scale bars, (A) 380 μm; (B-G) 150 μm.

PTHrP is expressed specifically within the forming mammary epithelium

We reasoned that, if PTHrP were to participate in regulating the differentiation of the dense mammary mesenchyme, it should be expressed early during the formation of the mammary bud. In mice, this process is initiated on E10 and is complete by E14-15. We have previously demonstrated that PTHrP is expressed within the mammary epithelium in the fully formed mammary bud (Dunbar et al., 1998; Wysolmerski et al., 1998). To detect the onset of PTHrP expression during the formation of the mammary bud, we performed whole-mount in situ hybridization on wild-type embryos from E10-E12. There was no expression of PTHrP in the ventral epidermis until late on E11, after the mammary buds had already begun to form and, by late E11-E12, there was strong and specific hybridization for PTHrP within the developing mammary buds (Fig. 4A). In situ hybridization on sections through developing mammary buds confirmed these findings, demonstrating that PTHrP was expressed in the mammary epithelial cells invaginating into the underlying mesenchyme (Fig. 4B-D). There was little, if any, expression of PTHrP within the ventral epidermis apart from the mammary buds at these stages. These findings are identical to those obtained by other investigators in whole-mount in situ experiments performed on E13 embryos (K. Lee and G. Segre, personal communication). PPR1 expression was found throughout the ventral mesenchyme both underlying the epidermis and surrounding the mammary buds (Fig. 4E-G).

Re-expression of PTHrP re-establishes sexual dimorphism

PTHrP and PPR1 are both expressed within the embryo as early as the morula stage. Therefore, it is possible that the changes that we observed in the knockout embryos were not
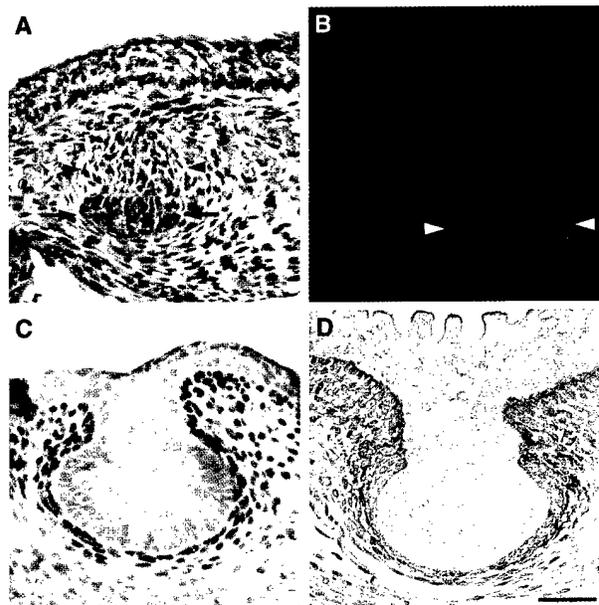

Fig. 5. Return of sexual dimorphism in K14-PTHrP/PTHrP-null embryos. (A) Histology of a mammary bud taken from a male K14-PTHrP/PTHrP-null embryo at E15. Note the typical mesenchymal condensation around the epithelial stalk (between arrowheads) and the degenerating epithelial remnant (between arrows) (compare with Fig. 1B). (B) TUNEL assay performed on a male K14-PTHrP/PTHrP-null embryo at E15. Note the return of the apoptotic response in both the mammary mesenchyme and the epithelial remnant (arrowheads). (C) Androgen receptor staining in a female K14-PTHrP/PTHrP-null embryo at E15. (D) Tenascin C staining in a female K14-PTHrP/PTHrP-null embryo at E15. Note that with the restoration of PTHrP to the mammary epithelium, both molecules are again induced within the mammary mesenchyme. Scale bar,100 μm.

the result of the loss of PTHrP-signaling from mammary epithelium to mammary mesenchyme during the formation of the mammary bud, but were instead the consequence of earlier changes in mesenchymal patterning (de Stolpe et al., 1993; Behrendtsen et al., 1995). In order to demonstrate a direct link between PTHrP production by the mammary epithelium and mesenchymal cell differentiation, we utilized transgenic mice overexpressing PTHrP under the control of the keratin 14 promoter (K14-PTHrP mice) to restore PTHrP to the mammary epithelium of PTHrP-knockout embryos (Wysolmerski et al., 1998). We have previously shown that this promoter directs transgene expression to the epithelial cells of the fetal mammary gland and have recently observed that a K14-driven β-galactosidase transgene is expressed within the mammary bud as early as E12 (P. R. D. and J. J. W., unpublished results). Therefore, we reasoned that a K14-transgene replacement strategy would be expected to duplicate the normal expression of PTHrP within the mammary bud. The K14-PTHrP transgene was bred onto a homozygous PTHrP-null background, producing embryos (K14-PTHrP/PTHrP-null) that were devoid of PTHrP in all tissues except for the sites of K14 expression (such as mammary epithelial cells). As depicted in Fig. 5, the reintroduction of PTHrP expression within the mammary epithelium resulted in a return of the androgen-mediated destruction of the mammary buds and re-established androgen and tenascin C expression within the dense mammary mesenchyme. On a gross level, at E15, male K14-PTHrP/PTHrP-null embryos possessed only remnants of mammary buds. Histologically, these buds demonstrated the typical features of the androgen-mediated response (Fig. 5A), and TUNEL staining revealed a return of the apoptotic response (Fig. 5B). Androgen receptor (Fig. 5C) and tenascin C (Fig. 5D) staining of female K14-PTHrP/PTHrP-null mammary buds at E15 showed the expected pattern of expression of these markers in the mammary mesenchyme (compare Fig. 5C,D with Fig. 3A,D). These results demonstrate that it is the expression of PTHrP within the epithelium during mammary bud formation that is critical for the normal pattern of sexual dimorphism and suggest that PTHrP signaling from the epithelium to the mesenchyme during early mammary gland development is required for full differentiation of the mammary mesenchyme.

Ectopic expression of PTHrP induces ectopic expression of mammary mesenchyme markers

The mammary phenotypes of the PTHrP- and PPR1-knockout embryos, the specific expression of PTHrP within the mammary epithelial buds and the general expression of the PPR1 within the subepidermal mesenchyme suggested that PTHrP might serve as a dominant signal regulating the fate or course of differentiation of the ventral mesenchyme. We hypothesized that the presence of PTHrP in the mammary bud might lead to the acquisition of the mammary mesenchyme phenotype, while the absence of PTHrP within the general epidermis would be associated with a dermal mesenchyme phenotype. If this were true, ectopic expression of PTHrP within the epidermis might lead to the formation of mammary mesenchyme instead of dermis. In order to test this idea, we again turned to K14-PTHrP transgenic mice. As demonstrated in Fig. 6A, K14 is expressed not only within the mammary epithelium but also within the basal keratinocytes of the fetal

epidermis (Kopan and Fuchs, 1989). Therefore, we stained the epidermis of K14-PTHrP transgenic and wild-type littermates for the expression of androgen receptor and tenascin C. In wild-type embryos, we could not detect androgen receptor expression within the ventral dermal mesenchyme but, in the ventral epidermis of K14-PTHrP transgenics, there was clear expression of androgen receptor within the nuclei of the dermal mesenchymal cells closest to the epidermis (Fig. 6B,C). As has been previously reported, there was some tenascin C expression within the basement membrane of the ventral epidermis in wild-type embryos, especially around developing hair follicles (Fig. 6E). However, there was a dramatic accumulation of tenascin C within the basement membrane of the ventral epidermis in K14-PTHrP transgenic embryos as well as an accumulation of tenascin C within the extracellular matrix of the dermal mesenchyme just beneath the epidermis (Fig. 6F). Despite the widespread expression of the PPR1 beneath the epidermis (Fig. 4F,G), only those cells nearest the epidermal source of PTHrP expressed androgen receptor (Fig. 6C) or tenascin C (Fig. 6F), demonstrating that PTHrP acted only over a range of a few cell diameters. Interestingly, these effects also appeared to be limited to the ventral epidermis of the K14-PTHrP embryos. Staining of the dorsal epidermis did not reveal expression of androgen receptor or tenascin C within the dermal mesenchyme (Fig. 6D,G). This was surprising, since both the PPR1 gene and the K14-PTHrP transgene were expressed in both dorsal and ventral epidermis (data not shown). Thus, ectopic overexpression of PTHrP within the ventral epidermis leads to the ectopic expression of androgen receptor and tenascin C within mesenchymal cells that should posses a dermal phenotype, suggesting that, at least on the ventral surface of the embryo, PTHrP may act as a dominant signal to induce the differentiation of the mammary mesenchyme.

DISCUSSION

In this report, we demonstrate that PTHrP, signaling through the PPR1, is essential for the sexual dimorphism in normal murine mammary development. In PTHrP- or the PPR1-knockout embryos, the androgen-mediated destruction of the mammary bud in male embryos fails, due to the absence of androgen receptors in the mammary mesenchyme. In addition, the mammary buds of both types of knockout mice lack tenascin C, an extracellular matrix constituent which is highly expressed within the mammary mesenchyme but not within the dermis (Sakakura, 1987). PTHrP is expressed within the epithelial cells of the forming mammary bud beginning late on E11, however it is not expressed within the mesenchyme or within the epidermis at this stage. In contrast to PTHrP, the PPR1 is expressed within the mesenchyme and its expression is not restricted to the developing mammary bud. Rather, it is expressed both within the mesenchyme surrounding the mammary bud and within the mesenchyme underlying the epidermis. The expression of androgen receptor and tenascin C are directly dependent on PTHrP expression during the formation of the mammary bud and are not a consequence of earlier PTHrP expression, for transgenic expression of PTHrP within the forming mammary epithelial bud in otherwise PTHrP-null (K14-PTHrP/PTHrP-null) embryos leads to the

Fig. 6. Ectopic expression of PTHrP in the epidermis induces dermal expression of androgen receptor and tenascin C.
(A) Immunohistochemistry for keratin 14 in the fetal epidermis at E18. Note that K14 is expressed both within the fetal mammary epithelial cells within the mammary duct below the epidermis and within the basal keratinocytes of the epidermis. (B-D) Androgen receptor staining of ventral epidermis from a wild-type embryo at E18 (B), and of ventral (C) and dorsal (D) epidermis from a K14-PTHrP transgenic embryo, also at E18. There is no androgen receptor staining in the wild-type dermis (B), but there is nuclear androgen receptor staining in the dermal cells close to the epidermal basement membrane in the ventral surface of the K14-PTHrP transgenic. However, this is not true for the dorsal aspect of the K14-PTHrP embryos as seen in D. (E-G) A similar pattern is seen for tenascin C. In the ventral surface of wild-type embryos at E18 (E), there is some tenascin expression along the basement membrane, especially in the vicinity of developing hair follicles. However, there is a dramatic upregulation of tenascin within the basement membrane and within the extracellular matrix of the upper dermis on the ventral surface of K14-PTHrP embryos at E18 (F), but not on the dorsal surface of K14-PTHrP transgenic embryos (G). Scale bar, 150 μm.
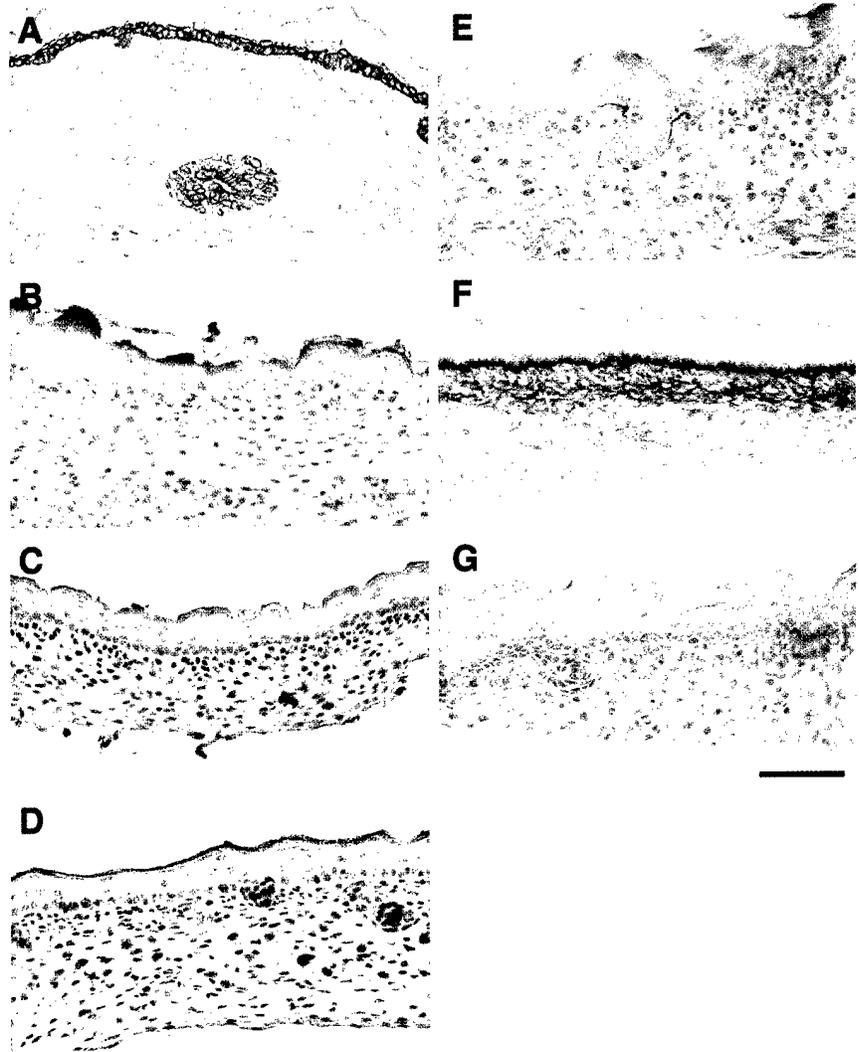
restoration of the expression of both androgen receptor and tenascin C, and thus the androgen-mediated destruction of the mammary bud. Finally, transgenic expression of PTHrP in the basal epidermis leads to the induction of androgen receptor and tenascin C expression within the ventral dermis, suggesting that ectopic expression of PTHrP may induce an ectopic mammary mesenchyme phenotype.

