DEVELOPMENTAL BIOLOGY **203,** 75–89 (1998)
ARTICLE NO. DB989029

Stromal Cells Are Critical Targets in the Regulation of Mammary Ductal Morphogenesis by Parathyroid Hormone-Related Protein

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Parathyroid hormone-related protein (PTHrP) was originally identified as the tumor product responsible for humoral hypercalcemia of malignancy. It is now known that PTHrP is produced by many normal tissues in which it appears to play a role as a developmental regulatory molecule. PTHrP is a normal product of mammary epithelial cells, and recent experiments in our laboratory have demonstrated that overexpression or underexpression of PTHrP in the murine mammary gland leads to severe disruptions in its development. The nature of these phenotypes suggests that PTHrP acts to modulate branching growth during mammary development by regulating mammary stromal cell function. We now demonstrate that throughout mammary development, during periods of active ductal-branching morphogenesis, PTHrP is produced by epithelial cells, whereas the PTH/PTHrP receptor is expressed on stromal cells. In addition, we show that mammary stromal cells in culture contain specific binding sites for amino terminal PTHrP and respond with an increase in intracellular cAMP. Finally, we demonstrate that the mammary mesenchyme must express the PTH/PTHrP receptor in order to support mammary epithelial cell morphogenesis. These results demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal signaling circuit that is necessary for mammary morphogenesis and that stromal cells are a critical target for PTHrP's action in the mammary gland.

Key Words: **mammary gland development; branching morphogenesis; mammary mesenchyme; mammary stroma; tissue recombination experiments.**

INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was initially discovered because of its pathogenic role in a common paraneoplastic syndrome known as humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). It derives its name from the fact that its gene and the parathyroid hormone (PTH) gene are both descended from a common ancestor through a process of gene duplication (Broadus and Stewart, 1994). As a result, the two genes share sequence homology and structural characteristics that allow amino-terminal species of PTH and PTHrP to signal through the same receptor, termed the type I PTH/PTHrP receptor (Jüppner *et al.,* 1991). Despite these similarities, these two peptides have evolved to serve very different functions. PTH is made solely by the parathyroid chief cells and is secreted into the systemic circulation where it functions as a classical peptide hormone regulating calcium homeostasis. In contrast, PTHrP is made by a wide variety of cell types, does not circulate, and appears to

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0012-1606/98 $25.00

function as a local autocrine or paracrine factor influencing cell growth and differentiation (Broadus and Stewart, 1994).

As noted above, amino-terminal PTHrP signals through a seven-transmembrane-spanning, G-protein-coupled receptor known as the type I PTH/PTHrP receptor (Jüppner *et al.*, 1991). This receptor subserves the calcium-regulating functions of PTH and PTHrP in classical PTH-target organs, but, like PTHrP, is also widely expressed in tissues not involved in calcium homeostasis (Orloff *et al.*, 1989). In these sites the receptor is often expressed in cells in close proximity to those expressing PTHrP, suggesting that it also mediates many of the normal physiologic functions of PTHrP (Lee *et al.*, 1995). However, it is also known that PTHrP is a polyprotein which, through a series of posttranslational processing steps, gives rise to several biologically active peptides not containing the amino terminus (Broadus and Stewart, 1994; Soifer *et al.*, 1992; Wu *et al.*, 1996). These peptides presumably signal through distinct, but as yet unidentified, receptors (Kovacs *et al.*, 1996; Orloff *et al.*, 1996).

PTHrP's role in mature organisms remains unclear, but in recent years evidence has accumulated to suggest that this peptide functions as an important developmental regulatory molecule (Dunbar *et al.*, 1996; Philbrick *et al.*, 1996). In fact, they have been reported to be one of the earliest peptide hormone/receptor pairs to be detected during mouse development and appear to participate in the formation of parietal endoderm (Behrendsten *et al.*, 1995; deStolpe *et al.*, 1993). Because PTHrP-knockout mice suffer from a fatal form of chondrodysplasia (Karaplis *et al.*, 1994), the most widely studied aspect of PTHrP's developmental functions has been its role in regulating chondrocyte differentiation during fetal bone development. In the absence of PTHrP, chondrocytes within the fetal growth plate appear to differentiate too rapidly, and the fetal bones ossify prematurely (Amizuka *et al.*, 1994; Karaplis *et al.*, 1994). In contrast, mice overexpressing PTHrP in chondrocytes are born with a cartilaginous skeleton, resulting from a profound delay in chondrocyte differentiation (Weir *et al.*, 1996). Recent studies have demonstrated that PTHrP acts in a feedback loop with Indian Hedgehog and BMP's, in a pathway involved in controlling the rate at which immature chondrocytes progress through their program of differentiation (Kretzschmar *et al.*, 1997; Lanske *et al.*, 1996; Vortkamp *et al.*, 1996).

Another site in which PTHrP has clearly been shown to have an important developmental role is the mammary gland. Soon after its discovery, PTHrP was noted to be expressed in the pregnant and lactating mammary gland and to be present in large quantities in milk (Budayr *et al.*, 1989; Thiede and Rodan, 1988). Its function during pregnancy and lactation is still unknown but more recent data from transgenic models of PTHrP underexpression and overexpression have shown that it participates in the regulation of ductal branching morphogenesis during embryonic development as well as during sexual maturation and early pregnancy (Wysolmerski *et al.*, 1996; 1998). In the absence of PTHrP or the PTH/PTHrP receptor, mammary epithelial buds form, but fail to initiate ductal branching morphogenesis. Instead, the fetal epithelial cells degenerate and mammary glands do not form (Wysolmerski *et al.*, 1998). Overexpression of PTHrP or PTH within the mammary gland also perturbs ductal branching morphogenesis (Wysolmerski *et al.*, 1996). In this case, an excess of PTHrP results in severe defects in ductular proliferation and side branching during puberty and the inhibition of terminal ductule formation during early pregnancy. Hence, amino-terminal PTHrP, acting through the PTH/PTHrP receptor, appears to contribute to the regulation of ductal branching morphogenesis at several different stages of mammary development.

Like many other epithelial organs, the mammary gland is dependent on the sequential and reciprocal exchange of information between epithelial cells and neighboring mesenchymal cells for its proper morphogenesis (Cunha and Hom, 1996; Sakakura, 1991; Thesleff *et al.*, 1995). These epithelial–mesenchymal interactions are especially critical for the regulation of ductal branching morphogenesis. The studies cited above suggest that PTHrP might act to regulate this process during mammary development by serving as an epithelial signal acting to regulate mammary stromal cell function. In this study, we present data to support such a role for PTHrP. We demonstrate that during periods of active ductal branching morphogenesis, the PTHrP gene is expressed in the mammary epithelium, and the PTH/PTHrP receptor gene is expressed in mammary stroma. In addition, we show that mammary stromal cells bind and respond to amino-terminal PTHrP. Finally, we demonstrate that the presence of the PTH/PTHrP receptor in mammary mesenchyme is critical to the ability of the mesenchymal cells to support the initiation of ductal growth.

MATERIALS AND METHODS

In Situ Hybridization

In situ hybridization was performed on 5-μm paraffin sections as described previously (Wysolmerski *et al.*, 1998). 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 (Weir *et al.*, 1996). Sense and antisense probes 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 min 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 min. Sections were then rinsed in 2× SSC and incubated for 30 min in 0.66% *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) in 2× SSC, rinsed in 2× SSC, dehydrated in graded alcohol, treated with chloroform for 5 min, rehydrated, and then air-dried. The probes (1.5×10^7 cpm/ml) were then hybridized to the samples for 17 h 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 min at 52°C, rinsed in 2× SSC, and treated with 30 μg/ml RNase A in 2× SSC at 37°C for 30 min. Following two rinses in 2× SSC, sections were again washed in 2× SSC/50% formamide at 52°C for 5 min, dehydrated through graded ethanol, air-dried, and dipped in a mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development of the emulsion, sections were counterstained with hematoxylin and mounted for microscopic examination.

