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

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

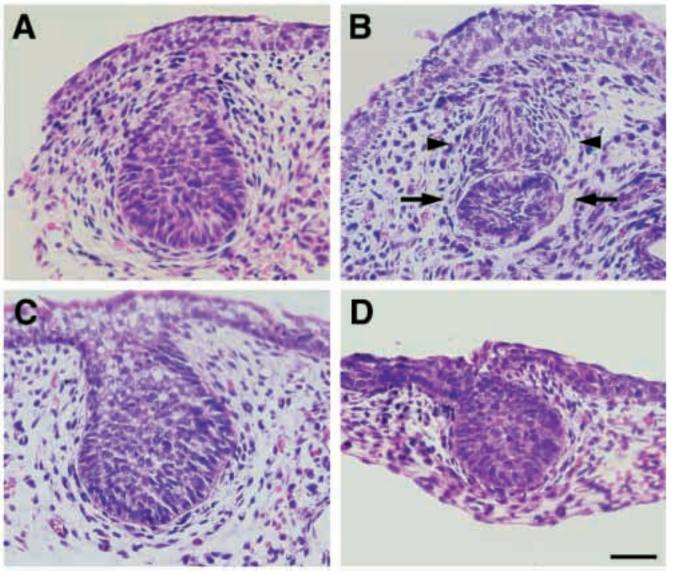

Fig. 1. Failure of the androgen-mediated destruction of the mammary buds in PTHrP- and PPR1-knockout embryos. Hematoxylin- and eosin-stained sections through E15 mammary buds taken from a wild-type female embryo (A), a wild-type male embryo (B), a PTHrP-knockout male embryo (C) and a PPR1-knockout male 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.

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

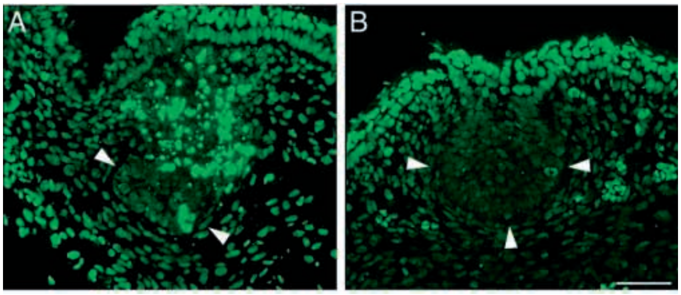

Fig. 2. Programmed cell death in male mammary buds at E15. (A) Results of a TUNEL assay performed on a section through a wild-type male mammary bud. Apoptotic nuclei stain bright green whereas normal nuclei stain pale green. Note the multitude of apoptotic nuclei in the region of the stalk and mesenchymal 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 [^{3}H]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).

