Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development

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SUMMARY

Prior reports have demonstrated that both parathyroid hormone-related protein (PTHrP) and the type I PTH/PTHrP receptor are necessary for the proper development of the embryonic mammary gland in mice. Using a combination of loss-of-function and gain-of-function models, we now report that PTHrP regulates a series of cell fate decisions that are central to the survival and morphogenesis of the mammary epithelium and the formation of the nipple. PTHrP is made in the epithelial cells of the mammary bud and, during embryonic mammary development, it interacts with the surrounding mesenchymal cells to induce the formation of the dense mammary mesenchyme. In response, these mammary-specific mesenchymal cells support the maintenance of mammary epithelial cell fate, trigger epithelial morphogenesis and induce the overlying epidermis to form the nipple. In the absence of PTHrP signaling, the mammary epithelial cells revert to an epidermal fate, no mammary ducts are formed and the nipple does not form. In the presence of diffuse epidermal PTHrP signaling, the ventral dermis is transformed into mammary mesenchyme and the entire ventral epidermis becomes nipple skin. These alterations in cell fate require that PTHrP be expressed during development and they require the presence of the PTH/PTHrP receptor. Finally, PTHrP signaling regulates the epidermal and mesenchymal expression of LEF1 and β-catenin, suggesting that these changes in cell fate involve an interaction between the PTHrP and Wnt signaling pathways.

Key words: PTHrP, PTH/PTHrP receptor, Keratinocyte differentiation, Epithelial-mesenchymal interactions, Nipple, Mammary development, Mouse

INTRODUCTION

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

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

Mammary glands are epidermal appendages and, in the mouse, their embryonic development is a two-step process (Sakakura, 1987). The first step is the bud-like invagination of epithelial cells into the underlying mesenchyme at 10 characteristic locations along the milk line, a thickened ridge of embryonic skin stretching between the limb buds on the ventral surface of the embryo. The mammary buds are first discernible on embryonic day 10 (E10) and are fully formed by E12. Each bud consists of a pear-shaped collection of epithelial cells surrounded by a condensed mesenchyme that is distinct from the surrounding dermis. In male embryos, the
mammary buds are destroyed at E14 by the actions of androgens on the mammary mesenchyme. In female embryos, the buds remain quiescent until E16, when the second step of development is initiated. During this stage, the primary duct is formed. It grows away from the primary mammary mesenchyme into another stromal compartment known as the mammary fat pad, and 10 to 15 secondary ducts are formed within the fat pad before birth. Concurrent with these changes, the nipple sheath forms. This is a modification of the skin that occurs in the immediate vicinity of the mammary duct. The epidermis thickens and projects down into the dermis in an umbrella-like fashion, forming a ridge that surrounds the origin of the primary epithelial duct.

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

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

**MATERIALS AND METHODS**

**Mouse strains**

PTHRp knockout, PTHR1 knockout and K14-PTHRp transgenic embryos were harvested and genotyped as previously reported (Wysolmerski et al., 1998; Dunbar et al., 1999). The appearance of the vaginal plug was considered to mark day 0 of gestation. Wild-type littermates were used as controls. K14-tTA/TetO-PTHRp double transgenic mice were created by crossing K14-tTA transgenic mice with TetO-PTHRp transgenic mice. As outlined in Fig. 4, the K14-tTA transgene was generated by inserting a 2.1 kb portion of the human K14 promoter upstream of the tetracycline transactivator gene (Schreck et al., 1995). The TetO-PTHRp transgene was generated by inserting a 568 bp human PTHrP 1-141 cDNA and 2.2 kb of human growth hormone sequences downstream of a minimal CMV promoter containing 7 concatamered tetracycline repressor binding sites (Wysolmerski et al., 1994; Schreck et al., 1995). TetO-PTHRp mice were identified by amplification of a 171 bp sequence of the human growth hormone gene (Dunbar et al., 1999). K14-tTA mice were identified by amplification of a 159 bp sequence of the tetracycline transactivator gene using the following primers: forward, aacacccgaaaaactecgc; reverse, aactcggcagcattcc. TetO-β-gal mice were a gift from Dr Lothar Hennighausen, and were genotyped by amplification of a 192 bp fragment of the lacZ gene using the following primers: forward, aatgattagtcgccacagc; reverse, aattcggcatactgac. TetO-PTHRp transgene expression was suppressed by feeding double transgenic mice 150 μg/ml of tetracycline hydrochloride (Roche, Indianapolis, IN) in 5% sucrose water.