Preparation of Mammary Stromal Cell Cultures

Mammary epithelial and stromal cells were isolated using a modification of a previously described procedure (Haslam and Levely, 1985). Briefly, the No. 4 inguinal mammary glands were dissected from 11- to 14-day pregnant CD-1 mice, minced with razor blades, and incubated overnight at 37°C in a digestion buffer containing DMEM/F12, 5% FBS, 0.2% dispase grade II, 0.2% collagenase type III, 50 μg/ml gentamycin, 100 units/ml nystatin, and 2.5 μg/ml amphoteracin B. Following digestion, the cells were pelleted by centrifugation at 1500 rpm, washed with DMEM, and then filtered through a 70-μm nitex mesh filter to remove mammary epithelial organoids. To enrich the remaining cells for mammary fibroblasts, we used the differential centrifugation method of Voyles and McGrath (1976). The flow-through following filtration was spun at 80*g* for 30 s to pellet epithelial cells, and the resulting supernatant, containing the mammary fibroblasts, was removed and plated in DMEM medium supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 units/ml nystatin, 2.5 μg/ml amphoteracin B, and 5 μg/ml each insulin and hydrocortisone. Finally, to remove any remaining epithelial cells, the medium was changed 4 h after plating, and the resulting cultures of mammary stromal cells were grown at 37°C in 5% CO_2 for 5 days.

To assess the purity of the mammary stromal cultures, we performed immunohistochemistry using anti-vimentin and anti-keratin 14 and anti-keratin 8,18 antibodies as stromal cell and epithelial cell markers, respectively. The anti-mouse vimentin antibody is a monoclonal antibody and was purchased from Boehringer Mannheim (Indianapolis). The K14 antibody is an affinity-purified rabbit polyclonal antibody and was a kind gift of Dr. D. Roop (Houston, TX). The anti-keratin 8,18 antibody is a mouse monoclonal and was purchased from Nova-Castra (Burlingame, CA). Immunohistochemistry was performed using standard techniques 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 hematoxlyn.

RNA Isolation and RNase Protection Analysis

Total RNA was isolated from cells using Trizol reagent (Gibco, Gaithersville, MD). RNase protection analysis was performed as described previously (Daifotis *et al.,* 1992) using 2×10^5 cpm of labeled antisense cRNA probes corresponding to a 349-bp *Avr*II *Pvu*II genomic fragment of the mouse PTHrP gene and a 283-bp *Sau*3a–*Pvu*II fragment of the mouse PTH/PTHrP receptor gene. For an internal standard, 5×10^4 cpm of labeled antisense probe corresponding to a 220-bp *Sau*3a–*Sau*3a fragment of the mouse cyclophilin gene was used.

Receptor Binding Assay

The receptor binding assays were performed as described previously (Orloff *et al.,* 1992) using 30,000 cpm/well of ^{125}I-labeled [Tyr36]hPTHrP-(1–36) NH_2 in a final volume of 0.15 ml/well in 24-well plates for 4 h at 4°C in the presence or absence of increasing concentrations of competing unlabeled PTHrP(1–36).

cAMP Assay

Cells were grown to confluence in 12-well plates at 37°C in 5% CO_2. Prior to the experiment, the cells were washed once with serum-free medium containing 0.1% BSA and then incubated with serum-free medium/0.1% BSA with or without PTHrP (1–36) at various concentrations and for various time periods. The medium was then aspirated, and the cells were treated with ice cold 90% *n*-propanol for 24 h at −70°C. The samples were then lyophilized, and intracellular cAMP content was measured using a commercially available RIA (Biomedical Technologies, Stoughton, MA).

Tissue Recombination Experiments

Mammary gland rudiments were dissected from E13 PTH/PTHrP receptor knockout (ko) and wild-type (wt) embryos which were identified by their genotype as determined by PCR as described previously (Wysolmerski *et al.,* 1998). The mammary rudiments were incubated for 1.5 h in 1% Bacto-trypsin in calcium–magnesium-free Hanks' salt solution at 4°C. Following neutralization of the enzyme with 10% fetal bovine serum in DMEM, the epithelium and mesenchyme were teased apart with watchmakers forceps. For PTH/PTHrP receptor knockout embryos, the four possible tissue recombinants were prepared with mammary epithelium (MGE) and mammary mesenchyme (MGM) from wt and ko mice: wt-MGM + wt-MGE, wt-MGM + ko-MGE, ko-MGM + ko-MGE, and ko-MGM + wt-MGE (Cunha *et al.,* 1995). All tissue recombinants were transplanted beneath the renal capsule of female athymic nude mouse hosts (see web site http://mammary.nih.gov/tools/Cunha001/index.html for technical details). Following 1 month of growth, the grafts were harvested for histological analysis (Cunha *et al.,* 1995).

RESULTS

Expression of PTHrP and the PTH/PTHrP Receptor during Ductal Morphogenesis

To begin to study the mechanisms by which PTHrP and the PTH/PTHrP receptor regulate ductal growth and branching morphogenesis, we examined their temporal and spatial patterns of expression during fetal life, during sexual maturation, and during early to mid-pregnancy, three periods of active ductal growth during mammary development. We first sought to determine the temporal pattern of PTHrP and PTH/PTHrP receptor mRNA expression in the whole mammary gland by RNase protection analysis. Due to the small size of the mammary glands, this analysis was not possible for fetal time points and, therefore, we initiated these studies by examining the temporal pattern of PTHrP and PTH/PTHrP receptor expression in the preadolescent gland (3-week-old virgin), during puberty (6-week-old vir-

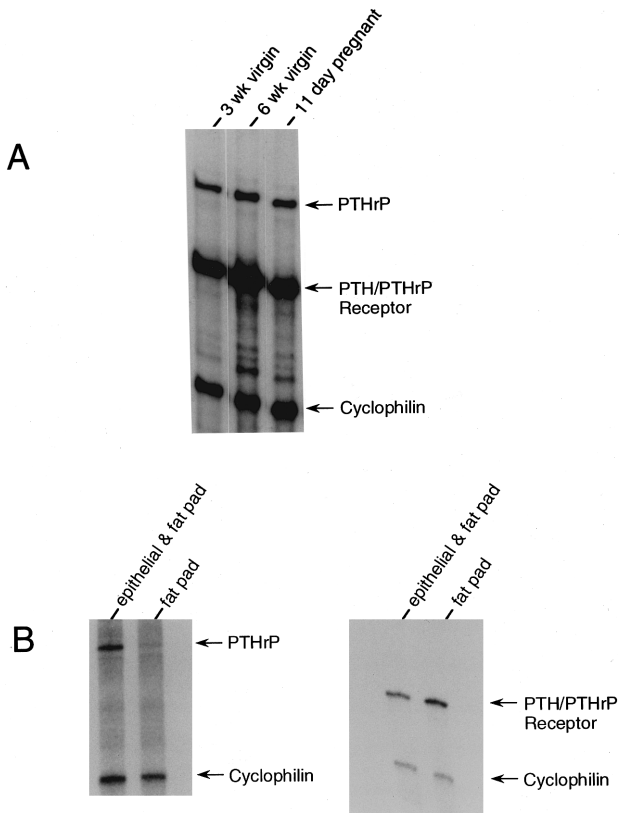

FIG. 1. (A) Analysis of PTH and PTH/PTHrP receptor RNA in the mammary gland during preadolescence, sexual maturation, and pregnancy. 50 μg of total cellular RNA prepared from mammary tissue from preadolescent (3-week-old virgin), adolescent (6-week-old virgin), and pregnant (11 days postcoitus) mice was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. The murine cyclophilin RNA was included as a loading control. Note that both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. (B) Analysis of PTHrP and PTH/PTHrP receptor mRNA expression in proximal and distal segments of preadolescent mammary glands. Mammary glands from 3-week-old virgin mice were dissected and separated into proximal and distal segments. Whole mount analysis confirmed that the proximal segment contained both epithelial and stromal components, while the distal segment contained only stroma (data not shown). 50 μg of total cellular RNA prepared from either the proximal component (epithelial + fat pad) or the distal component (fat pad) was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that the proximal component with both epithelial and stromal cells contains both PTHrP and the PTH/PTHrP receptor mRNA, but the distal component, with stromal cells alone, contains only the PTH/PTHrP receptor.