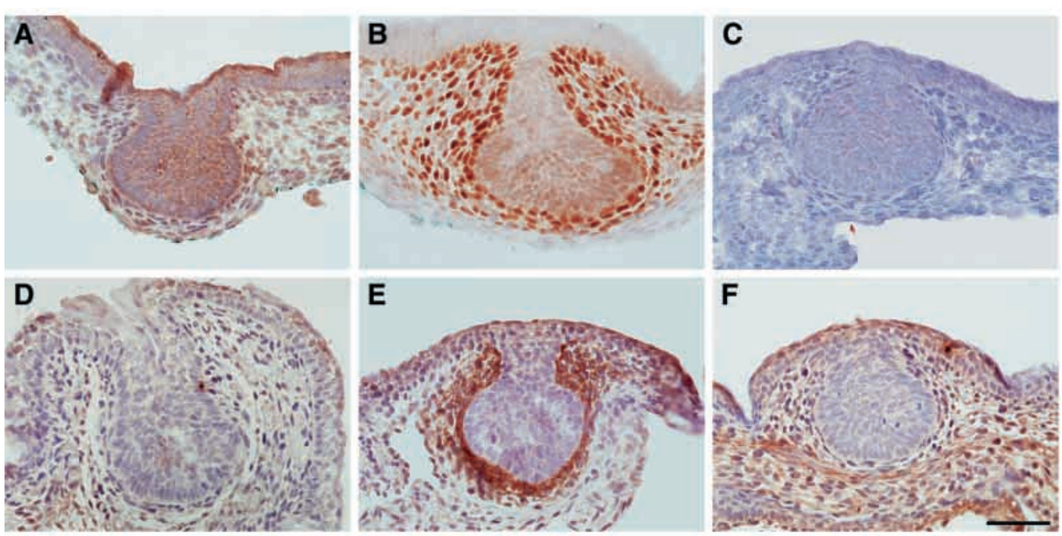

Fig. 3. Androgen receptor and tenascin C staining of E15 mammary buds. (A-C) Sections stained for androgen receptor and (D-F) represent sections stained for tenascin C. (A,D) Sections through male PTHrP-knockout mammary buds. (B,E) Sections through female wild-type buds. (C,F) Sections through male PPR1-knockout 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.

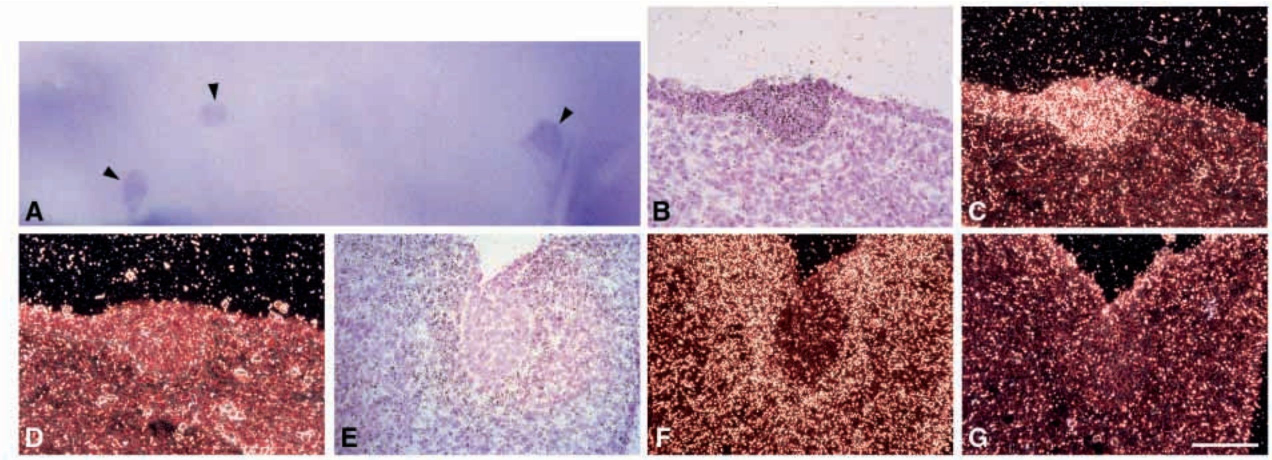

Fig. 4. Initiation of PTHrP expression in developing mammary buds. (A) A representative photograph of whole-mount in situ hybridization experiments using PTHrP antisense probe on a late E11 embryo. The photograph shows a magnified view of the ventral-lateral surface of the embryo. PTHrP expression is 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