The expression of androgen receptors and tenascin C has classically distinguished the dense mammary mesenchyme from the surrounding dermal mesenchyme (Sakakura, 1987). It has been known for many years that the mesenchymal expression of both molecules was dependent on short-range inductive tissue interactions with the mammary epithelium, but the nature of the inductive signal(s) sent from epithelium to mesenchyme was not known (Heuberger et al., 1982; Inaguma et al., 1988; Kalembey et al., 1997). Our findings suggest that PTHrP is a vital component in these interactions. However, both of these molecules are expressed elsewhere and their expression is not universally dependent on PTHrP. Likewise, the ability of epidermal overexpression of PTHrP to induce the production of these molecules does not appear to extend to all the dermal mesenchyme, for we did not observe their induction

within the dorsal subcutis. Therefore, it is unlikely that PTHrP generally regulates the expression of these molecules. Rather, our hypothesis is that PTHrP, expressed exclusively within the developing epithelial bud, acts as a short-range dominant signal to a receptive ventral mesenchyme to differentiate into dense mammary mesenchyme. This results in the induction of mammary mesenchyme-specific genes (e.g. tenascin and androgen receptor) and the ability of the mesenchyme to support mammary epithelial morphogenesis.

In addition to the failure of androgen responsiveness, the loss of PTHrP-signaling also renders the mammary mesenchyme incapable of supporting the initiation of branching morphogenesis associated with the primary growth spurt on E16 (Dunbar et al., 1998; Wysolmerski et al., 1998). It is unlikely that the loss of either androgen receptor or tenascin C expression explains the inability of the mammary mesenchyme to support the outgrowth of the mammary epithelium in female PTHrP- or PPR1 knockouts because Tfm mice with inactivating mutations of the androgen receptor as well as tenascin C-knockouts both carry out these processes normally (Kratochwil and Schwartz, 1977; Saga et al., 1992). Recently, a series of additional molecules such as BMP-4,

preprotachykinin, Msx 2, Fgf 7, Hoxa9, Hoxb9 and Hoxd9 have been described to be expressed in the mammary mesenchyme (Phippard et al., 1996; Weil et al., 1995; Cunha and Hom, 1996; Robinson et al., 1999; Chen and Capecchi, 1999). However, there is no evidence to date to suggest that the deletion of any of these molecules phenocopies the changes in mammary development noted in the PTHrP- and PPR1-knockouts (Robinson et al., 1999; Chen and Cappechi, 1999). The mammary phenotype of LEF-1-deficient mice remains the closest to that of the PTHrP and PPR1 knockouts (van Genderen et al., 1994; Kratochwil et al., 1996). However, LEF-1 is expressed in the mammary epithelium prior to the onset of PTHrP expression and the failure of mammary development in LEF-1-knockout embryos occurs at an earlier stage than does the failure of mammary development in PTHrP- or PPR1-knockouts. Thus, if LEF-1 and PTHrP are in a common genetic pathway, LEF-1 most likely resides upstream of PTHrP within this pathway (van Genderen et al., 1994; Kratochwil et al., 1996). Except for the expression of androgen receptors and the androgen-mediated destruction of the mammary bud, the nature of the other PTHrP-induced mesenchymal changes that allow the mammary mesenchyme to support morphogenesis remains obscure.

It is also apparent from our results that, although mammary mesenchyme is dependent on PTHrP for its ability to support morphogenesis, the morphological appearance of the mammary mesenchyme is not dependent on PTHrP. In both PTHrP- and PPR1-knockout embryos, the mammary mesenchyme is histologically indistinguishable from that in normal littermates. The most-likely explanation for these findings is that the condensation or "structural" differentiation of the mammary mesenchyme precedes its functional differentiation (which is dependent on PTHrP). In support of this concept, we have recently observed that syndecan 1, which has been reported to be important to the condensation of tooth mesenchyme, continues to be expressed normally within the mammary mesenchyme of PTHrP and PPR1 knockouts (P. R. D., unpublished observations; Salmivirta et al., 1991; Thesleff et al., 1995). It is likely that there are one or more reciprocal exchanges between the mammary epithelium and the mammary mesenchyme that precede the actions of PTHrP and it will be important to examine the mechanisms leading to activation of PTHrP expression within the developing epithelial bud.

In summary, we have found that PTHrP and the PPR1 are necessary for sexual dimorphism during murine mammary development. PTHrP is an inductive signal from the epithelium to the mesenchyme that is necessary for androgen receptor and tenascin C expression within the mesenchyme. We propose that PTHrP participates in the regulation of mesenchymal cell fate decisions leading to a distinct mammary mesenchyme with the ability to support early mammary morphogenesis. The specific initiation of PTHrP gene expression within the epithelium of the forming mammary bud, the more general expression of the PPR1 within the subepidermal mesenchyme, the ability of ectopic epidermal expression of PTHrP to induce the inappropriate dermal expression of androgen receptor and tenascin C, the short-range nature of this signaling and the inability of the mesenchyme to support morphogenesis in the absence of PTHrP or the PPR1 all support this model. PTHrP has been shown to participate in fetal bone morphogenesis in part by directly regulating the differentiation of proliferating chondrocytes within the growth plate (Chung et al., 1998). In addition, PTHrP has been shown to regulate the morphogenesis of several epithelial organs other than the mammary gland, such as tooth, hair follicles and lung and, at these sites, it most likely also contributes to the regulation of epithelial-mesenchymal interactions (Philbrick et al., 1998; Rubin et al., 1994; Wysolmerski et al., 1994). The current data suggest that PTHrP regulates epithelial morphogenesis in the fetal mammary gland by regulating mesenchymal cell fate decisions and we anticipate that this will be the case in other organs as well.

REFERENCES

Behrendtsen, O., Alexander, C. M. and Werb, Z. (1995). Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos. *Development* 121, 4137-4146.

Birchmeier, C. and Birchmeier, W. (1993). Molecular aspects of mesenchymal-epithelial interactions. *Ann. Rev. Cell Biol.* 9, 511-540.

Chen, F. and Capecchi, M. D. (1999). Paralogous mouse Hox genes Hoxa 9, Hoxb 9, and Hoxd 9, function together to control development of the mammary gland in response to pregnancy. *Proc. Natl Acad. Sci. USA* 96, 541-546.

Chung, V., Lanske, B., Lee, K., Li, E. and Kronenberg, H. (1998). The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc. Natl Acad. Sci. USA* 95, 13030-13035.

Cunha, G. R. (1994). Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* 74, 1030-1044.

Cunha, G. R., Young, P., Christov, K., Guzman, R., Nandi, J., Talamsutes, F. and Thorderson, G. (1995). Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. *Acta Anat.* 152, 195-204.

Cunha, G. R. and Hom, Y. K. (1996). Role of mesenchymal-epithelial interactions in mammary gland development. *J. Mamm. Gland Biol. Neopl.* 1, 21-35.

de Stolpe, A., Karperian, M., Lowik, C. W. G. M., Jüppner, H., Segre, G. V., Abou-Samra, A. B., deLaat, S. W. and Defize, L. H. K. (1993). Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell Biol.* 120, 235-243.

Dunbar, M. E., Young, P., Zhang, J. P., McCaughern-Carucci, J., Lanske, B., Orloff, J. J., Karaplis, A., Cunha, G. and Wysolmerski, J. J. (1998). Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein. *Dev. Biol.* 203, 75-89.

Erickson, H. P. and Bourdon M. A. (1989). Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annual Rev. Cell Biol.* 5, 71-92.

Gilbert, S. F. (1994). Sex determination. In *Developmental Biology* pp. 754-787. Sunderland, MA: Sinauer Associates.

Grumbach, M. M. and Conte, F. A. (1992). Disorders of sex differentiation.

In *Williams Textbook of Endocrinology 8th Edition.* (ed. J. D. Wilson and D. W. Foster) pp. 853-952. Philadelphia. PA: W. B. Saunders Co.

Heuberger, B., Fitzka, I., Wasner, G. and Kratochwil, K. (1982). Induction of androgen receptor formation by epithelial-mesenchymal interaction in embryonic mouse mammary gland. *Proc. Natl Acad. Sci. USA* **79**, 2957-2961.

Hoshino, K. (1965). Development and function of mammary glands of mice prenatally exposed to testosterone propionate. *Endocrinology* **76**, 789-794.

Inaguma, Y., Kusakabe, M., Mackie, E. J., Pearson, C. A., Chiquet-Ehrisman, R. and Sakakura, T. (1988). Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev. Biol.* **128**, 245-255.

Jüppner, H., Abou-Samra, A. B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F., Hock, J., Potts, J. T., Kronenberg, H. M. and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254**, 1024-1026.

Kalembey, I., Yoshida, T., Iriyama, K. and Sakakura, T. (1997). Analysis of tenascin mRNA expression in the murine mammary gland from embryogenesis to carcinogenesis: an in situ hybridization study. *Int. J. Dev. Biol.* **41**, 569-573.

Kopan, R. and Fuchs, E. (1989). A new look at an old problem: keratins as tools to investigate determination morphogenesis and differentiation in skin. *Genes Dev.* **3**, 1-15.

Kratochwil, K. (1977). Development and loss of androgen responsiveness in the embryonic mammary rudiment of the mouse mammary gland. *Dev. Biol.* **61**, 358-365.

Kratochwil, K and Schwartz, P. (1977). Tissue interaction in androgen response of embryonic mammary rudiment of mouse: identification of target tissue for testosterone. *Proc. Natl Acad. Sci. USA* **73**, 4041-4044.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J. and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**,1382-1394.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinol.* **136**, 453-463.

Majdic, G., Millar, M. R. and Saunders, P. T. K. (1995). Immunolocalization of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *J Endocrinol.* **147**.285-293.

Neumann, F., Berswordt-Wallrabe, Elger, W., Steinbech, H., Hahn, J. D. and Kramer, M. (1970). Aspects of androgen-dependent events as studied by anti-androgens. *Recent Prog. Horm. Res.* **26**, 337-410.

Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Orloff, J. J., Yang, K. H., Vasavada, R. C., Weir, E. C., Broadus, A. E. and Stewart, A. F. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol. Rev.* **76**, 127-173.

Philbrick, W. M., Dreyer, B. E., Nakchbandi, I. A. and Karaplis A. C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc. Natl Acad. Sci. USA* **95**, 11846-11851.

Phippard, D. J., Weber-Hall, S. J., Sharpe P. T., Naylor, H., Jayatalake, H., Mass, R., Woo, D., Roberts-Clark, D., Francis-West, P. H., Liu, Y. H., Maxson, R., Hill, R. E. and Dale, T. C. (1996). Regulation of Msx-1, Msx-2. Bmp-2 and Bmp-4 during foetal and postnatal mammary gland development. *Development* **122**, 2729-2737.

Propper, A. and Gomot, L. (1967). Interactions tissulaires au cours de l'organogenèse de la gland mammaire de l'embryon de Lapin. *C. R. Acad. Sci. Paris* **264**, 2573-2575.

Raynaud, A. (1949). Nouvelles observations sur l'appareil mammaire des foetus de souris provent de mères ayant reçu des injections de testostérone pendant la gestation. *Annals Endocrin.* **10**, 54-62.