gin), and during early to mid-pregnancy (11 days postcoitus). As shown in Fig. 1A, both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. In addition, despite the dramatic changes in cellular composition of the mammary gland at these different time points, whole-gland levels of PTHrP and PTH/PTHrP receptor mRNA expression remained relatively constant.

Our prior studies had suggested that, during embryonic mammary development, PTHrP mRNA is expressed in mammary epithelial cells and PTH/PTHrP receptor mRNA is expressed in the mammary mesenchyme (Wysolmerski *et al.*, 1998). To determine if this pattern was also present during the later stages of ductal morphogenesis, we first took advantage of the directional growth of mammary epithelial ducts during puberty. Prior to the initiation of adolescence the mammary ducts occupy only a small portion of one end of the mammary fat pad and, in response to the hormonal stimulation of puberty, they grow toward the opposite end of the mammary fat pad until they completely fill out this stromal compartment. As a result of this growth pattern, at the initiation of puberty, one can divide the murine mammary gland into a proximal segment that contains both epithelial and stromal components and a distal segment that contains only stroma. As shown in Fig. 1B, the proximal component with both epithelial and stromal cells, contains both PTHrP and PTH/PTHrP receptor mRNA, but the distal component, that is stroma alone, contains only PTH/PTHrP receptor mRNA. These data suggest that PTHrP mRNA is expressed in the mammary epithelium, and that PTH/PTHrP receptor mRNA is expressed within the fat pad stroma.

To examine this possibility directly, we next determined the spatial localization of PTHrP and PTH/PTHrP receptor mRNA expression by *in situ* hybridization during fetal life, during adolescence and during early pregnancy. As shown in Fig. 2, the PTHrP gene was expressed in epithelial cells during periods of mammary ductal growth. At E12, we found PTHrP expression to be very intense in the epithelial cells of the embryonic mammary bud, especially in the cells located peripherally, adjacent to the basement membrane (Figs. 2A and 2B). At E18, at a time when the mammary bud is elongating and initiating ductal branching morphogenesis, PTHrP expression continued to be intense and was localized to mammary epithelial cells (Figs. 2C and 2D). Once again, expression of the PTHrP gene was most obvious in epithelial cells located on the outer most layer of the developing mammary ducts. These results are in agreement with our previous findings that demonstrated PTHrP mRNA expression in epithelial cells of the embryonic mammary bud at E16 (Wysolmerski *et al.*, 1998).

In the postnatal mammary gland (Figs. 2E through 2M), PTHrP mRNA expression continued to be localized to epithelial cells. However, overall, expression appeared to be less intense than during fetal development and it appeared to be restricted to epithelial cells located within terminal end buds. End buds are specialized structures that form at the leading edge of growing ducts, and they serve as the sites of active cellular proliferation and differentiation during phases of ductular proliferation (Daniel and Silberstein, 1987). As seen in Figs. 2E–2I, we found that, during puberty, PTHrP mRNA was present in the epithelial cells of end buds (Figs. 2E through 2G), but was undetectable in

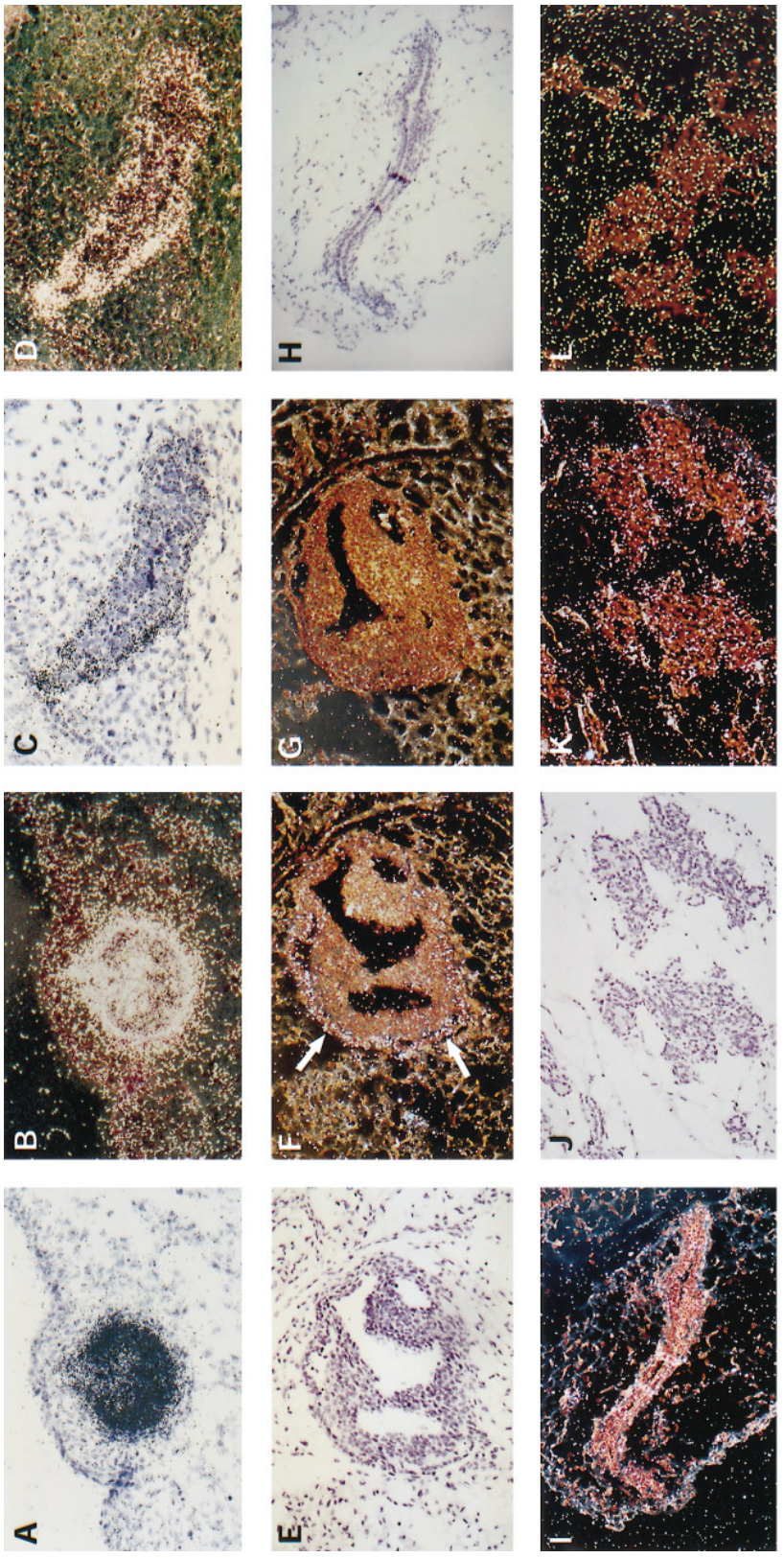

FIG. 2. Localization of PTHrP mRNA expression in mammary glands of embryonic, adolescent, and pregnant mice. (A–D) *In situ* hybridization for PTHrP mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images, respectively, of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTHrP mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of an adolescent mammary gland hybridized with an antisense probe. (J–M) *In situ* hybridization for PTHrP mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