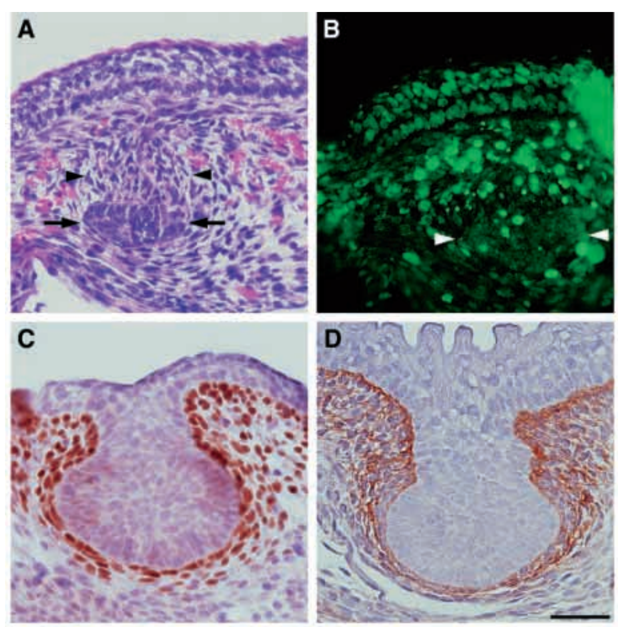

Fig. 5. Return of sexual dimorphism in K14-PTHrP/PTHrP-null embryos. (A) Histology of a mammary bud taken from a male K14-PTHrP/PTHrP-null embryo at E15. Note the typical mesenchymal condensation around the epithelial stalk (between arrowheads) and the degenerating epithelial remnant (between arrows) (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

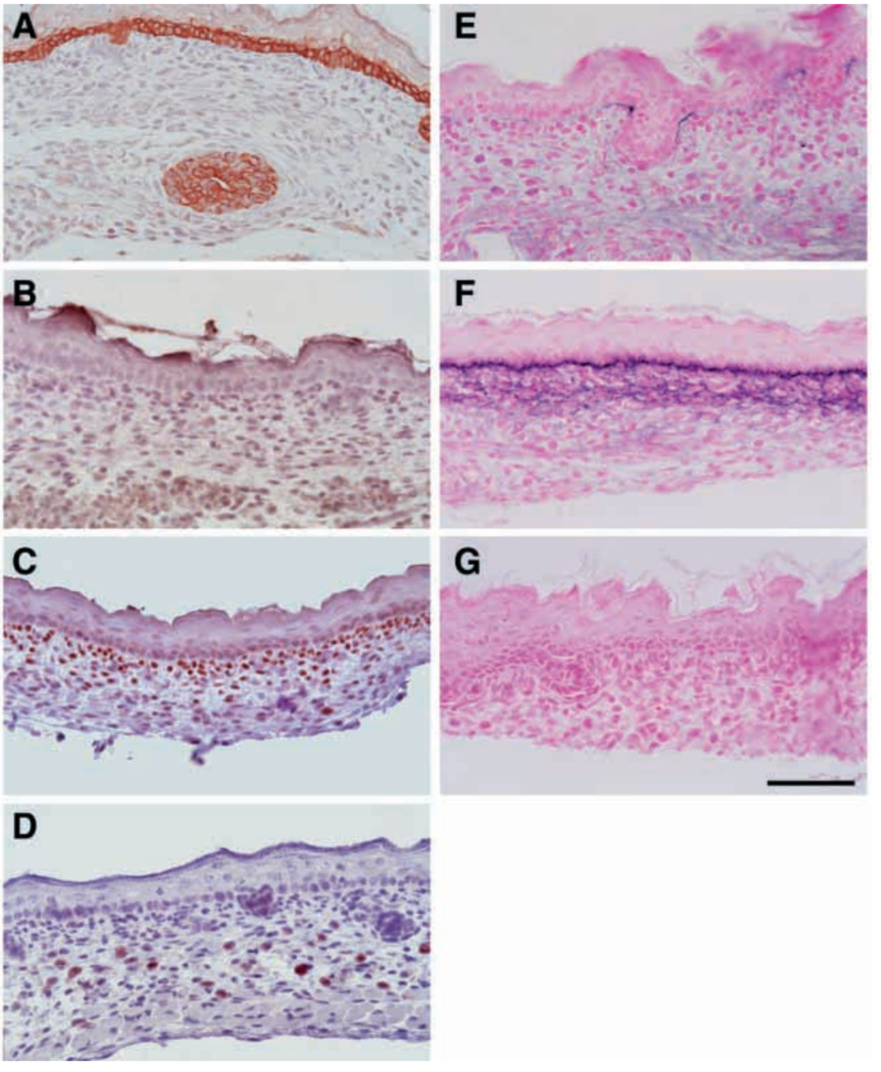

Fig. 6. Ectopic expression of PTHrP in the epidermis induces dermal expression of androgen receptor and tenascin C. (A) Immunohistochemistry for keratin 14 in the fetal epidermis at E18. Note that K14 is expressed both within the fetal mammary epithelial cells within the mammary duct below the epidermis 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 the 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.

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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.