Raynaud, A. and Frilley, M. (1947). Destruction des glandes genitales de l'embryon de souris per une irradiation au moyen des rayon x, a l'aye de treizejours. *Ann. Endocrinol. Paris* **8**, 400-419.

Robinson, G. W., Karpf, A. B. C. and Kratochwil, K. (1999). Regulation of mammary gland development by tissue interaction. *J. Mamm. Gland Biol. Neopl.* **4**,

Rubin, L. P., Kifor, O., Hua, J., Brown, E. M. and Torday, J. S. (1994). Parathyroid hormone (PTH) and PTH-related protein stimulate surfactant phospholipid synthesis in rat fetal lung, apparently by a mesenchymal-epithelial mechanism. *Biochim. Biophys. Acta* **1223**, 91-100.

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992). Mice develop normally without tenascin. *Genes Dev.* **6**, 1821-1831.

Salmivirta, M., Elenius, K., Vaino, S., Hofen, V., Chiquet-Ehrisman, R., Thesleff, I. and Jalkanen, M. (1991). Syndecan from embryonic tooth mesenchyme binds tenascin. *J. Biol. Chem.* **266**, 7733-7739.

Sakakura, T. (1987). Mammary embryogenesis. In *The Mammary Gland, Development, Regulation and Function,* (ed. M. C. Neville, C. W. Daniel) pp 37-65. New York: Plenum Press.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol.* **39**, 35-50.

Turner, C. W. and Gomez, E. T. (1933). The normal development of the mammary gland of the male and female albino mouse. I. Intra-uterine. *Bull. Mo. Agric. Exp. Stn* **182**, 3-20.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R., Parslow, T. G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8**, 2691-2703.

Weil, M., Itin, A. and Keshet, E. (1995). A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis. *Development* **121**, 2419-2428.

Wysolmerski, J. J., Broadus, A. E., Zhou, J., Fuchs, E., Milstone, L. M. and Philbrick, W. M. (1994). Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl Acad. Sci. USA* **91**, 1133-1137.

Wysolmerski, J. J., McCaughern-Carucci, J. F., Daifotis, A. G., Broadus, A. E. and Philbrick, W. M. (1995). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* **121**, 3539-3547.

Wysolmerski, J. J. and Stewart, A. F. (1998). The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. *Ann. Rev. Physiol.* **60**, 431-460.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H., Karaplis, A. and Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**, 1285-1294.

Appendix 3

Title: Parathyroid Hormone-Related Protein Maintains Mammary Epithelial Fate and Triggers Nipple Skin Differentiation During Embryonic Breast Development.

Authors: John Foley[1,2,3] , Pamela Dann[4], James Hong[1], Jason Cosgrove[1], Barbara Dreyer[4], David Rimm[5], Maureen E. Dunbar[4], William Philbrick[4] and John J Wysolmerski[4]

1. Medical Sciences, Indiana University School of Medicine, Bloomington, IN 47405

2. Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN 46202

3. Institute for Molecular Biology, Indiana University, Bloomington, IN 47405

4. Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520

5. Department of Pathology, Yale University School of Medicine, New Haven, CT 06520

Running title: PTHrP regulates mammary and nipple fate.

Key words: PTHrP/ PTH/PTHrP receptor/ keratinocyte-differentiation/ epithelial-mesenchymal interactions /nipple / mammary development

Corresponding Author:

John J. Wysolmerski, M.D.
Section of Endocrinology and Metabolism
Department of Internal Medicine
Yale University School of Medicine
FMP 102
333 Cedar St.
New Haven, CT   06520
Phone: (203) 785-7447
Fax: (203) 785-6015
Email: john.wysolmerski@yale.edu

Accepted in Development -
revised version sent 11/29/00

1

Summary

Prior reports have demonstrated that parathyroid hormone-related protein (PTHrP) and the type I PTH/PTHrP receptor are both necessary for the proper development of the embryonic mammary gland in mice. Utilizing a combination of loss-of-function and gain-of-function models, we now report that PTHrP regulates a series of cell fate decisions central to the survival and morphogenesis of the mammary epithelium and the formation of the nipple. PTHrP is made in the epithelial cells of the mammary bud and, during embryonic mammary development, it interacts with the surrounding mesenchymal cells to induce the formation of the dense mammary mesenchyme. In response, these mammary-specific mesenchymal cells support the maintenance of mammary epithelial cell fate, trigger epithelial morphogenesis and induce the overlying epidermis to form the nipple. In the absence of PTHrP signaling, the mammary epithelial cells revert to an epidermal fate, no mammary ducts are formed and the nipple does not form. In the presence of diffuse epidermal PTHrP signaling, the ventral dermis is transformed into mammary mesenchyme and the entire ventral epidermis becomes nipple skin. These alterations in cell fate require that PTHrP be expressed during development and they require the presence of the PTH/PTHrP receptor. Finally, PTHrP signaling regulates the epidermal and mesenchymal expression of LEF-1 and β-catenin, suggesting that these changes in cell fate involve an interaction between the PTHrP and Wnt signaling pathways.

Introduction

Parathyroid hormone related-protein (PTHrP) was first identified as a tumor-derived factor causing humoral hypercalcemia of malignancy (Strewler, 2000). We now know that the PTHrP gene is expressed in a wide variety of normal tissues in which PTHrP appears to act as an autocrine, paracrine or intracrine growth factor (Philbrick et al., 1996; Strewler, 2000; Wysolmerski and Stewart, 1998). PTHrP signals through a G protein-coupled receptor known as the type I PTH/PTHrP receptor (PTHR1), so named because it can be stimulated by the homologous N-terminal regions of either PTHrP or parathyroid hormone (PTH) (Jüppner et al., 1991). This receptor is also widely distributed in normal tissues, often on cells adjacent to those producing PTHrP (Lee et al., 1995).

Recently, attention has focused on the developmental functions of PTHrP. Thus far, two principal roles for PTHrP have been documented in mammalian development. First, PTHrP, signaling via the PTHR1, has been shown to be a critical regulator of chondrocyte differentiation during endochondral bone formation (Vortkamp et al., 1996; Lanske et al., 1996). Second, again acting via the PTHR1, PTHrP has been shown to participate in epithelial-mesenchymal interactions during the formation of epithelial organs such as the skin, mammary glands, and teeth (Wysolmerski et.al., 1994; Wysolmerski et al., 1995; Foley et al., 1998; Philbrick et al. 1998; Dunbar et al., 1999).

Mammary glands are epidermal appendages and, in the mouse, their embryonic development is a two-step process (Sakakura, 1987). The first is the bud-like invagination of epidermal cells into the underlying mesenchyme at 10 characteristic locations along the milk line, a thickened ridge of embryonic skin stretching between the limb buds on the ventral surface of the embryo. The mammary buds are first discernable on embryonic day 10 (E10) and are fully formed by E12. Each bud consists of a pear-shaped collection of epithelial cells surrounded by a condensed mesenchyme that is distinct from the surrounding dermis. In male embryos, the mammary buds are destroyed on E14 by the actions of androgens on the mammary mesenchyme. In female embryos, the buds remain quiescent until E16 when the second step of development is

initiated. In this stage, the primary duct is formed, it grows away from the primary mammary mesenchyme into another stromal compartment known as the mammary fat pad, and 10 to 15 secondary ducts are formed within the fat pad before birth. Concurrent with these changes, the nipple sheath forms. This is a modification of the skin that occurs in the immediate vicinity of the mammary duct. The epidermis thickens and projects down into the dermis in an umbrella-like fashion, forming a ridge that surrounds the origin of the primary epithelial duct.

Classical experiments have demonstrated that the formation of the embryonic mammary gland depends on reciprocal and sequential exchanges of information between the developing epithelium and the mammary mesenchyme (Sakakura, 1987; Hennighausen and Robinson, 1998). However, at present our knowledge regarding the nature of these epithelial-mesenchymal communications is limited. Recent experiments have demonstrated that PTHrP and the PTHR1 comprise one important signaling pathway involved in this exchange. Both are necessary for mammary development, and in their absence, although the mammary buds initially form, they fail to undergo the expected androgen-mediated destruction in males or the initiation of ductal branching morphogenesis in females (Wysolmerski et al., 1998; Dunbar et al., 1999). Instead, the mammary epithelial cells disappear and the nipple sheath fails to form, leaving neonates without mammary glands or nipples. During the early stages of mammary bud formation, PTHrP is expressed within mammary epithelial cells and the PTHR1 is expressed in the condensed mammary mesenchyme as well as in the presumptive dermis (Wysolmerski et al., 1998; Dunbar et al., 1999). These data suggest that PTHrP represents an epithelial signal critical to the development of the mammary mesenchyme's morphogenetic capacity.

In this report, we demonstrate that PTHrP signaling is central to a series of cell fate decisions needed to form the mammary gland and nipple. PTHrP, apparently interacting with the Wnt-signaling cascade, determines the fate of the mammary mesenchyme. In turn, the mammary mesenchyme supports both the maintenance of mammary epithelial fate and the subsequent morphogenesis of the mammary epithelium, and it triggers the overlying epidermal cells to form a nipple sheath.

4

Materials and Methods

Mouse Strains

PTHrP knockout, PTHR1 knockout and K14-PTHrP transgenic embryos were harvested and genotyped as previously reported (Wysolmerski et al., 1998; Dunbar et al., 1999). The appearance of the vaginal plug was considered day 0 of gestation. Wild-type littermates were used as controls. K14-tTA/tet_{O7}-PTHrP double transgenic mice were created by crossing K14-tTA transgenic mice with tet_{O7}-PTHrP transgenic mice. As outlined in Fig. 4, the K14-tTA transgene was generated by inserting a 2.1 kb portion of the human K14 promoter upstream of the tetracycline transactivator gene (Schockett et al., 1995). 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 7 concatamerized tetracycline repressor binding sites (Wysolmerski et al., 1994; Schockett et al., 1995). Tet_O-PTHrP mice were identified by amplification of a 171 bp sequence of the human growth hormone gene (Dunbar et al., 1999). K14-tTA mice were identified by amplification of a 159 bp sequence of the tetracycline transactivator gene using the following primers: forward, **aacaacccgtaaactcgcc** and reverse, **aaatcttgccagctttccc**. Tet_O-β-gal mice were the gift of Dr. Lothar Hennighausen, and were genotyped by amplification of a 192 bp fragment of the lacZ gene using the following primers: forward, **aattgaattatggcccacac** and reverse, **aattccgccgatactgac**. Tet_O-PTHrP transgene expression was suppressed by feeding double transgenic mice 150 µg/ml of tetracycline hydrochloride (Roche, Indianapolis, IN) in 5% sucrose water.

Histology and Immunohistochemistry

Whole embryos were fixed in Bouins or 4% paraformaldehyde at 4°C for 12 hours. Mammary glands and strips of skin were dissected and embedded in paraffin, and mammary buds were identified by serial sectioning as previously described (Dunbar et al., 1999). Longitudinal strips of ventral or dorsal adult mouse skin were either processed for frozen sections or fixed and

5

embedded as described above. Immunohistochemistry was performed using standard techniques. Antigen retrieval was accomplished by heating sections in 7 mM citrate under pressure. Anti-Lef1 antibodies were the gift of Dr. R. Grosschedl; primary incubation was 12 hours at 4°C after antigen retrieval. Nuclear staining was performed using an anti-sm antisera (Y12) that was the gift of Dr. Joan Steitz; primary incubation was performed for 1 hour at room temperature after antigen retrieval. Antibodies for smooth muscle α-actin were from Sigma (St. Louis, Mo); primary incubation was for 10 minutes at room temperature. Vimentin antibodies were from Chemicon (Temicola, CA); primary incubation was performed on frozen sections for 1 hour at room temperature. The anti-keratin 9 antibody was the gift of Dr. Lutz Langbein; primary incubation was performed on frozen sections for 1 hour at room temperature. Staining for K14, K1, K10, K6, involucrin, loracrin, fillagrin, tenascin C, androgen receptor and β-catenin were performed as previously described (Dillon et al., 1998; Foley et al., 1998; Dunbar et al., 1999). Antibodies to androgen receptor, tenascin C, LEF-1, K14, K1, K10, K6, K9, involucrin, loracrin, fillagrin, smooth muscle α-actin, and vimentin were detected using Vector Elite ABC kits (Vector laboratories, Burlingame, CA) and 3,3' diaminobenzidine as chromagen. β-Catenin antibodies were detected with a goat anti-rabbit Cy3-conjugated secondary (Amersham Pharmacia, Piscataway, NJ) and anti-sm antibodies were detected with a goat anti-mouse, alexafluro 488-conjugated secondary (Molecular Probes, Eugene, OR). Confocal laser-scanning microscopy was performed at the Center for Cell Imaging at Yale University.