epithelial cells of mature ducts (Figs. 2H and 2I). Specifically, the PTHrP mRNA signal appeared to localize to the peripheral, or cap cells of the end buds, a pattern similar to the peripheral location of the PTHrP signal seen during fetal life. During early pregnancy, there appeared to be a very low level of PTHrP mRNA expression within the epithelial cells of developing lobuloalveolar units (Figs. 2J through 2M) but, similar to puberty, we could not detect PTHrP mRNA in mature mammary ducts (data not shown). However, we did detect a similar pattern of epithelial PTHrP expression in the occasional end bud we observed at the periphery of early pregnant glands (data not shown). Therefore, it appears that similar to embryonic life, in the postnatal mammary gland, the major site of PTHrP expression is within epithelial cells. During puberty the PTHrP gene is expressed most prominently in end buds, and during early pregnancy there appears to be a low level of PTHrP expression within the developing lobuloalveolar units.

In contrast to the epithelial pattern of PTHrP mRNA expression, PTH/PTHrP receptor mRNA appeared to be expressed in mammary stromal cells (Fig. 3). In the embryonic mammary gland, at E12, PTH/PTHrP receptor mRNA was expressed throughout the ventral mesenchyme, including the dense mammary mesenchyme (Figs. 3A and 3B). At E18, when the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in stromal cells enveloping the growing mammary ducts (Figs. 3C and 3D). During puberty, PTH/PTHrP receptor mRNA was expressed at a low level throughout the mammary stroma, but the most prominent PTH/PTHrP receptor expression was in stromal cells immediately surrounding terminal end buds (Figs. 3E through 3G). This expression appeared to be most intense at the neck regions of the end buds and decreased rapidly along the more mature portions of the duct so that the majority of the periductal stroma demonstrated a level of receptor mRNA expression indistinguishable or just above the background expression of the fat pad stroma (Figs. 3H and 3I). During early to mid-pregnancy PTH/PTHrP receptor mRNA also appeared to be expressed at a low level throughout the fat pad stroma both within the periductal stroma and surrounding the developing lobuloalveolar units (Figs. 3J through 3M). However, the signal intensity was very low in the pregnant tissue, and there was not striking pattern of hybridization such as that seen during puberty. The exception to this diffuse low level of hybridization was in the occasional gland with a few remaining end buds during early pregnancy, where there was a strong signal within the stroma surrounding the end buds (not shown).

Together, these studies demonstrate that during active mammary ductular branching morphogenesis, PTHrP is expressed by epithelial cells, and its receptor is expressed by surrounding mesenchymal cells. In addition, it appears that in the postnatal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor gene is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty.

Mammary Stromal Cells Express Functional PTH/PTHrP Receptors

Our *in situ* hybridization results demonstrate that the PTH/PTHrP receptor is expressed in mammary stromal cells and, therefore, suggest that these cells are a target for PTHrP's action in the mammary gland. To test this hypothesis, we prepared primary cultures of both epithelial and stromal cells from adult female mammary glands using previously described protocols (Haslam and Levely, 1985; Voyels and McGrath, 1976) and characterized their composition by immunocytochemistry, using anti-vimentin and anti-keratin 14 and anti-keratin 8, 18 antibodies to identify fibroblasts and epithelial cells, respectively. As defined by the expression of vimentin and the lack of keratin expression, we were able to consistently prepare primary cultures of mouse mammary stromal cells that were 90–95% pure, a level of stromal cell enrichment comparable to that of previous reports (data not shown, Haslam and Levely, 1985).

To determine whether stromal cells in culture continued to express PTH/PTHrP receptor mRNA, total RNA was prepared from our stromal cell cultures and from freshly isolated mammary epithelial organoids and assayed for steady-state levels of both PTHrP and PTH/PTHrP receptor mRNA by RNase protection analysis. As shown in Fig. 4, mammary stromal cells in culture contained PTH/PTHrP receptor mRNA, but not PTHrP mRNA. In contrast, mammary epithelial cells in freshly isolated organoids contained PTHrP mRNA but no PTH/PTHrP receptor mRNA. These results are identical to the results from our *in situ* analysis and lend further support to the epithelial–mesenchymal pattern of expression of PTHrP and the PTH/PTHrP receptor in the mammary gland.

We next examined mammary stromal cells for specific binding sites for amino-terminal PTHrP. For this purpose, receptor binding assays were performed on intact cells, using ^{125}I-labeled PTHrP(1–36) as a ligand. These experiments documented specific binding of ^{125}I-labeled PTHrP-(1–36) amide to mammary stromal cells with an apparent K_d of 8.9 ± 1.4 nM. Binding was specific, as it was effectively competed with unlabeled PTHrP (1–36) amide (Fig. 5). The number of binding sites per cell, as determined by Scatchard analysis, was calculated to be 126,000 ± 13,000. In addition, treatment of mammary stromal cells with PTHrP(1–36) caused a increase in intracellular cAMP over basal (Fig. 6). This cAMP response peaked at 2 min, as shown in Fig. 6a, and showed a dose-dependent increase in cAMP accumulation with maximal responses occurring with 10^{-6} and 10^{-7} M PTHrP (Fig. 6b). Together, these results indicate that mouse mammary stromal cells express the PTH/PTHrP receptor both *in vivo* and *in vitro,* and cultured stromal cells display high-affinity binding sites for amino-terminal PTHrP at their cell surface and respond to PTHrP with an increase in intracellular cAMP.