**Histology and immunohistochemistry**

Whole embryos were fixed in Bouins or 4% paraformaldehyde at 4°C for 12 hours. Mammary glands and strips of skin were dissected and embedded in paraffin, and mammary buds were identified by serial sectioning as previously described (Dunbar et al., 1999). Longitudinal strips of ventral or dorsal adult mouse skin were either processed for frozen sections or fixed and embedded as described above. Immunohistochemistry was performed using standard techniques. Antigen retrieval was accomplished by heating sections in 7 mM citrate under pressure. Anti-Left1 antibodies were a gift from Dr R. Grosschedl; primary incubation was 12 hours at 4°C after antigen retrieval. Nuclear staining was performed using an anti-sm antisera (Y12) that was a gift from Dr Joan Steitz; primary incubation was performed for 1 hour at room temperature after antigen retrieval. Antibodies for smooth muscle α-actin were from Sigma (St Louis, MO); primary incubation was for 10 minutes at room temperature. Vimentin antibodies were from Chemicon (Temecula, CA); primary incubation was performed on frozen sections for 1 hour at room temperature. The anti-keratin 9 antibody was a gift from Dr Lutz Langbein; primary incubation was performed on frozen sections for 1 hour at room temperature. Staining for K14, K1, K10, K6, involucrin, loricrin, filaggrin, tenascin C, the androgen receptor and β-catenin were performed as previously described (Dillon et al., 1998; Foley et al., 1998; Dunbar et al., 1999). Antibodies to the androgen receptor, tenasin C, LEF1, K14, K1, K10, K6, K9, involucrin, loricrin, filaggrin, smooth muscle α-actin and vimentin were detected using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA) and 3,3’ diaminobenzidine as chromagen. β-catenin antibodies were detected with a goat anti-rabbit Cy3-conjugated secondary (Amersham Pharmacia, Piscataway, NJ) and anti-sm antibodies were detected with a goat anti-mouse, alexafluor 488-conjugated secondary (Molecular Probes, Eugene, OR). Confocal laser-scanning microscopy was performed at the Center for Cell Imaging at Yale University.

**β-Galactosidase assay**

β-Galactosidase was detected as previously described (Furth et al., 1994). Briefly, tissue was fixed in 2% paraformaldehyde and 0.02% gluteraldehyde in phosphate buffered saline (PBS) for 1 hour at room temperature and then washed twice in PBS. Samples were incubated in 0.1% 4-chloro-5-bromo-3-indolyl β-D-galactopyranoside, 2 mM MgCl2, 5 mM EDTA, 0.02% Nonidet P-40, 5 mM K3Fe(CN)6 and 5 mM K4Fe(CN)6·6H2O at 30°C for 12 hours. Tissue was then post-fixed in 4% paraformaldehyde at 4°C, embedded in paraffin wax, sectioned and counterstained with Eosin.

**RESULTS**

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

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

In order to test this hypothesis, we first examined mammary rudiments from PTHrP and PTHR1 knockout mice from E17-E19. By E17, the normal mammary duct has grown away from the overlying epidermis and the nipple sheath has formed (Fig. 1A). In contrast, in PTHrP (Fig. 1B) or PTHR1 knockout embryos, the mammary bud has not grown away from the skin and the nipple sheath is absent. We also noted that what appeared to be hair follicles could occasionally be seen budding off the neck of the knockout mammary buds or arising from the epidermis adjacent to the origin of the primary duct, an area that should have given rise to the nipple sheath (Fig. 1B). These findings were distinctly abnormal, as the nipple and mammary bud should be devoid of hair follicles. Finally, cells within the middle of the knockout buds had a vacuolated appearance similar to that of the supra-basal or spinous layer of the epidermis and, in fact, appeared to be continuous with that layer of the skin (Fig. 1B). These histological changes suggested that the knockout mammary buds were behaving more like epidermis than mammary epithelium. If this were true, we reasoned that the knockout epithelial cells might begin to express epidermal-specific proteins. Therefore, we stained E17 mammary glands from PTHrP and PTHR1 knockout and wild-type littermates with a battery of antibodies to epidermal markers, including K14, K1, K10, involucrin, loricrin and filaggrin (Kopan and Fuchs, 1989; Foley et al., 1998). As shown in Fig. 1C, K14 antibodies labeled all of the mammary epithelial cells but only the basal epidermal cells of wild-type embryos. In PTHrP (Fig. 1D) and PTHR1 knockouts, K14 labeled just the cells along the perimeter of the knockout mammary buds, a pattern similar to wild-type skin. Antibodies to suprabasal epidermal markers, such as K1, K10 and involucrin, labeled the epidermis and just a very thin component of the upper lactiferous duct in wild-type embryos. However, the entire central portion of the mammary rudiment in the knockout embryos expressed these suprabasal keratinocyte markers (Fig. 1E,F – K10 and involucrin not shown). Antibodies to the granular markers loricrin and filaggrin labeled the outer layers of the epidermis in both the knockout and wild-type embryos but did not label the mammary epithelial cells in either (Fig. 1G,H – loricrin not shown).
Taken together, these results suggest that the absence of PTHrP signaling leads the epithelial cells of the mammary bud to undergo an epidermal pattern of differentiation.