β-galactosidase Assay

β-galactosidase was detected as described by Furth et al. (1994). Briefly, tissue was fixed in 2% paraformaldehyde and 0.02% gluteraldehyde in phosphate buffered saline (PBS) for 1 hour at room temperature and then washed twice in PBS. Samples were incubated in 0.1% 4-chloro-5-bromo-3-indoyl β-D-galactopyranoside, 2mM $MgCl_2$, 5mM EGTA, 0.02% Nonidet P-40, 5mM $K_3Fe(CN)_6$ and 5mM $K_4Fe(CN)_6.6H_2O$ at 30°C for 12 hr. Tissue was then post-fixed in 4% paraformaldehyde at 4°C, embedded in paraffin, sectioned and counterstained with eosin.

Results

Loss of PTHrP signaling leads to squamous differentiation of mammary epithelial cells.

Previous studies suggested that PTHrP signaling is necessary for the differentiation of the primary mammary mesenchyme (Dunbar et al., 1999). We therefore speculated that the failure of mammary epithelial cells to survive in PTHrP or PTHR1 knockout mice was related to a failure of mammary mesenchyme differentiation. Specifically, we hypothesized that in the absence of a functional mammary mesenchyme, mammary epithelial cells might differentiate along an epidermal pathway.

In order to test this hypothesis, we first examined mammary rudiments from PTHrP and PTHR1 knockout mice from E17 – E19. By E-17, the normal mammary duct has grown away from the overlying epidermis, and the nipple sheath has formed (Fig 1A). In contrast, in PTHrP (Fig. 1B) or PTHR1 knockout embryos, the mammary bud has not grown away from the skin, and the nipple sheath is absent. We also noted that what appeared to be hair follicles could occasionally be seen budding off the neck of the knockout mammary buds or arising from the epidermis adjacent to the origin of the primary duct, an area that should have given rise to the nipple sheath . (Fig 1B). These findings were distinctly abnormal, as the nipple and mammary bud should be devoid of hair follicles. Finally, cells within the middle of the knockout buds had a vacuolated appearance similar to that of the supra-basal or spinous layer of the epidermis and, in fact, appeared to be continuous with that layer of the skin (Fig. 1B). These histological changes suggested that the knockout mammary buds were behaving more like epidermis than mammary epithelium. If this were true, we reasoned that the knockout epithelial cells might begin to express epidermal-specific proteins. Therefore, we stained E-17 mammary glands from PTHrP and PTHR1 knockout and wild-type littermates with a battery of antibodies to epidermal markers, including K14, K1, K10, involucrin, loricrin and filaggrin (Kopan and Fuchs, 1989; Foley et al., 1998). As shown in Figure 1 C, K14 antibodies labeled all of the mammary epithelial cells but only the basal epidermal cells of wild-type embryos. In PTHrP (Fig. 1D) and PTHR1 knockouts, K14 labeled just the cells along

7

the perimeter of the knockout mammary buds, a pattern similar to wild-type skin. Antibodies to suprabasal epidermal markers, such as K1, K10 and involucrin, labeled the epidermis and just a very thin component of the upper lactiferous duct in wild-type embryos. However, the entire central portion of the mammary rudiment in the knockout embryos expressed these suprabasal keratinocyte markers (Fig. 1E&F, K10 and involucrin not shown). Antibodies to the granular markers loricrin and filaggrin labeled the outer layers of the epidermis in both the knockout and wild-type embryos but did not label the mammary epithelial cells in either (Fig. 1G&H, loricrin not shown). Taken together, these results suggest that the absence of PTHrP signaling leads the epithelial cells of the mammary bud to undergo an epidermal pattern of differentiation.

Overexpression of PTHrP Leads to Nipple-Like Differentiation of the Ventral Epidermis.

Given the epidermal-like differentiation of the mammary epithelial cells in the knockout buds, we next asked if PTHrP overexpression in the developing skin would lead to mammary-like changes in the epidermis. Consistent with this possibility, we had previously demonstrated that overexpression of PTHrP in the basal keratinocytes of transgenic mice, utilizing the keratin 14 promoter (K14-PTHrP mice), induces the inappropriate expression of androgen receptor and tenascin C, two mammary mesenchyme markers, in the ventral dermis (Dunbar et al., 1999). In addition, we had previously reported that K14-PTHrP mice failed to develop hair follicles on their ventral surface and demonstrated an altered expression pattern of keratinocyte differentiation markers (Wÿsolmerski et al., 1994; Foley et al., 1998). With the above question in mind, we carefully re-examined the epidermis in K14-PTHrP transgenic mice and found that it strikingly resembles the normal nipple sheath.

In mice, nipples are only present on females and, grossly, are composed of wrinkled, flaky and hairless skin, all characteristics shared with the ventral skin of K14-PTHrP transgenic mice (Montagna, 1970; Toyoshima et al., 1998). Histologically, in comparison to wild-type skin, both nipple skin and K14-PTHrP skin demonstrate a thickened, hyperkeratotic epidermis without hair follicles which overlies a cellular dermis containing small bundles of uniformly-sized collagen (see

8

Fig. 2 A-C, and data not shown). As in other mammals, the epidermis of the murine nipple sends an umbrella-like projection down into the dermis in a circumferential fashion around the primary lactiferous duct (2B). The epidermis of K14-PTHrP mice also sends projections down into the dermis, although these folds tend to be shallower and more rounded and none are associated with a mammary duct (Fig. 2C). Another hallmark of nipple skin in the mouse is the smooth muscle beds found at the base of the nipple which extend as a group of thin fibers along the lactiferous duct (Montagna, 1970; Toyoshima et al., 1998). In K14-PTHrP skin, similar bundles of elongated cells were found distributed beneath the entire ventral epidermis. These cells stained for alpha-actin and vimentin, confirming their identity as smooth muscle (see Fig. 2D-F, and data not shown). Finally, nipple skin is characterized by an abundance of elastic fibers in the underlying connective tissue (Montagna, 1970; Toyoshima et al., 1998). As shown in Fig. 2G-I, elastic fibers are not abundant in the dermis of wild-type mice, but are dramatically increased in the connective tissue underneath the nipple and within the K14-PTHrP dermis. Although the nipple-like features described above were found throughout the hairless ventral skin of the K14-PTHrP mice, the dorsal skin of these animals was similar to dorsal skin of wild-type controls, even though the transgene was also expressed in dorsal skin.

We next examined the pattern of expression of several markers of epidermal differentiation in normal nipple skin and compared these patterns to those in normal and K14-PTHrP transgenic skin. As shown in Fig. 3A, in normal ventral skin, antibodies to keratin 14 stain the entire basal keratinocyte layer, but not the upper layers of keratinocytes. In both the K14-PTHrP transgenic skin and the normal nipple skin, the layer of cells staining for keratin 14 appears expanded (Fig. 3B&C). This is because the basal keratinocytes are elongated and more columnar in appearance, and also because some suprabasal cells appear to retain keratin 14 protein expression. As expected, staining for the suprabasal marker, K1, intensely labeled the upper layers of keratinocytes as well as lightly staining some basal keratinocytes in wild-type ventral skin (Fig. 3D). Similar to the pattern in K14-PTHrP skin, labeling for K1 in nipple skin was restricted to the outermost suprabasal layers (Fig. 3E&F). Similar staining was seen with an antibody to keratin 10, another suprabasal marker,

9

(not shown). Next, we examined the pattern of expression of the granular layer marker, filaggrin. This protein was minimally expressed in what appears to be a single thin layer within wild-type ventral skin, whereas in the nipple and in K14-skin it was expressed at high levels in multiple layers (see Fig 3G-I). As with the histological findings described in the previous paragraph, the dorsal skin of the K14-PTHrP mice did not exhibit any alterations in epidermal marker expression as compared to wild-type dorsal skin (data not shown). Finally, given their similar lack of hair growth, we also investigated the expression of keratin 9 and keratin 6, two keratins that are found in the specialized epidermis of the footpad (Schwietzer and Baust, 1989; Rothangle et al., 1999). Neither of these keratins was expressed in the nipple or K14-PTHrP transgenic skin (data not shown). Thus, it appears that the alterations in keratinocyte differentiation marker expression seen in the ventral skin of K14-PTHrP transgenic mice closely mimic the natural pattern observed in the nipple. In both sites, as compared to normal ventral skin, the basal keratinocyte layer appears to be expanded, the acquisition of K1 and K10 expression appears to be delayed and there is an increase in the number of granular layers within the upper epidermis.

Embryonic overexpression of PTHrP is necessary and transient overexpression is sufficient to induce nipple-like skin changes in K14-PTHrP transgenic mice.

In order to investigate if the nipple-like changes in the K14-PTHrP transgenic skin required expression of PTHrP at a specific time, we created a binary K14-PTHrP transgenic mouse that would enable us to regulate PTHrP production in a temporal fashion. As shown in Fig. 4A, we made two transgenic lines, one bearing the K14-tTA transgene and other bearing the Tet_o-PTHrP transgene, and bred them together to generate double transgenic mice. Since this is a tet-off system, when double transgenic mice are fed tetracycline no excess PTHrP is produced but withdrawal of tetracycline allows PTHrP overexpression. In order to test the ability of the K14-tTA transgene to properly activate target transgene expression, we first bred the K14-tTA line to a Tet_o-βGal mouse (Furth et al., 1994). The resulting double transgenic mice (K14-tTA/ Tet_o-βGal) should express β-galactosidase in a K14-dependent manner, only when tetracycline is absent. Fig. 4 demonstrates

that this is the case. In the presence of tetracycline, there is no transgene expression (Fig 4B).
However, in the absence of tetracycline, β-galactosidase activity is present in the basal keratinocytes
and developing hair follicles as would be expected for a transgene being driven by the K14
promoter (Fig. 4C).

We next examined the consequences of different periods of PTHrP overexpression on
ventral skin development in K14-tTA/ Tet_0-PTHrP double transgenic mice. As expected, if the
transgene was kept off throughout the life of the animal, the ventral skin was normal, and if the
transgene was kept on continuously, the double transgenic skin displayed changes identical to those
seen in the original K14-PTHrP transgenic mice. Nipple-like changes were also seen in the skin of
double transgenic mice if PTHrP overexpression was allowed to begin before birth, but was
subsequently turned off at birth. As seen in Fig. 5, if PTHrP was overexpressed before birth, the
K14-PTHrP transgenic phenotype was reproduced; ventral hair follicle development was
suppressed and keratinocyte differentiation was altered as described earlier. Furthermore, these
changes persisted even after the transgene was turned off. In contrast to these findings, no nipple-
like skin characteristics ever appeared if PTHrP expression was delayed until after birth. In this
case, double transgenic mice appeared normal and there were no defects in hair follicle development
or keratinocyte differentiation (see Fig.5). Therefore, the nipple-like changes seen in the epidermis
of K14-PTHrP mice are the consequence of PTHrP acting on the developing skin before birth.

*PTHrP Signaling Modulates LEF-1 and β-Catenin Expression in the Developing Mammary
Gland and Epidermis*

Disruption of the gene for LEF-1 has been shown to interrupt embryonic mammary
development (van Genderen et al., 1994). We therefore sought evidence that LEF-1 might be in the
same signaling pathway as PTHrP and the PTHR1. It has been reported that LEF-1 is expressed in
the epithelial cells of the mammary bud early during its formation. However, it was not known if, as
has been described for tooth and hair follicle development, LEF-1 was also expressed in the
mesenchyme at later stages (van Genderen et al., 1994; Kratochwil et al., 1996). Therefore, we

11

performed immunohistochemistry for LEF-1 at different times during embryonic mammary development. At E11-12, we found that LEF-1 is expressed in the basal cells of the developing epidermis and in the epithelial cells of the mammary bud (not shown). By E14-E15, two changes occur. First, LEF-1 expression is induced within the mammary mesenchyme as it is fading away in the mammary epithelium. Second, in the epidermis destined to become the nipple sheath, LEF-1 expression becomes undetectable (Fig 6A). We next examined the expression of LEF-1 in mammary buds from PTHrP- and PTHR1-null mice and in skin from K14-PTHrP transgenic embryos. As seen in Fig.6B&C, in knockout mice, LEF-1 expression was preserved in mammary epithelial cells but was absent in the mammary mesenchyme. In addition, in these mice, LEF-1 continued to be expressed in the basal epidermal cells closest to the primary mammary duct. In contrast to these findings, ectopic overexpression of PTHrP in the epidermis of K14-PTHrP mice led to the loss of LEF-1 expression in the epidermis and its inappropriate expression in the dermis (Fig.6D&E). Interestingly, while this was true for the ventral skin of K14-PTHrP embryos, no such changes were observed in skin from the dorsal aspect of the embryos (not shown).