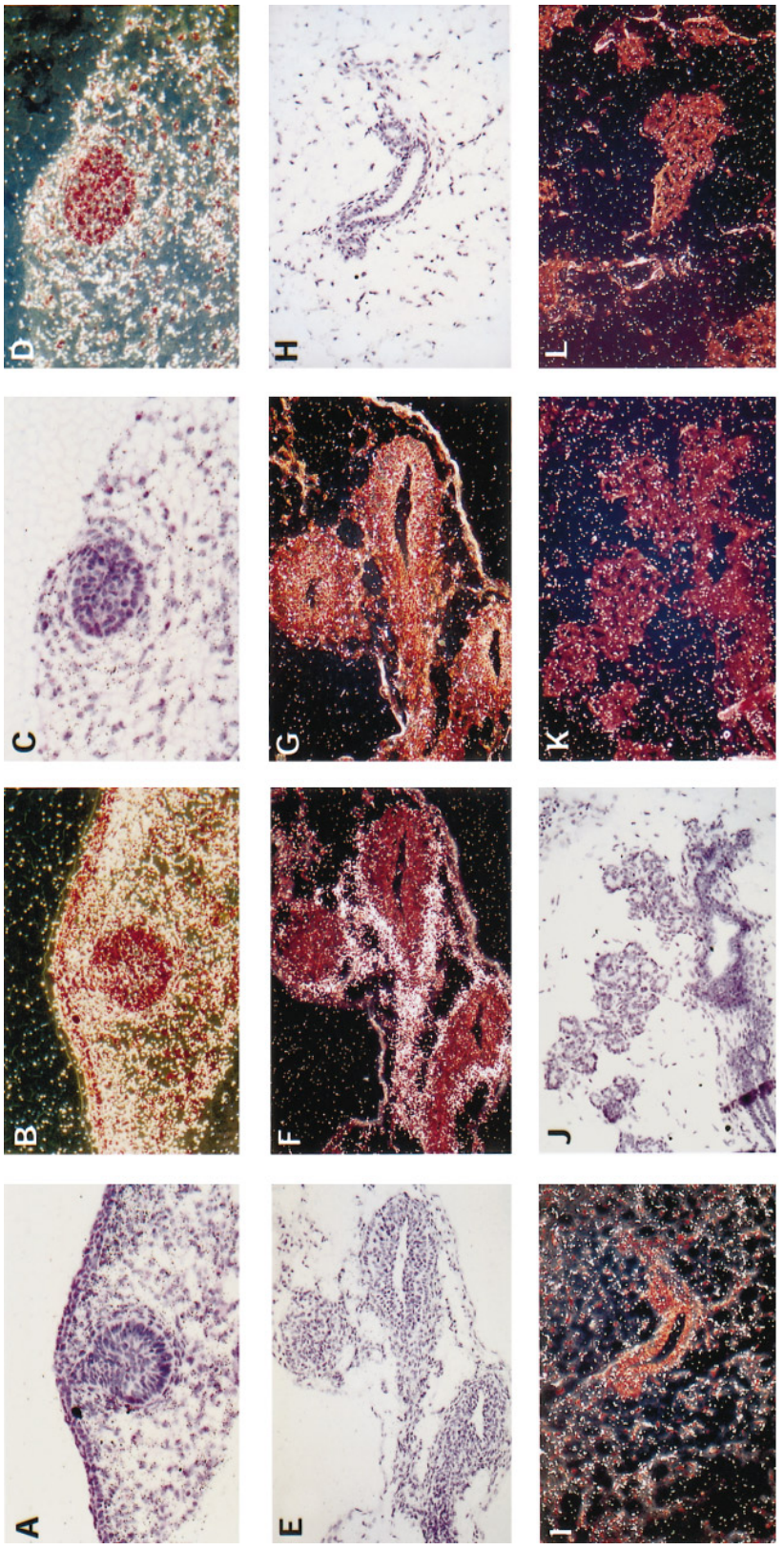

FIG. 3. Localization of PTH/PTHrP receptor mRNA during mammary development. *In situ* hybridization for PTH/PTHrP receptor mRNA in embryonic mammary rudiments at E12 (A and B) and E18 (C and D). A and C represent brightfield images and B and D represent darkfield images of the same sections hybridized with antisense probe. (E–I) *In situ* hybridization for PTH/PTHrP receptor mRNA in the adolescent mammary gland. E and F represent brightfield and darkfield images, respectively, of a section through an end bud of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H and I represent brightfield and darkfield images, respectively, of a section through a mature duct of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. (J–M) *In situ* hybridization for PTH/PTHrP receptor mRNA in the pregnant mammary gland. J and K represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days postcoitus) mouse hybridized with an antisense probe. L and M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

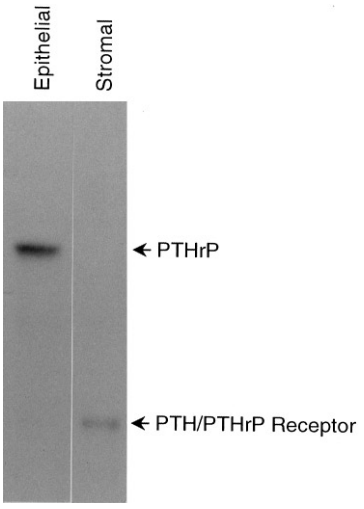

FIG. 4. Analysis of PTHrP and PTH/PTHrP receptor expression in freshly isolated mammary epithelial cells and mammary stromal cells in culture. 50 μg of total cellular RNA from freshly isolated mammary organoids and cultured mammary stromal cells was assayed for both PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that mammary stromal cells in culture express the PTH/PTHrP receptor but not PTHrP mRNA, whereas mammary epithelial cells express PTHrP mRNA but not PTH/PTHrP receptor mRNA.

The Mammary Mesenchyme Must Express the PTH/PTHrP Receptor to Support the Outgrowth of the Mammary Epithelium

We have previously reported that PTHrP and the PTH/PTHrP receptor are required for the outgrowth of the mammary epithelium during embryonic development. In mice lacking either PTHrP or the PTH/PTHrP receptor, mammary buds form but subsequent mammary development fails. In the absence of PTHrP or its receptor, mammary epithelial cells fail to undergo the initial stage of branching morphogenesis known as the primary growth spurt and, instead, degenerate and die (Wysolmerski *et al.*, 1998). These data, together with the expression patterns of PTHrP and the PTH/PTHrP receptor and the presence of functional PTH/PTHrP receptors in mammary stromal cells, as described above, suggest that epithelial-derived PTHrP, acting through stromal PTH/PTHrP receptors, plays an important role in regulating ductal morphogenesis during fetal life and perhaps also at later stages of development (Wysolmerski *et al.*, 1996; 1998). To test this hypothesis directly, we performed a series of tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild-type and PTH/PTHrP receptor-knockout embryos. In these experiments, knockout and wild-type mammary epithelial buds and mammary mesenchyme were recombined in the four possible combinations (see Table 1) and grown under the kidney capsule of recipient females. We reasoned that if the above hypothesis was valid, and PTHrP and the PTH/PTHrP receptor do represent an epithelial–mesenchymal signaling circuit, the PTH/PTHrP receptor-null phenotype would be expected to segregate with mesenchymal tissue. That is, receptor-knockout mesenchyme should be unable to support the outgrowth of either receptor-knockout or normal epithelial buds, but receptor-knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Table 1 and Fig. 7 summarize the results of these experiments. As expected, wild-type epithelial buds paired with wild-type mammary mesenchyme (wt-MGE + wt-MGM) consistently gave rise to a series of branched epithelial ducts contained within a fatty stroma (see Fig. 7A). In contrast, ductal outgrowth was never detected when PTH/PTHrP receptor-knockout epithelial buds were paired with PTH/PTHrP receptor-knockout mesencluyme (ko-MGE + ko-MGM, see Fig. 7B). These transplants gave rise to a fatty stroma that was devoid of mammary epithelial cells, reproducing the phenotype of the PTH/PTHrP receptor-knockout embryos. Recombinations consisting of PTH/PTHrP receptor-knockout epithelium paired with wild-type mesenchyme (ko-MGE + wt-MGM) uniformly gave rise to branched epithelial ducts within a fatty stroma (Fig. 7C). However, although the receptor-knockout epithelial buds consistently grew out and formed a rudimentary branching ductal structure, the growth of the resulting ducts appeared stunted compared with the ducts produced by wild-type epithelial buds paired with wild-type mesenchyme. Nonetheless, the PTH/PTHrP receptor-knockout epithelial cells survived and had the capacity to initiate branching morpho-

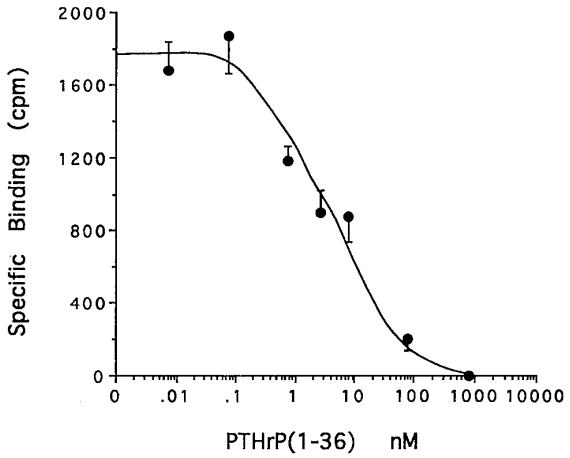

FIG. 5. Binding of PTHrP(1–36) to mammary stromal cells in culture. Receptor binding assays were performed with ^{125}I-labeled PTHrP(1–36) amide as a ligand for 4 h at 4°C with increasing concentrations of unlabeled PTHrP(1–36) as competitor. The data represent the mean ± SE. A representative of three independent experiments is shown.