**Overexpression of PTHrP leads to nipple-like differentiation of the ventral epidermis**

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

In mice, nipples are only present on females and, grossly, are composed of wrinkled, flaky and hairless skin, all characteristics shared with the ventral skin of K14-PTHrP transgenic mice (Montagna, 1970; Toyoshima et al., 1998). Histologically, in comparison with wild-type skin, both nipple skin and K14-PTHrP skin demonstrate a thickened, hyperkeratotic epidermis without hair follicles that overlies a cellular dermis containing small bundles of uniformly-sized collagen (see Fig. 2A-C, and data not shown). As in other mammals, the epidermis of the murine nipple sends an umbrella-like projection down into the dermis in a circumferential fashion around the primary lactiferous duct (Fig. 2B). The epidermis of K14-PTHrP mice also sends projections down into the dermis, although these folds tend to be shallower and more rounded, and none is associated with a mammary duct (Fig. 2C). Another hallmark of nipple skin in the mouse is the smooth muscle beds found at the base of the

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**Fig. 2.** (A,D,G) Sections from wild-type ventral skin. (B,E,H) Wild-type nipple skin. (C,F,I) K14-PTHrP transgenic ventral skin. All sections are from 8-week-old mice. (A-C) Hematoxylin and Eosin staining. Note the thickened epidermis, hyperkeratosis and lack of hair follicles (HF) in the nipple (B) and transgenic (C) skin, when compared with wild-type (A). (D-F) Smooth muscle α-actin staining. Bands of smooth muscle extend along the primary lactiferous duct (‘D’) under the nipple in E (arrows). Transgenic dermis (F) also contains similar bands of cells (arrows). In the wild type, staining is limited to the vasculature and erector pili muscles (arrow in D). (F-H) Sections stained with aldehyde fuchsin. Note the abundant elastic fibers (staining in blue) in the transgenic (I) and nipple (H) dermis when compared with wild type (G). Scale bar: 15 μm in A-C; 25 μm in D-F; 15μm in G-I.
nipple, which extend as a group of thin fibers along the lactiferous duct (Montagna, 1970; Toyoshima et al., 1998). In K14-PTHrP skin, similar bundles of elongated cells were found distributed beneath the entire ventral epidermis. These cells stained for α-actin and vimentin, confirming their identity as smooth muscle (see Fig. 2D-F, and data not shown). Finally, nipple skin is characterized by an abundance of elastic fibers in the underlying connective tissue (Montagna, 1970; Toyoshima et al., 1998). As shown in Fig. 2G-I, elastic fibers are not abundant in the dermis of wild-type mice, but are dramatically increased in number in the connective tissue underneath the nipple and within the K14-PTHrP dermis. Although the nipple-like features described above were found throughout the hairless ventral skin of the K14-PTHrP mice, the dorsal skin of these animals was similar to dorsal skin of wild-type controls, even though the transgene was also expressed in dorsal skin.

We next examined the pattern of expression of several markers of epidermal differentiation in normal nipple skin and compared these patterns with those in normal and K14-PTHrP transgenic skin. As shown in Fig. 3A, in normal ventral skin, antibodies to keratin 14 stain the entire basal keratinocyte layer, but not the upper layers of keratinocytes. In both the K14-PTHrP transgenic skin and the normal nipple skin, the layer of cells staining for keratin 14 appears expanded (Fig. 3B,C). This is because the basal keratinocytes are elongated and more columnar in appearance, and also because some suprabasal cells appear to retain keratin 14 protein expression. As expected, staining for the suprabasal marker, K1, intensely labeled the upper layers of keratinocytes as well as lightly staining some basal keratinocytes in wild-type ventral skin (Fig. 3D). Similar to the pattern in K14-PTHrP skin, labeling for K1 in nipple skin was restricted to the outermost suprabasal layers (Fig. 3E,F). Similar staining was seen with an antibody to keratin 10, another suprabasal marker (not shown). Next, we examined the pattern of expression of the granular layer marker, filaggrin. This protein was minimally expressed in what appears to be a single thin layer within wild-type ventral skin, whereas in the nipple and in K14 skin it was expressed at high levels in multiple layers (see Fig. 3G-I). As with the histological findings described in the previous paragraph, the dorsal skin of the K14-PTHrP mice did not exhibit any alterations in epidermal marker expression when compared with wild-type dorsal skin (data not shown). Finally, given their similar lack of hair growth, we also investigated the expression of keratin 9 and keratin 6, two keratins that are found in the