LEF-1 has been shown to participate in the Wnt-signaling pathway by complexing with nuclear β-catenin to form a binary transcription factor that modulates the expression of Wnt-responsive genes (Behrens et al., 1996; Eastman et al., 1999). Given the fact that Wnt signaling affects mammary development (Uyttendaele et al., 1998; Brisken et al., 2000) and given the findings described in the previous paragraph, we also examined β-catenin expression in the embryonic mammary bud. As expected, in the wild-type mammary bud (Fig. 7A&B), we observed the expected pattern of peripheral β-catenin expression within the mammary epithelial cells where it interacts with cadherins at the cell membrane (Miller and Moon, 1996). However, we also saw a patchy and somewhat punctate pattern of what appeared to be membrane-associated β-catenin staining in the mammary mesenchyme. There was also some low-level cytoplasmic staining within these cells. β-Catenin staining was especially prominent in the mesenchymal cells surrounding the neck of the bud, corresponding to the area of maximal LEF-1 staining (compare Fig. 7A&B and Fig. 6A). As can be seen in Fig. 7B, there was no evidence for co-localization of β-catenin and a

nuclear antigen, suggesting that there was no appreciable nuclear β-catenin in these cells. In contrast to these findings, although epithelial β-catenin expression was unchanged, in PTHrP and PTHR1 knockout buds the level of β-catenin expression in the mammary mesenchyme was dramatically reduced (Fig. 7C&D).

We also compared beta-catenin expression in skin samples taken from K14-PTHrP transgenic embryos with those taken from wild-type littermates. As seen in Fig 7E&F, in normal embryos, we detected prominent peripheral β-catenin staining in keratinocytes, similar to that seen in mammary epithelial cells. However, we saw little overall β-catenin staining in the normal dermal mesenchyme. In K14-PTHrP transgenic embryos, the pattern in keratinocytes was identical, but there was a clear induction of β-catenin expression in the dermis extending about 4 – 5 cell layers down from the epidermal basement membrane (Fig. 7 G&H). As in the mammary mesenchyme, the staining was patchy and mostly peripheral. As with LEF-1 expression, this pattern was not seen in the dorsal skin of the K14-PTHrP embryos, which stained in a pattern indistinguishable from wild-type skin. These data demonstrate that, at least on the ventral surface of the developing embryo, PTHrP-signaling is able to induce LEF-1 and β-catenin expression in the mammary and dermal mesenchymes.

PTH/PTHrP receptors are required for the nipple-like skin changes in K14-PTHrP mice.

Because the PTHR1 is expressed in the dermis but not on keratinocytes, our working hypothesis is that the nipple-like skin changes in the K14-PTHrP transgenic mice are the result of paracrine interactions. We believe that PTHrP acts on the dermis, which, in turn, alters the differentiation of the epidermis. However, PTHrP has also been shown to act via a nuclear pathway (Henderson et al., 1995; Massfelder et al., 1997; Lam et al., 1999; Aarts et al., 1999), and it has been suggested that there are PTHrP receptors other than the PTHR1 that exist on squamous cells (Orloff et al., 1995). Therefore, it is possible that the skin phenotype of the K14-PTHrP mice is caused by an autocrine effect of PTHrP. In order to distinguish between these possibilities, we generated mice that were hemizygous for the K14-PTHrP transgene and homozygous for the

13

disruption of the PTH/PTHrP receptor gene (K14-PTHrP/PTHR1$^{-/-}$ mice), so that we could overexpress PTHrP in the absence of type I receptors. These mice died shortly after birth due to skeletal complications resulting from disruption of the PTHR1 gene. As expected, there was a failure of mammary development in these mice identical to that seen in PTHrP and PTHR1 knockout mice. Disruption of the PTHR1 gene also prevented the induction of mammary mesenchyme markers in the ventral dermis in K14-PTHrP/PTHR1$^{-/-}$ mice. Fig. 8 demonstrates that, despite bearing the K14-PTHrP transgene, these embryos failed to express androgen receptor, tenascin C, LEF-1 or β-catenin in the dermal mesenchyme. Finally, there was no evidence of nipple-like changes in the ventral epidermis of the K14-PTHrP/PTHR1$^{-/-}$ embryos. LEF-1 expression was retained in the basal keratinocytes of the ventral surface and developing hair follicles were found throughout the ventral epidermis. In addition, the K14-PTHrP/PTHR1$^{-/-}$ epidermis lacked the alterations in epidermal marker expression seen in the ventral epidermis of K14-PTHrP neonates (Fig. 8G&H). At this age, the K14-PTHrP transgenic epidermis is only slightly thicker than wild-type epidermis, and the layer of cells staining for K14 appear slightly elongated compared to those cells staining for K14 in the wild-type. However, comparing Fig. 8A with 8B and Fig. 8K with 8L, one can see that these changes do not occur in a PTHR1-null background. Therefore, the changes in the ventral dermis and epidermis that result from overexpression of PTHrP in basal keratinocytes depend on the presence of the PTHR1. Furthermore, because the PTHR1 is expressed in the developing dermis but not in the epidermis, the nipple-like changes in the epidermis of the K14-PTHrP transgenic mice would appear to be mediated by PTHrP's effects on the dermis.

Discussion

In this report, we demonstrate that changes in PTHrP signaling result in significant alterations in cell fate during the development of the embryonic mammary gland and epidermis. As outlined in Fig. 9, we believe these data suggest that PTHrP acts as a critical patterning signal during the development of the ventral surface of the embryo, and we propose the following model. The PTHR1 is expressed in all the mesenchymal cells underlying the epidermis. During the formation of the normal mammary gland (Fig 9A) the epithelial cells of the bud, but not the surrounding keratinocytes, express the PTHrP gene at high levels. PTHrP is secreted by these cells and acts over short distances to induce several layers of mesenchymal cells surrounding the mammary epithelium to differentiate into mammary-specific mesenchyme. In turn, the cells of the mammary mesenchyme act back on the epithelium to maintain the mammary fate of the epithelial cells and to trigger ductal morphogenesis. In addition, the mammary mesenchyme instructs the overlying epidermis to become nipple skin. When this signaling pathway is interrupted, as in PTHrP- and PTHR1-knockout mice (Fig 9B), no mammary-specific mesenchyme forms. As a consequence, the mammary epithelial cells cannot maintain a mammary fate, and they differentiate into squamous-like cells that become resorbed into the forming epidermis. Furthermore, in the absence of a functioning mammary mesenchyme, the nipple sheath fails to form. Conversely, when the PTHrP signaling pathway is diffusely activated by overexpression of PTHrP in the skin of K14-PTHrP transgenic embryos (Fig. 9C), the ventral dermis takes on the characteristics of mammary-specific mesenchyme. Although no extra mammary epithelial ducts are formed, the entire ventral surface is transformed into nipple skin.

As reflected in this model, in addition to directing morphogenetic events, PTHrP signaling to the mammary mesenchyme appears to be necessary for the maintenance of mammary epithelial identity. Prior experiments have suggested that the mammary mesenchyme from E12-E13 embryos possesses the ability to induce *de novo* mammary bud formation from the epidermis (Propper, 1973; Cunha et al., 1995). Our data suggest that once formed, the epithelial cells continue to

15

require cues from the mammary mesenchyme to prevent them from reverting to an epidermal identity. Thus, even up to the bud stage of mammary development, the epithelial cells remain plastic and are not yet irreversibly committed to a mammary fate. Our data also suggest that, as a consequence of PTHrP signaling, the mammary mesenchyme alters the differentiation of the overlying epidermis to form the nipple, a response that requires embryonic exposure to PTHrP and the presence of the PTHR1 in the dermal mesenchyme. Since PTHrP is produced only in the mammary epithelium at this stage, this signaling pathway, in effect, determines the sites on the ventral surface of the embryo at which nipple skin develops.

One of the most intriguing aspects of the K14-PTHrP transgenic phenotype is the abrupt loss of virtually all nipple-like aspects of the transgenic skin at the transition from the ventral to the dorso-lateral aspects of the trunk. This line of demarcation is almost identical to the position of the nipple, or milk lines, thickened ridges of epidermis that exist between the limb buds from E10-E11 and along which the mammary buds form (Sakakura, 1987). With the exception of the developing mammary gland, we have not detected differences in native PTHrP, PTHR1 or K14-PTHrP transgene expression between the dorsal and ventral skin of mouse embryos (Dunbar et al., 1999). Therefore, the ability of PTHrP to induce mammary mesenchyme and nipple differentiation is restricted to the ventral surface even though the dermal mesenchyme on the dorsum also expresses PTHrP receptors. Many vertebrates have distinct skin appendages on their dorsal versus ventral surfaces, including the presence of mammary glands on the ventrum of placental mammals. It is likely that these patterning differences, at least in part, reflect underlying differences in the mesenchymal tissue beneath the epidermis. In fact, the dorsal and ventral dermal mesenchymes have distinct embryological origins, with the ventral cells arising from the somatopleural mesoderm and the dorsal cells arising from the dermatomyotome (Sengel, 1976). Whether it is these differences in mesenchymal lineage or other differences in growth or transcription factor expression that explain the differential response to PTHrP, it is clear that only the ventral surface is competent to generate mammary mesenchyme in response to PTHrP signaling.

Our data demonstrate a correlation between the changes in mesenchymal and epidermal cell fate resulting from manipulation of PTHrP signaling and changes in the patterns of mesenchymal and/or epidermal LEF-1 and β-catenin expression. LEF-1 and β-catenin expression are normally induced in the mammary mesenchyme, and LEF-1 expression is normally lost in the keratinocytes destined to give rise to the nipple sheath. When PTHrP-signaling is disrupted, LEF-1 and β-catenin are no longer expressed in the mesenchymal cells around the mammary bud, and LEF-1 continues to be expressed in the keratinocytes that should give rise to the nipple. Conversely, when PTHrP is ectopically overexpressed in the epidermis, LEF-1 and β-catenin are inappropriately expressed in the ventral dermis and, in the ventral epidermis, LEF-1 expression is lost. In the classical Wnt-signaling cascade, β-catenin and LEF-1 interact to form a transcription factor complex that is responsible for stimulating the expression of Wnt-responsive genes (Behrens et al., 1996; Eastman and Grosschedl, 1999). Both have been found to regulate skin patterning, keratinocyte proliferation, and hair follicle development (Zhou et al., 1995; Gat et al., 1998; Zhu and Watt, 1999; Widelitz et al., 2000). In this regard, it is likely that the loss of LEF-1 expression in the developing nipple skin is important in suppressing hair follicle development, as LEF-1-deficient mice are hairless, and forced expression of LEF-1 induces hair-follicle development in normally hairless squamous epithelia (vanGenderen et al., 1994; Zhou et al., 1995).

LEF-1 is also critical for the formation of the mammary gland. As in PTHrP or PTHR1 knockout mice, mammary development in LEF-1-deficient mice does not proceed past the bud stage. At E14-E15, we have found that, in addition to being expressed in the mammary epithelium, LEF-1 is prominently expressed in the primary mammary mesenchyme. Our data also indicate that, *in vivo*, PTHrP, acting through the PTHR1, is both necessary for the induction of mesenchymal LEF-1 expression, and at least on the ventral surface of the embryo, sufficient for mesenchymal LEF-1 expression. Given these findings and the similarities between the mammary phenotype upon disruption of the PTHrP, PTHR1 and LEF-1 genes, it is possible that LEF-1 acts downstream of PTHrP-signaling in the mammary mesenchyme. Furthermore, since changes in β-catenin expression upon manipulation of PTHrP-signaling parallel the changes in LEF-1 expression, it is

17

tempting to speculate that PTHrP-signaling might interact directly with wnt-signaling in these cells. Wnt 10b is expressed in the developing mammary bud, and mesenchymal cells have been shown to be targets of wnt signaling during hair follicle morphogenesis (Christiansen et al., 1995; DasGupta and Fuchs, 1999; Kishimoto et al., 2000). Therefore, it is possible that the mammary mesenchyme might also be a target of wnt signaling.

In conclusion, in this report we demonstrate that PTHrP regulates a series of cell fate decisions central to the embryonic development of the murine mammary gland. PTHrP acts as an epithelial signal that induces the mesenchyme around the epithelial bud to become mammary-specific. As a result, the mammary mesenchyme acts on the epithelial bud to maintain the mammary identity of the epithelium and to support ductal morphogenesis. It also acts upon the epidermis around the mammary bud to suppress hair follicle formation and trigger nipple sheath formation. Only the mesenchymal cells underlying the ventral epidermis are competant to respond to the mammary mesenchyme-inducing functions of PTHrP. Finally, these changes in cell fate correlate with changes in beta-catenin and LEF-1 expression within the mesenchyme and epidermis, implicating the involvement of the wnt-signaling cascade. We hope that future work aimed at unraveling the mechanisms by which PTHrP alters these cell fates will lead to a better understanding of mammary development and epidermal patterning.