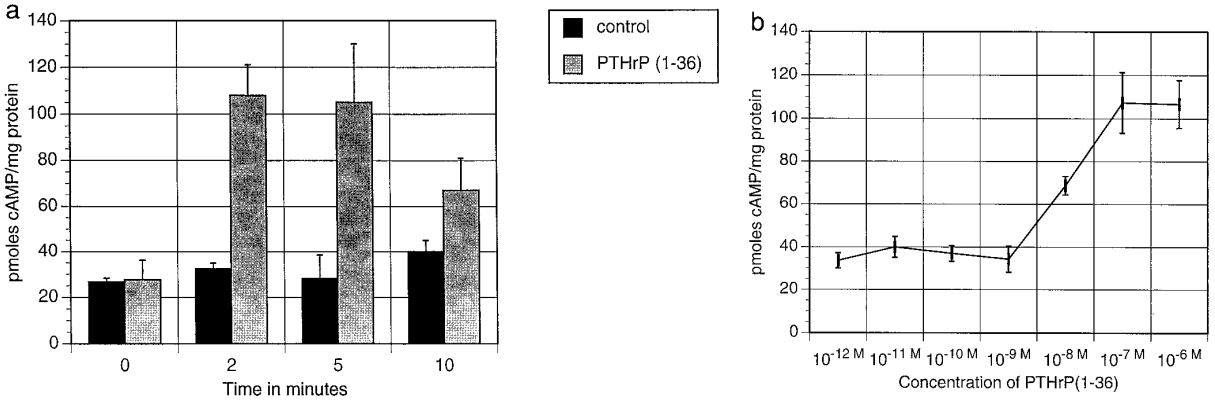

FIG. 6. cAMP response of mammary stromal cells in culture in response to PTHrP(1–36). (a) Time course of cAMP accumulation in primary cultures of mouse mammary stromal cells following treatment with PTHrP(1–36). Mammary stromal cells were incubated for the indicated times at 37°C in serum-free medium with or without 10^{-7} M PTHrP(1–36), and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE for duplicate samples from three separate experiments. (b) Dose-dependent effects of PTHrP(1–36) on cAMP accumulation in mammary stromal cells. Mammary stromal cells were treated with varying concentrations of PTHrP(1–36) for 2 min in serum-free medium, and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ± SE of three experiments each run in duplicate.

genesis when paired with normal mesenchyme. As expected, similar to the results seen with knockout buds paired with knockout mesenchyme, all recombinants composed of wild-type epithelial buds paired with receptor-knockout mesenchyme (wt-MGE + ko-MGM) lacked any evidence of epithelial ductal outgrowth and consisted of fatty stroma alone (see Fig. 7D), suggesting that PTH/PTHrP receptor-knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. These results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PTH/PTHrP receptor null mice segregate with mesenchymal tissue and suggest that the mesenchyme is a critical target for the actions of PTHrP during mammary ductal morphogenesis.

DISCUSSION

Mammary gland morphogenesis is dependent on the interplay of systemic endocrine signals and short-ranged epithelial–mesenchymal interactions (Cunha, 1994; Sakakura, 1991). Although the morphological and endocrine aspects of mammary development have been well defined, the paracrine molecules and signaling mechanisms that are influenced by systemic hormones and that regulate mammary epithelial–mesenchymal interactions have only recently begun to be understood (Friedmann and Daniel, 1996; Kratochwil *et al.*, 1996; Phippard *et al.*, 1996; Hennighausen and Robinson, 1998). Mammary ductal growth during puberty is strictly dependent upon estrogens such as estradiol signaling through the estrogen receptor-α, a conclusion derived from the analysis of estrogen receptor-knockout (ERKO) mice (Korach, 1994). In addition, recent analysis of ERKO/wild-type tissue recombinants have shown that stromal estrogen receptors are especially important for the effects of estrogens on ductal growth and branching (Cunha *et al.*, 1997). Several stromal cell factors (some of which are estrogen-responsive) have now been identified that appear to regulate epithelial cell morphogenesis and differentiation (Alexander *et al.*, 1996; Jones *et al.*, 1996; Pollard and Henninghausen, 1994; Sympson *et al.*, 1994; Weil *et al.*, 1995; Witty *et al.*, 1995; Yang *et al.*, 1995). However, less is known about epithelial signals that are involved in regulating stromal cell function during mammary development. Prior experiments in transgenic mice have suggested that amino-terminal PTHrP, acting through the PTH/PTHrP receptor, might play a role in the epithelial–mesenchymal interactions that govern mammary ductal morphogenesis. In this report we provide a series of observations lending further support to the notion that PTHrP is an epithelial signal received by the mammary mesenchyme that is critical for the mesenchyme's ability

TABLE 1
Summary of Tissue Recombination Experiments

Mesenchyme	Epithelium	*n*	Stroma only	Ducts
wt	wt	4	0	4
Receptor-ko	Receptor-ko	4	4	0
wt	Receptor-ko	2	0	2
Receptor-ko	wt	3	3	0