Acknowledgements

References

Aarts, M.M., Levy, D., He, B., Stregger, S., Chen, T., Richard, S. and Henderson, J.E. (1999) Parathyroid hormone-related protein interacts with RNA. *J Biol Chem* **274**:4832-4838.

Behrens, J., von Kries, J., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996) Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**:638-642.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon J.A., McMahon, A.P. and Weinberg, R.A. (2000) Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes & Dev.* **14**:650-654.

Christiansen, J.H., Dennis, C.L., Wicking, C.A., Monkley, S.J., Wilkinson, D.G. and Wainwright, B.J. (1995) Murine Wnt-11 and Wnt-12 have temporally and spatially restricted expression patterns during embryonic development. *Mech Devel* **51**:341-350.

Cunha, G.R., Young, P., Christov, K., Guzman, R., Nandi, J., Talamsutes, F. and Thorderson, G. (1995) Mammary phenotypic expression induced in epideraml cells by embryonic mammary mesenchyme. *Acta Anat* **152**:195-204.

DasGupta, R. and Fuchs, E. (1999) Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**:4557-4568.

Dillon, D.A., Daquila, T., Reynolds, A.B., Fearon, E.R., and Rimm, D.L. (1998) The

Expression Of P120ctn Protein In Breast Cancer Is Independent Of Alpha- and Beta-Catenin

and E-Cadherin. *American Journal Of Pathology* **152**:75-82.

Dunbar, M.E., Young, P., Zhang, J.P., McCaughern-Carucci, J., Lanske, B., Orloff, J.J.,

Karaplis, A., Cunha, G. and Wysolmerski, J.J. (1998) Stromal cells are critical targets in

the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein. *Dev.*

Biol **203**:75-89.

Dunbar, M.E., Dann, P.R., Robinson, G., Hennighausen, L., Zhang, J.P. and

Wysolmerski, J.J. (1999) Parathyroid hormone-related protein is necessary for sexual

dimorphism during embryonic mammary development. *Development* **126**: 3485-93.

Eastman, Q., and Grosschedl, R. (1999) Regulation of Lef/TCF transcription factors by Wnt

and other signals. *Current Opinion Cell Biol* **11**:233-240.

Foley, J., Longely, B.J., Wysolmerski, J.J., Dreyer, B.E., Broadus, A.E. and Philbrick,

W.M. (1998) Regulation of epidermal differentiation by PTHrP: evidence from PTHrP-null

and PTHrP-overexpressing mice. *J. Investigative Dermatology* **111**: 1122-1128.

Furth, P.A., St Onge, L., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. and

Hennighausen, L. (1994) Temporal control of gene expression in transgenic mice by a

tetracycline-responsive promoter. *Proc Natl Acad Sci USA* **91**:9302-9306.

Gat, U., DasGupta, R., Degenstein, L. and Fuchs, E. (1998) De Novo hair follicle

morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell.*

95:605-14.

Henderson, J.E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske B.M.K., Goltzman, D. and Karaplis, A.C. (1995) Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol. Cell. Biol.* **15**:4064-4075.

Hennighausen, L. and Robinson, G.W. (1998) Think globally, act locally: the making of a mouse mammary gland. *Genes & Dev* **12**:449-455.

Jüppner, H., Abou-Samra, A.B., Freeman, M., Kong, X.F., Schipani, E., Richards, J., Kolakowski, L.F., Hock, J., Potts, J.T., Kronenberg, H.M. and Segre, G.V. (1991) A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* **254**:1024-1026.

Kishimoto, J., Burgeson, R.E. and Morgan, B.A. (2000) Wnt signaling maintains the hair-inducing activity of the dermal papilla. *Genes & Dev.* **14**:1181-1185.

Kopan, R. and Fuchs, E. (1989) A new look at an old problem: keratins as tools to investigate determination morphogenesis and differentiation in skin. *Genes & Dev* **3**:1-15.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J. and Grosschedl, R. (1996) Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes & Dev.* **10**:1382-1394.

Lam, M.H.C., House, C.M., Tiganis, T., Mitchelhill, K.I., Sarcevic, B., Cures, A., Ramsay, R., Kemp, B.E., Martin, T.J. and Gillespie, M.T. (1999) Phosphorylation at the cyclin-

dependent kinases site (Thr^{85}) of parathyroid hormone-related protein negatively regultes its nuclear localization. *J Biol Chem* **274**:18559-66.

Lanske, B., Karaplis, A., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L., Ho, C., Mulligan, R., Abou-Samra, A., Jüppner, H., Segre, G. and Kronenberg, H. (1996) PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**: 663-666.

Lee, K., Deeds, J.D. and Segre, G.V. (1995) Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**:453-463.

Massfelder, T., Dann, P., Wu, T.L., Vasavada, R., Helwig, J.J. and Stewart, A.F. (1997) Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting, *Proc Nat Acad Sci*, **94**:13630-35.

Montagna, W. (1970) Histology and cytochemistry of human skin. XXXV. The nipple and areola. *British Journal of Dermatology*. **83**:Suppl:2-13.

Miller, J.R., and Moon, R.T. (1996) Signal transduction through b-catenin and specification of cell fate during embryogenesis. *Genes & Dev.* **10**:2527-2539.

Orloff, J.J., Kats, J., Urena, P., Schipani, E., Vasavada, R.C., Philbrick, W.M., Behal, A., Abou-Samra, A.B., Segre, G.V. and Juppner, H. (1995) Further evidence for a novel receptor for amino-terminal PTHrP on keratinocytes and squamous carcinoma cell lines. *Endocrinology* **136**:3016-3023.

Philbrick, W.M., Wysolmerski, J.J., Galbraith, S., Orloff, J.J., Yang, K.H., Vasavada, R.C., Weir, E.C., Broadus, A.E. and Stewart, A.F. (1996) Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol Rev* **76**:127-173.

Philbrick, W.M., Dreyer, B.E., Nakchbandi, I.A. and Karaplis A.C. (1998) Parathyroid hormone-related protein is required for tooth eruption. *Proc Natl Acad Sci USA* **95**:11846-11851.

Propper, A. (1973) Evolution en culture in vitro de l'epithelium mammaire d'embryon de lapin associe au mesoderme·d'osieau. *CR Acad. Sci. Paris* **277**, 2409-2412.

Rothnagel, J. A., Seki, T., Ogo, M., Longley, M. A., Wojcik , S. M., Bundman, D. S., Bickenbach, J. R. and Roop, D. R. (1999) The mouse keratin 6 isoforms are differentially expressed in the hair follicle, footpad, tongue and activated epidermis. Differentiation. 65:119-30.

Sakakura, T. (1987) Mammary embryogenesis. In: *The Mammary Gland, Development, Regulation and Function,* (ed. M.C. Neville, C.W. Daniel) pp 37-65. Plenum Press, New York, NY.

Schockett, P., Difilippantonio, M., Hellman, N. and Schatz, D.G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc Natl Acad Sci USA* **92**:6522-6526.

Schockett, P.E. and Schatz, DG. (1996) Diverse strategies for tetracycline-regulated inducible gene expression. *Proc Natl Acad Sci USA* **93**:5173-5176.

Schweizer, J. and Baust, I. (1989) Winter H. Identification of murine type I keratin 9 (73 kDa) and its immunolocalization in neonatal and adult mouse foot sole epidermis. *Experimental Cell Research.* 184:193-206.

Sengel P. (1976) Morphogenesis of Skin. Cambridge University Press: London,.

Strewler GJ. (2000) The physiology of parathyroid hormone-related protein. *N. Eng. J. Med.* **342**: 177-185.

Toyoshima, Y., Ohsako, S., Nagano, R., Matsumoto, M., Hidaka, S. and Nishinakagawa, H. (1998) Histological changes in mouse nipple tissue during the reproductive cycle. Journal of Veterinary Medical Science. 60:405-11.

Uyttendaele, H., Soriano, J.V., Montesano, R. and Kitajewski, J. (1998) Notch 4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. *Dev. Biol* **196**:204-217.

van Genderen, C., Okamura, R.M., Farinas, I., Quo, R., Parslow, T.G., Bruhn, L. and Grosschedl, R. (1994) Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes & Dev* **8**:2691-2703.

Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M. and Tabin, C.J. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**:613-622.

Weir, E.C., Philbrick, W.M., Amling, M., Neff, L.A., Baron, R. and Broadus, A.E. (1996) Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc Natl Acad Sci USA* **93**:10240-10245.

Widelitz R. B., Jiang, T.X., Lu, J. and Chuong, C.M. (2000) beta-catenin in epithelial morphogenesis: conversion of part of avian foot scales into feather buds with a mutated beta-catenin. *Developmental Biology.* **219**:98-114.

Wysolmerski, J.J., Broadus, A.E., Zhou, J., Fuchs, E., Milstone, L.M. and Philbrick, W.M. (1994) Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc Natl Acad Sci USA* **91**:1133-1137.

Wysolmerski, J.J., McCaughern-Carucci, J.F., Daifotis, A.G., Broadus, A.E. and Philbrick, W.M. (1995) Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development* **121**:3539-3547.

Wysolmerski, J.J. and Stewart, A.F. (1998) The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. *Annual Rev. Physiol* **60**:431-460.

Wysolmerski, J.J., Philbrick, W.M., Dunbar, M.E., Lanske, B., Kronenberg, H., Karaplis, A. and Broadus, A.E. (1998) Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**: 1285-1294.

Zhou, P., Byrne, C., Jacobs, J. and Fuchs, E. (1995) Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes & Dev* **9**:700-13.

Zhu, A. J. and Watt F. M. (1999) beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development.* **126**:2285-98.

Figure Legends

Figure 1. Wild type (A, C, E, G) and PTHrP knockout (B, D, F, H) mammary rudiments at E18. In the wild type (A), there is a primary duct (arrow) and a nipple sheath (arrowheads) forming on either side of the duct. In contrast, the PTHrP knockout mammary gland (B) remains bud-like, and no nipple sheath is present. Note that the epithelial cells in the center of the bud resemble supra-basal cells in the epidermis, and that hair follicles are forming from the neck of the mammary bud (arrows). C&D are stained for keratin 14. All mammary epithelial cells in the wild type duct stain (C, arrow), but only peripheral cells in the PTHrP knockout bud (D). E&F are stained for K1. There is no K1 staining in the wild-type duct (E, arrow) but the central cells in the knockout bud stain (F). G&H are stained for fillagrin. Neither wild type (G, arrow) nor knockout (H) mammary epithelial cells stain. Scale bar represents 15 μm.

Figure 2. A,D & G are sections from wild-type ventral skin. B, E & H are from wild-type nipple skin. C, F & I are from K14-PTHrP transgenic ventral skin. All sections are from 8 week old mice. A-C are stained with hematoxylin and eosin. Note the thickened epidermis, hyperkeratosis and lack of hair follicles (HF) in the nipple (B) and transgenic (C) skin as compared with wild-type (A). D-F are sections stained for smooth muscle α-actin. Bands of smooth muscle extend along the primary lactiferous duct (D), under the nipple in E (arrows). Transgenic dermis (F) also contains similar bands of cells (arrows). In the wild type, staining is limited to the vasculature and erector pili muscles (arrow in D). F-H are sections stained with aldehyde fuchsin. Note the abundant elastic fibers (staining in blue) in the transgenic (I) and nipple (H) dermis as compared with wild-type (G). Scale bar represents 15 μm in A-C, 25 μm in D-F and 15μm in G-I.

Figure 3. A, D & G are sections from wild-type ventral skin. B, E & H are from wild-type nipple skin. C, F & I are from K14-PTHrP transgenic ventral skin. All sections are from 8-week-old

28

mice. A-C are stained for keratin 14, D-F for keratin 1 and G-I for filaggrin. For each marker, the pattern of staining in transgenic skin is similar to that in nipple skin and both are different from wild type. Nipple and transgenic skin demonstrate an expanded basal pattern of K14 staining, a shift of K1 staining to the upper layers of the epidermis and an augmentation of filaggrin staining. The dotted lines in E & F are at the dermal-epidermal border. The scale bar represents 15 μm.

Figure 4. (A) The K14-tTA transgene consists of the human keratin 14 promoter driving expression of the tetracycline transactivator cDNA. 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 promoter containing 7 tet operon binding sites. In double transgenic mice, the tTa protein is produced in tissues expressing keratin 14. This is a "tet-off" system, so that 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 does not bind and the Tet_o-PTHrP transgene remains silent. (B&C) K14-tTA transgenic mice were bred to Tet_o-β-gal mice, and double transgenic mice were raised on (B) or off (C) of tetracycline. There is no expression of β-galactosidase in the presence of tetracycline (B). In the absence of tetracycline, β-galactosidase activity produces a blue color only in basal keratinocytes and developing hair follicles, cells known to express keratin 14. Scale bar represents 15 μm.

Figure 5. Each panel represents a section of ventral skin taken from a K14-tTA/ Tet_o-PTHrP double transgenic mouse exposed to different patterns of tetracycline before being sacrificed at 7 weeks of age. Sections A, E, I & M are from double transgenic mice always on tetracycline. Therefore, the transgene was always off (Tg-off). Sections B,F,J & N are from double transgenic mice always off tetracycline. Therefore, the transgene was always on (Tg-on). Sections C,G,K & O are from double transgenic mice off tetracycline during gestation, but given tetracycline at birth. Therefore, the transgene was on before birth, but was shut off after birth (Tg-on/off). Sections D, H, L & P are from double transgenic mice exposed to tetracycline during gestation but taken off of

tetracycline at birth. Therefore, the transgene was off before birth and was turned on at birth. A-D are stained with H&E, E-H for keratin 14, I-L for keratin 1 and M-P for filaggrin. When double transgenic mice are left off tetracycline (Tg-on, B, F, J, N), hair follicle (HF) development is suppressed and keratinocyte marker expression is altered as in the original K14-PTHrP transgenics. When these mice are reared on tetracycline (Tg-off, A, E, I, M) these changes do not occur. These same nipple-like skin changes occur when PTHrP overexpression occurs before birth, but not after birth (Tg-on/off, C, H, K, O). However, when PTHrP overexpression is initiated after birth (Tg-off/on, D, H, L, P), none of these alterations takes place. Scale bar represents 20 μm.

Figure 6. Immunohistochemistry for LEF-1 in mammary buds and ventral skin at E15. (A) is a wild-type mammary bud. Note the intense nuclear staining for LEF-1 in the mesenchymal cells surrounding the epithelial bud (EB). LEF-1 is also expressed in the mammary epithelial cells, but not in the basal keratinocytes near the mammary bud. (B) is a PTHR1 knockout bud and (C) is a PTHrP knockout bud. In the knockouts, LEF-1 expression in the mammary mesenchyme is lost, but it is retained within the basal keratinocytes near the buds. (D) is wild-type ventral skin at E15. LEF-1 is normally expressed in basal keratinocytes, but not within the dermis. (E) is ventral skin from a K14-PTHrP transgenic embryo at E15. LEF-1 expression is induced in the transgenic dermis but is lost in the keratinocytes, reproducing the pattern normally seen in the developing nipple. Scale bar represents 10 μm.

Figure 7. Immunohistochemistry for β-catenin and anti-sm nuclear antigen in mammary buds and skin from E15 embryos. Sections are double-stained, with red representing the β-catenin and green the nuclei. The confocal images in A, C, E & G show just the β-catenin staining and B, D, F & H show the merged images. A & B represent a wild-type mammary bud. Note the typical peripheral-staining pattern for β-catenin in the epithelial cells of the bud (EB). β-Catenin is also expressed in the mammary mesenchyme (MM) although in a more patchy and punctate pattern. Note the lack of nuclear β-catenin on the merged view. Arrowheads in A outline the border between the mammary

epithelium and the mammary mesenchyme. C & D represent a PTHrP knockout mammary bud. Note that β-catenin expression is unchanged in the epithelial cells but is reduced in the mammary mesenchyme. E & F represent wild-type ventral skin. Note the epithelial pattern of β-catenin staining in the epidermis (E) and the lack of β-catenin expression in the dermis (D). G & H represent K14-PTHrP transgenic ventral skin. Note the dramatic induction of β-catenin expression in the dermis. As with the mammary mesenchyme there is no obvious nuclear β-catenin expression. Arrowheads in G mark the epidermal/dermal border. The scale bar represents 6 μm.

Figure 8. Comparison of K14-PTHrP transgenic mice and K14-PTHrP/PTHR1 [-/-] mice. Panels A, C, E, G, I & K are sections of ventral skin from a newborn K14-PTHrP female mouse. Panels B, D, F, H, J & L are sections of skin from a newborn K14-PTHrP/PTHR1^{-/-} mouse. A & B are stained with hematoxyln and eosin, C & D for tenascin E & F for androgen receptor, G & H for LEF-1. I & J for β-catenin, and K & L for keratin 14. In the absence of the PTHR1 (D, F, H, J), the dermis fails to express mammary mesenchyme markers normally induced in K14-PTHrP transgenic skin (C, E, G, I), hair follicle growth returns (arrows in B & L) and the epidermis reverts back to a normal thickness (compare B to A). In neonates, there are only subtle differences in keratinocyte marker expression in the K14 mice as compared to normal. Note the elongated appearance of the basal keratinocytes and the frequent suprabasal cells expressing keratin 14 in K. However, in the absence of the PTHR1, the basal keratinocytes are flatter and only rare suprabasal cells express keratin 14 (L). The scale bar represents 16 μm in all panels except I & J. where it represents 10 μm.

Figure 9. Model for the regulation of cell fate by PTHrP-signaling during mammary gland and nipple development. The different colors represent different cell fates: red circles represent mammary epithelial cells, yellow squares represent mammary mesenchyme, green ovals represent dermal mesenchyme, blue ovals represent typical epidermal cells and purple squares represent nipple cells. (A) Normally, the mammary epithelial cells express PTHrP after the bud starts to

31

form. PTHrP signals to the dermal mesenchyme near the developing bud and, as a result, these cells become mammary mesenchyme. The mammary mesenchyme maintains the mammary fate of the epithelial cells, triggers their morphogenesis and induces the overlying epidermis to become the nipple. (B) In the absence of PTHrP signaling no mammary mesenchyme is formed. Therefore, the mammary epithelial cells revert to an epidermal fate, no morphogenesis occurs and the nipple does not form. (C) In the presence of diffuse PTHrP signaling, the entire ventral dermis becomes mammary mesenchyme and the ventral epidermis becomes nipple sheath.

FIG 1

	Control	Nipple	Transgenic
A / D			
(41) / E			
/ F			

A — Control; Nipple; Transgenic

D; E; F

Fig. 3
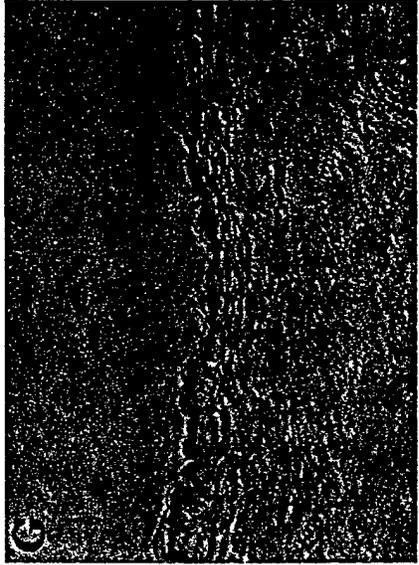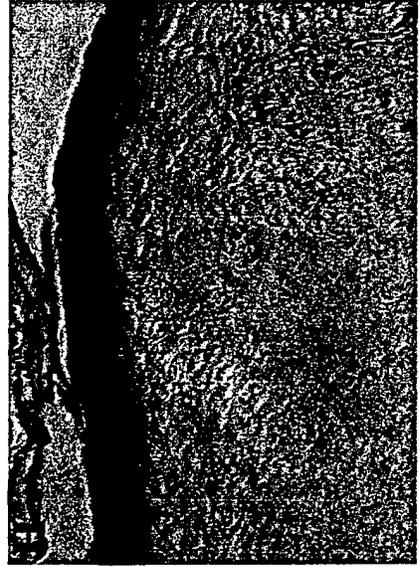
FIG 4