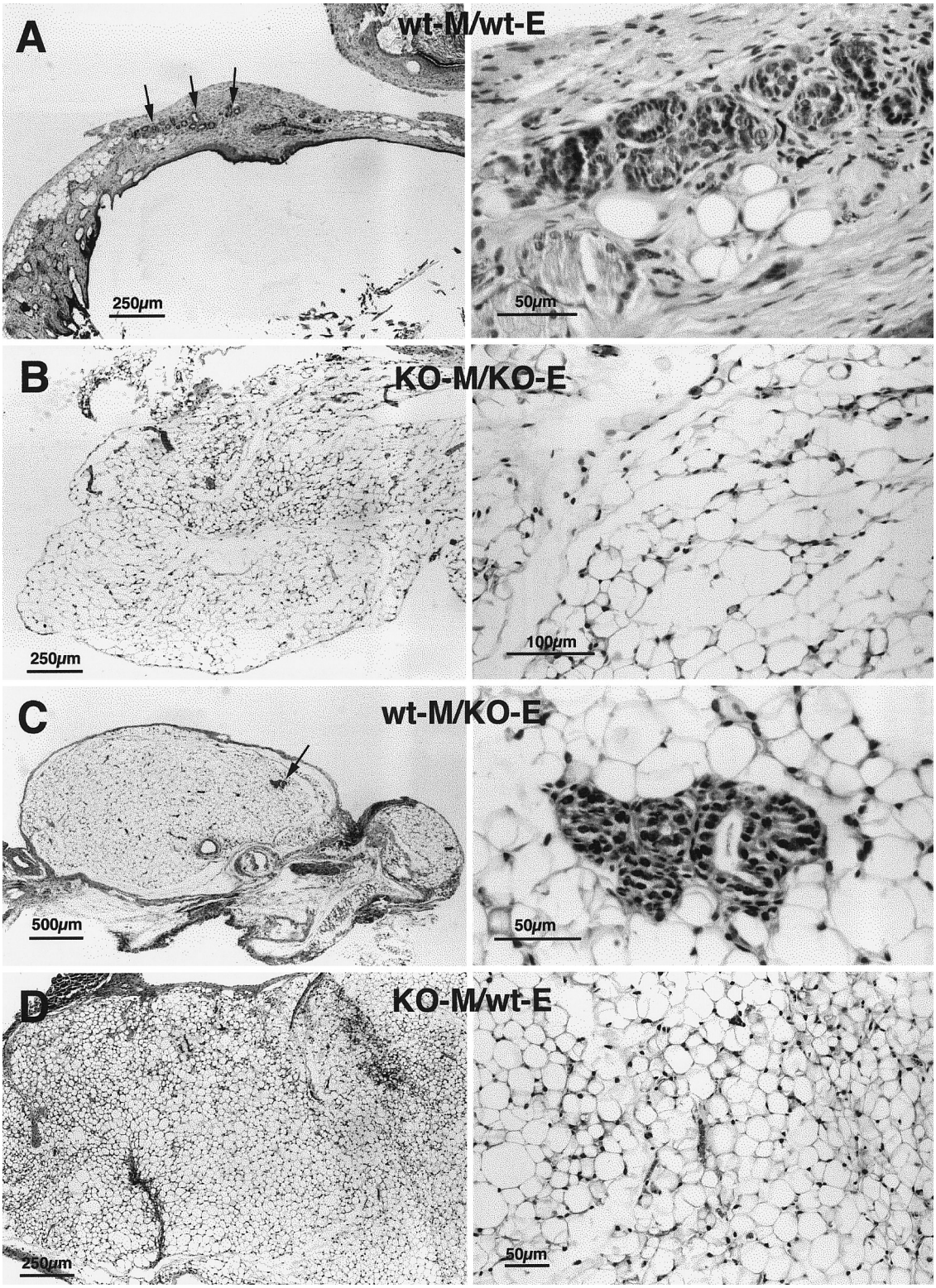

FIG. 7. Mesenchymal PTH/PTHrP receptor is necessary for the initiation of epithelial outgrowth. Mammary buds were dissected from wild-type and PTH/PTHrP receptor knockout embryos at E13, and the epithelium and mesenchyme were separated and then recombined in the four possible combinations and grown under the kidney capsule of recipient female mice for one month. Shown here are H&E-stained

to support epithelial cell outgrowth and ductal branching morphogenesis.

The first observation in support of this concept is that, during periods of active ductal morphogenesis, PTHrP and the PTH/PTHrP receptor are localized in epithelial and mesenchymal cells, respectively. Mammary ductal morphogenesis occurs in three distinct phases: during embryogenesis, during puberty and during early to mid-pregnancy. At each of these stages, we have shown that PTHrP is expressed in mammary epithelial cells and that the PTH/PTHrP receptor is expressed in mammary stromal cells. During embryogenesis, PTHrP mRNA was localized by *in situ* hybridization to the epithelial cells of the mammary bud and to the epithelial cells of rudimentary ducts as they progressed through the initial round of ductal branching morphogenesis from E16 to birth. The PTH/PTHrP receptor gene was expressed in the mesenchymal cells surrounding the mammary bud and in the mesenchyme that becomes associated with the branching epithelial ducts as they grow out into the developing mammary fat pad. This pattern of epithelial/mesenchymal expression was also observed during puberty. RNase protection analysis of PTHrP and PTH/PTHrP receptor expression demonstrated that PTHrP is found only within the portions of the mammary gland containing epithelial cells, but that PTH/PTHrP receptor mRNA is found both within the portions of the gland containing epithelium as well as within the fat pad alone. *In situ* hybridization demonstrated that this is due to the expression of the PTHrP gene in epithelial cells and its receptor gene in stromal cells. Furthermore, our *in situ* results demonstrated that, during puberty, both genes are most prominently expressed in terminal end buds. Interestingly, reminiscent of the fetal pattern of expression, we found that PTHrP mRNA appears to be localized to peripheral epithelial cells within the end buds which are known as cap cells (Daniel and Silberstein, 1987). PTH/PTHrP receptor message is found in the stromal cells immediately adjacent to the cap cells and enveloping the terminal end buds. Whereas we observed a low level of expression of the PTH/PTHrP receptor gene in the stroma of the fat pad and surrounding more mature ducts, we could not detect PTHrP mRNA within the epithelial cells of the mature ducts.

During pregnancy there was a low level of PTHrP mRNA expression in epithelial cells of the developing lobuloalveolar units, and it appeared that the PTH/PTHrP receptor gene was expressed throughout the stroma. These results are consistent with the findings of Rakopolous *et al.* (1992) who reported a diffuse, low level of PTHrP gene expression within mammary epithelial cells during pregnancy in rats. However, in our experiments, the levels of expression of both genes were just above the detection limit of our *in situ* hybridization technique and neither gene displayed an obvious pattern of expression as seen within the end buds of the adolescent gland. Despite this low level of hybridization, we are confident that PTHrP is expressed in epithelial cells and the PTH/PTHrP receptor is expressed in stromal cells during pregnancy for we obtained exactly these results using the more sensitive RNase protection assay. First, it is clear from Fig. 1A that both genes continue to be expressed on a whole gland level during pregnancy and, second, our analysis of epithelial and stromal cells isolated from pregnant mammary glands (Fig. 4) demonstrates PTHrP but no PTH/PTHrP receptor mRNA expression in epithelial cells and PTH/PTHrP receptor but not PTHrP mRNA in stromal cells.

The expression of PTHrP and its receptor in end buds in an epithelial/mesenchymal pattern is logical if PTHrP affects stromal function in a way that is important to the overall regulation of morphogenesis because the terminal end buds are the sites of active ductal growth and morphogenesis. During the embryonic development of the mammary gland, the initial round of branching morphogenesis occurs as a consequence of signaling between the mammary epithelial bud and its surrounding mesenchyme (Sakakura, 1991). Likewise, during puberty, epithelial–mesenchymal signaling at the terminal end bud influences the overall rate of ductal proliferation as well as the branching pattern of the growing duct system (Daniel and Silberstein, 1987; Silberstein and Daniel, 1987; Silberstein *et al.*, 1990). Therefore the recapitulation of the embryonic pattern of PTHrP and PTH/PTHrP receptor expression within the end buds suggests that PTHrP signaling to mesenchymal cells is most likely important to these processes during both phases of mammary gland development. This concept is supported

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sections through the resultant transplants after they were removed from beneath the kidney capsule. The left side of the figure represents low-power views and the right side represents the high-power magnifications of the same sections displayed on the left. Each transplant consists of a fragment of connective tissue containing varying amounts of fatty stroma, fibrous stroma, epidermal structures, and mammary epithelium. (A) Representative transplant resulting from wild-type epithelium recombined with wild-type mesenchyme (wt-MGE + wt-MGM). Note the mammary epithelial ducts (higher power view on right) located within a mixture of fibrous and fatty stroma. The structures at the lower left in the left-sided panel are hair follicles. (B) Representative transplant resulting from PTH/PTHrP receptor-knockout epithelium (ko-MGE) paired with PTH/PTHrP receptor-knockout mesenchyme (ko-MGM). Note that there are no epithelial ducts in this section, only fatty stroma. (C) Representative transplant resulting from wild-type mesenchyme (wt-MGM) paired with PTH/PTHrP receptor-knockout epithelium (ko-MGE). Note that epithelial ducts are present within the stroma (higher magnification on right), but that there are fewer ducts than in A. (D) Representative transplant resulting from knockout mesenchyme (ko-MGM) paired with wild-type epithelium (wt-MGE). As in B, note the complete absence of epithelial ducts. The arrows in A and C indicate mammary epithelial ducts. The scale bars in each panel demonstrate magnification as labeled.

by the results of our experiments in transgenic animals because disruption of the PTHrP gene results in the failure of the initial round of branching outgrowth during embryonic development and overexpression of PTHrP in the mammary gland in transgenic mice results in defects in ductular proliferation and branching during adolescence and pregnancy (Wysolmerski *et al.*, 1996; 1998).

We did not detect PTH/PTHrP receptor expression in epithelial cells by *in situ* hybridization during the time points we examined. This is in contrast to reports in the literature that have demonstrated PTH/PTHrP receptor expression in cultured myoepithelial cells and in several breast cancer cell lines (Birch *et al.*, 1995; Ferrari *et al.*, 1993; Seitz *et al.*, 1993). It may be that our *in situ* hybridization techniques cannot detect low levels of PTH/PTHrP receptor mRNA in myoepithelial cells, or perhaps the timing of PTH/PTHrP receptor expression in epithelial cells is not represented in our sampling. Alternatively, the presence of the receptor in myoepithelial cells may be specific to cultured or transformed cells. Our results cannot exclude that there may also be effects of PTHrP directly on some epithelial cells. However, these results clearly do demonstrate that the major location of PTH/PTHrP receptor expression is the stroma.

The second observation in support of stromal cells as a target of PTHrP's effects in the mammary gland is the ability of cultured stromal cells to express the PTH/PTHrP receptor, to bind amino-terminal PTHrP with high affinity, and to generate cAMP in response to PTHrP. These results, combined with our findings that freshly isolated mammary epithelial cells express the PTHrP gene but not the PTH/PTHrP receptor gene, represent a correlation of the *in situ* findings, *in vitro*, and underscore the concept that PTHrP, produced by epithelial cells, acts on stromal cells. This pattern is probably not unique to the mammary gland because dermal fibroblasts and lung fibroblasts have also been shown to respond to PTH and PTHrP, presumably via the PTH/PTHrP receptor (Rubin *et al.*, 1994; Shin *et al.*, 1997; Wu *et al.*, 1987). Furthermore, the ability of these cultured cells to retain their response to PTHrP now offers us an experimental system with which to begin to study the biological responses of stromal cells to PTHrP.

Our final observation in support of our working hypothesis is the demonstration that the expression of the PTH/PTHrP receptor in the mesenchyme is necessary for mammary epithelial cell survival and ductal morphogenesis. Using heterotypic tissue recombination and transplantation experiments, we have demonstrated that mammary mesenchymal cells require functional PTHrP signaling for these cells to support the survival and outgrowth of embryonic mammary epithelial cells. Unfortunately, experiments using tissues from PTHrP knockout embryos were uninformative, but mesenchymal cells taken from PTH/PTHrP receptor-knockout embryos were not able to support the survival or growth of either receptor-knockout or wild-type epithelial cells when these tissues were transplanted under the kidney capsule of recipient mice. These results mirror the failure of PTH/PTHrP receptor-knockout mammary buds to undergo the initial, embryonic round of ductal branching morphogenesis *in vivo* (Wysolmerski *et al.*, 1998). However, receptor-knockout epithelial cells were able to survive and initiate branching morphogenesis when recombined with normal mesenchyme, demonstrating directly that mesenchymal cells are critical targets of PTHrP's actions in promoting the outgrowth of the mammary epithelial bud. It should be noted that although receptor-knockout epithelium grew out and formed a rudimentary duct system when combined with normal mesenchyme, the growth of the resultant epithelial ducts was clearly not normal. These results may reflect the possibility that myoepithelial cells express a low level of PTH/PTHrP receptor which is below the detection limit of our *in situ* hybridizations (discussed above) but which is, nonetheless, important for the subsequent growth of the epithelial ducts following the initiation of branching morphogenesis. It may be that PTHrP signaling to the mesenchyme is sufficient to initiate branching growth during fetal life but that subsequent ductular proliferation requires a more complicated signaling cascade involving PTHrP's actions on both stromal and myoepithelial cells. To clarify this issue as well as the temporal requirements for PTHrP during ductular morphogenesis, it is likely that we will need to employ more sensitive localization techniques and to generate either conditional PTHrP-overexpressing or conditional PTHrP-knockout mice.

Despite the caveats noted in the previous paragraph, in the aggregate, it is clear that PTHrP's actions on mammary stromal cells have important consequences for ductal morphogenesis. Much work has demonstrated that the stromal mesenchyme plays a key role in determining the morphology of the epithelial duct system as well as in regulating the differentiation and functional activity of the mammary epithelium (Cunha *et al.*, 1995; Propper, 1973; Propper and Gomot, 1973). Recent experiments have suggested that the stromal regulation of epithelial function is complex. The stroma secretes growth factors such as KGF, HGF/SF, IGF1, and neuregulin which have been shown to regulate ductal morphogenesis (Hadsell *et al.*, 1996; Niranjan *et al.*, 1995; Ulich *et al.*, 1994; Yang *et al.*, 1995). In addition, the stroma contributes to the extracellular matrix, whose composition can have profound influences on epithelial behavior (Bissell and Hall, 1987; Sakakura, 1991). Finally, the stroma appears to be the principal source of matrix remodeling enzymes that also have been shown to have important effects on epithelial cell form, function, and survival (Alexander *et al.*, 1996; Sympson *et al.*, 1994; Witty *et al.*, 1995). All of these molecules are potential downstream stromal effectors of PTHrP's actions on epithelial development, and we are currently examining PTHrP's effects on their expression in our cultured mammary stromal cells. However, irrespective of the exact stromal response to PTHrP, our results underscore the truly reciprocal nature of the epithelial–mesenchymal interactions at play during the regulation of epithelial morphogenesis. Although stromal cells and their

products are critical to the regulation of epithelial form and function, it is clear that the epithelial cells participate in the regulation of their own fate, for without epithelial signals, such as PTHrP, the stromal cells are incompetent to direct epithelial morphogenesis.

In summary, our experiments demonstrate that PTHrP and the PTH/PTHrP receptor represent an epithelial/mesenchymal circuit that is necessary for mammary morphogenesis. Specifically, PTHrP produced by mammary epithelial cells must signal through the PTH/PTHrP receptor in mammary mesenchymal cells in order for the mesenchyme to support the initiation of mammary ductal morphogenesis. PTHrP has also been implicated in the development of other tissues that rely on epithelial/mesenchymal interactions for their development, including lung, teeth, and hair follicles (Philbrick *et al.*, 1998). Therefore, understanding PTHrP's role in regulating stromal cell function during the epithelial/mesenchymal interactions that govern mammary development should allow us to better understand the overall role of PTHrP in development.

ACKNOWLEDGMENTS

We thank Drs. Arthur Broadus and William Philbrick for their critical reading of the manuscript. We are grateful for the excellent technical assistance of Lina Golovyan. This work was supported by the DOD Grant DAMD17-96-1-6198 (J.J.W.) and NIH Grants CA 60498 (J.J.W.), CA 62114 (J.J.O.), CA 58207 (G.C.), and P01CA44768 (G.C.). M.E.D. is supported by the DOD Postdoctoral Fellowship DAMD17-97-1-7137. We are grateful for the use of the microscopy facilities of the Cell Biology Core of the Yale Diabetes and Endocrine Research Center.

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Received for publication February 23, 1998
Revised June 26, 1998
Accepted July 22, 1998