A

h K14   tTA   SV40

p/t

tet ●

Tet R VP16

minCMV

Tet R VP16

Tet$_{O7}$ PTHrP   1   2   3   4   5   hGH

p/t

Tet$_0$ - PTHrP

B

C

	Tg-off	Tg-on	Tg-on/off	Tg-off/on
H&E	A	B	C	D
K14	E	F	G	H
K1	I	J	K	L
Fil	M	N	O	P

FIG 6

A
MM
EB

B

C
MM
EB

D

E
E
D

F

G
E
D

H

A

B

C

D

E

F

G

H

I

J

K

L

A WILD TYPE

Localized PTHrP Signaling

Mammary Mesenchyme Differentiation

Mammary and Nipple Morphogenesis

B PTHrP and PTHR KO

No PTHrP Signaling

Failure of Mammary Mesenchyme Differentiation

No Nipple or Mammary Morphogenesis

C K14 - PTHrP Transgenics

Difffuse PTHrP Signaling

Inappropriate Mammary Mesenchyme Differentiation

Diffuse Ventral Nipple Formation
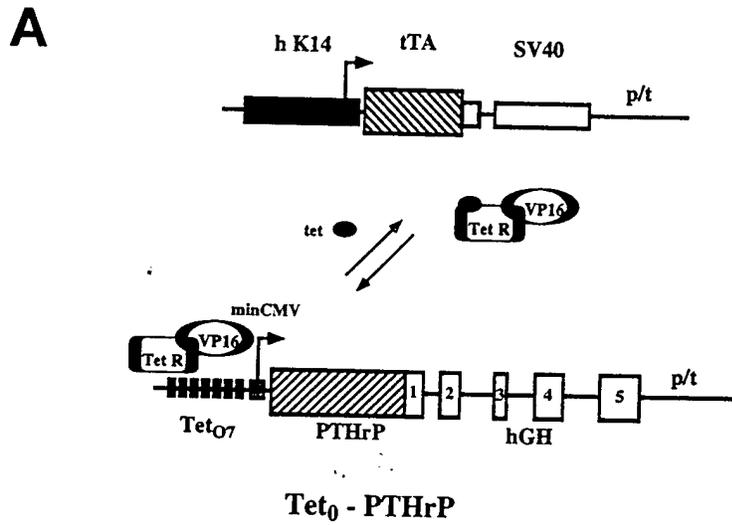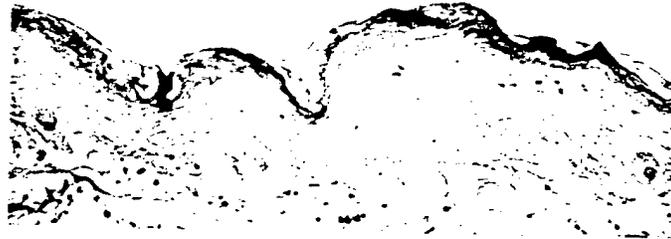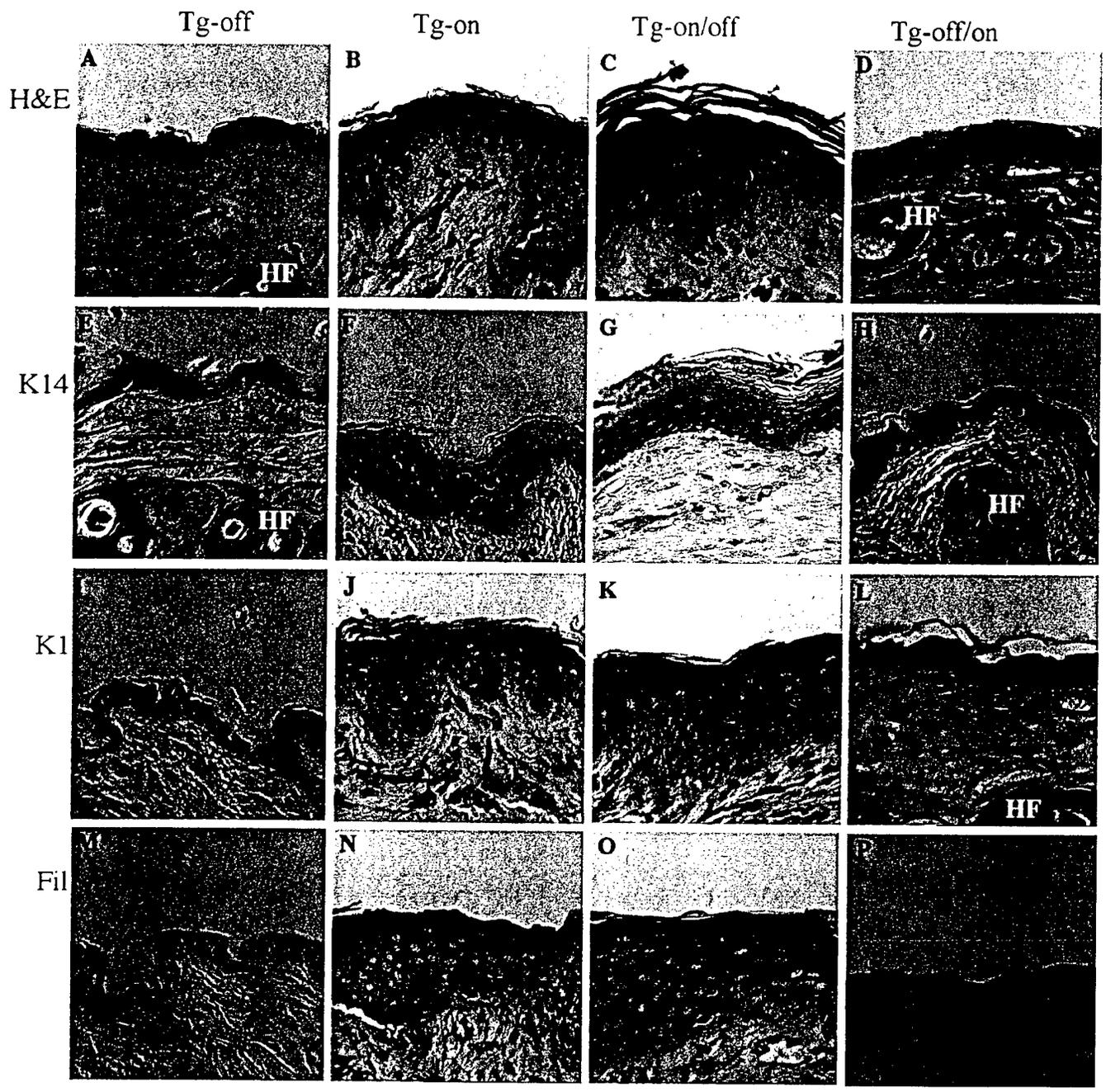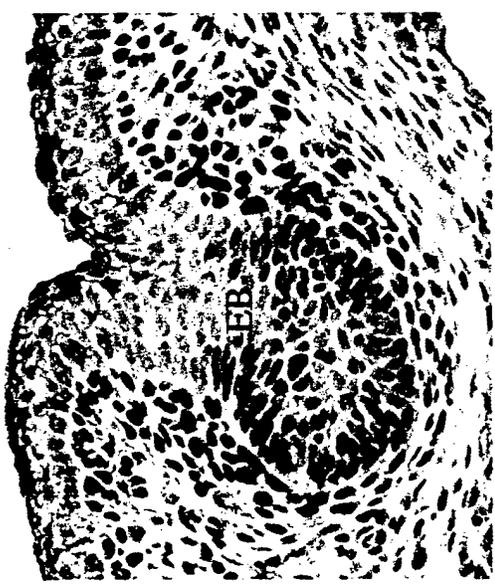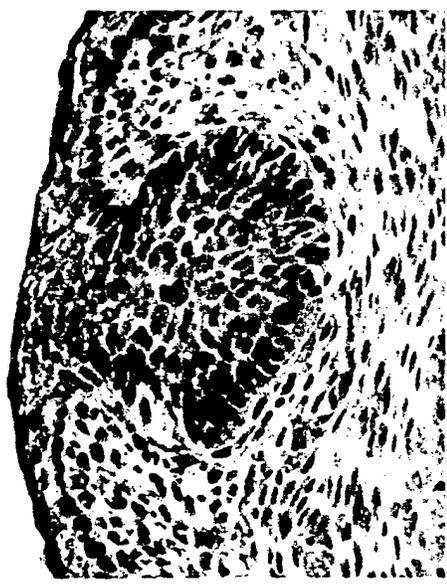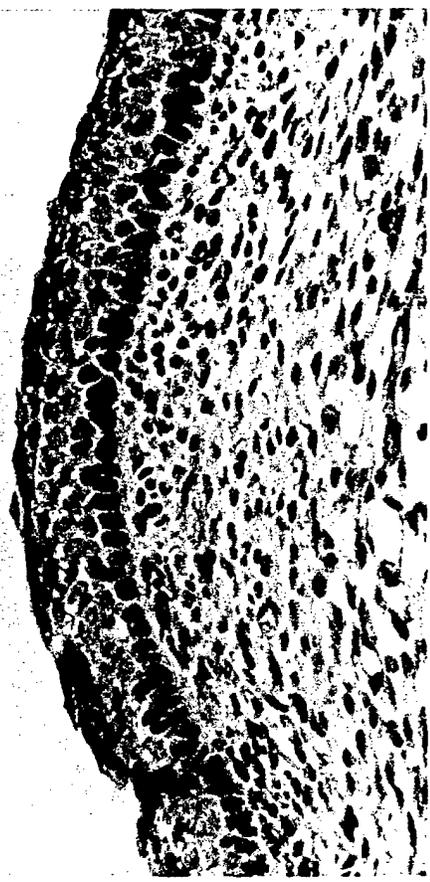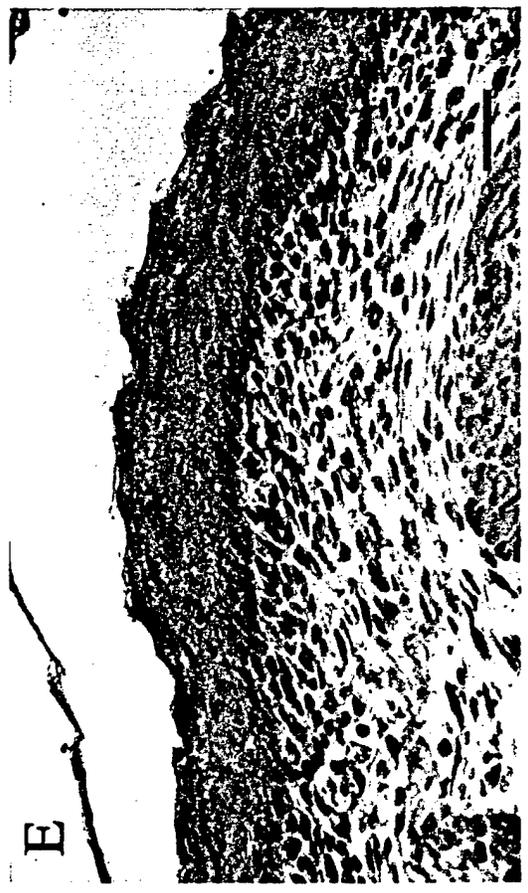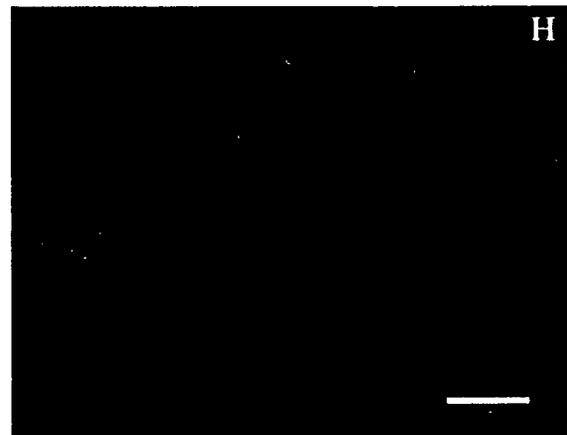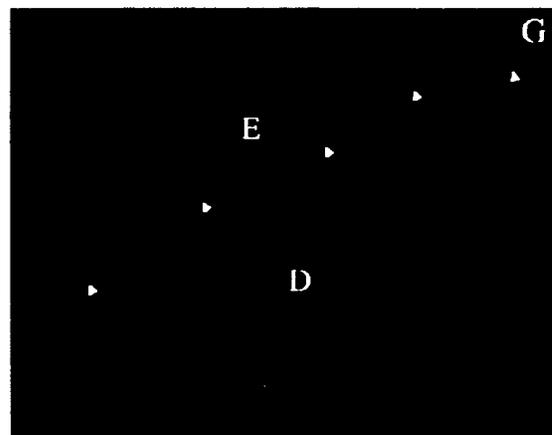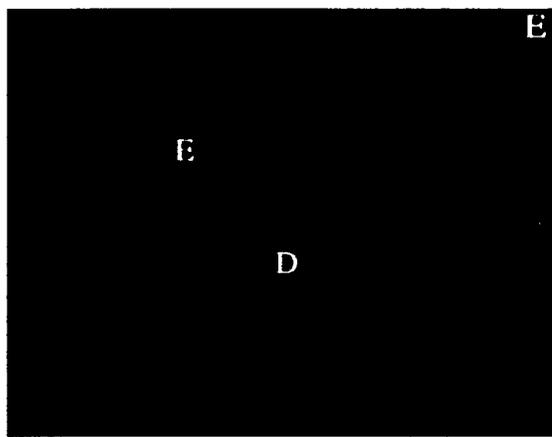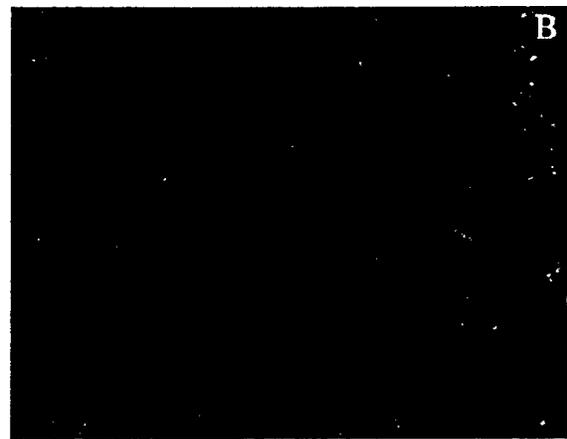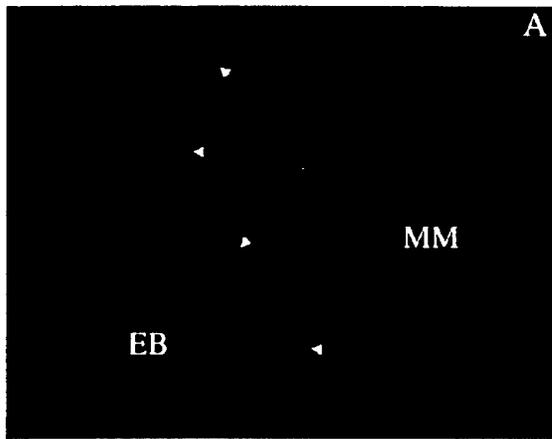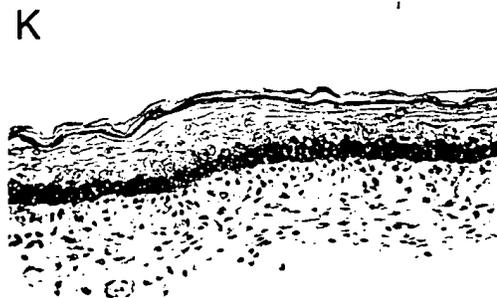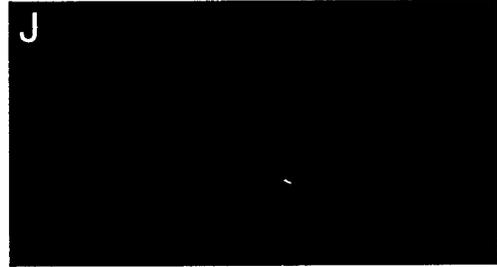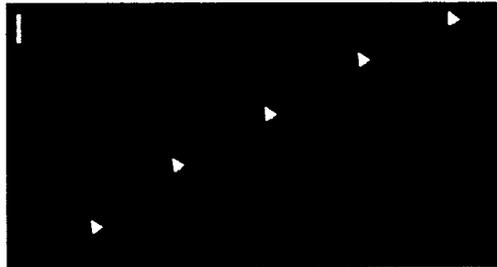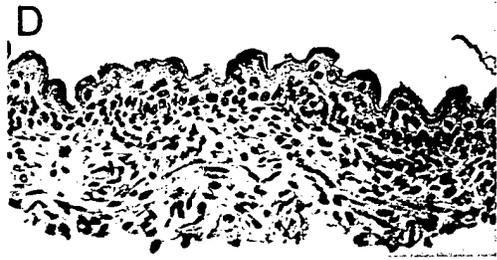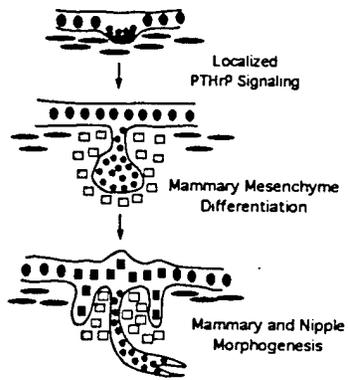
Reprints:

All papers from the last year of funded research are either in press or in preparation. Therefore we have no new reprints to include. Reprints of prior publications were included in the previous annual reports.