**Fig. 3.** (A,D,G) Sections from wild-type ventral skin. (B,E,H) Wild-type nipple skin. (C,F,I) K14-PTHrP transgenic ventral skin. All sections are from 8-week-old mice. (A-C) Keratin 14 staining; (D-F) keratin 1 staining; (G-I) Filaggrin staining. For each marker, the pattern of staining in transgenic skin is similar to that in nipple skin and both are different from wild type. Nipple and transgenic skin demonstrate an expanded basal pattern of K14 staining, a shift of K1 staining to the upper layers of the epidermis and an augmentation of filaggrin staining. The broken lines in E,F are at the dermal-epidermal border. Scale bar: 15 μm.
specialized epidermis of the footpad (Schwietzer and Baust, 1989; Rothangle et al., 1999). Neither of these keratins was expressed in the nipple or K14-PTHrP transgenic skin (data not shown). Thus, it appears that the alterations in keratinocyte differentiation marker expression seen in the ventral skin of K14-PTHrP transgenic mice closely mimic the natural pattern observed in the nipple. In both sites, as compared with normal ventral skin, the basal keratinocyte layer appears to be expanded, the acquisition of K1 and K10 expression appears to be delayed and there is an increase in the number of granular layers within the upper epidermis.

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

In order to investigate if the nipple-like changes in the K14-PTHrP transgenic skin required expression of PTHrP at a specific time, we created a binary K14-PTHrP transgenic mouse that would enable us to regulate PTHrP production in a temporal fashion. As shown in Fig. 4A, we made two transgenic lines, one bearing the K14-tTA transgene and the other bearing the TetO-PTHrP transgene, and bred them together to generate double transgenic mice. This is a ‘tet-off’ system: when double transgenic mice are fed tetracycline no excess PTHrP is produced but withdrawal of tetracycline allows PTHrP overexpression (Schockett and Schatz, 1996). In order to test the ability of the K14-tTA transgene to properly activate target transgene expression, we first bred the K14-tTA line to a TetO-βGal mouse (Furth et al., 1994). The resulting double transgenic mice (K14-tTA/TetO-βGal) should express β-galactosidase in a K14-dependent manner, only when tetracycline is absent. Figure 4 demonstrates that this is the case. In the presence of tetracycline, there is no transgene expression (Fig. 4B). However, in the absence of tetracycline, β-galactosidase activity is present in the basal keratinocytes and developing hair follicles, as would be expected for a transgene being driven by the K14 promoter (Fig. 4C).

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

**PTHrP signaling modulates LEF1 and β-catenin expression in the developing mammary gland and epidermis**

Disruption of the gene for LEF1 has been shown to interrupt
PTHrP regulates mammary and nipple fate

embryonic mammary development (van Genderen et al., 1994). We therefore sought evidence that LEF1 might be in the same signaling pathway as PTHrP and the PTHR1. It has been reported that LEF1 is expressed in the epithelial cells of the mammary bud early during its formation. However, it is not known if, as has been described for tooth and hair follicle development, LEF1 is also expressed in the mesenchyme at later stages (van Genderen et al., 1994; Kratochwil et al., 1996). Therefore, we performed immunohistochemistry for LEF1 at different times during embryonic mammary development. At E11-E12, we found that LEF1 is expressed in the basal cells of the developing epithelium and in the epithelial cells of the mammary bud (not shown). By E14-E15, two changes occur. First, LEF1 expression is induced within the

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**Fig. 5.** Each panel represents a section of ventral skin taken from a K14-tTA/Teto-PTHrP double transgenic mouse exposed to different patterns of tetracycline before being sacrificed at 7 weeks of age. (A,E,I,M) Double transgenic mice always on tetracycline: the transgene was always off (Tg-off). (B,F,J,N) Double transgenic mice always off tetracycline: the transgene was always on (Tg-on). (C,G,K,O) Double transgenic mice off tetracycline during gestation, but given tetracycline at birth: the transgene was on before birth, but was shut off after birth (Tg-on/off). (D,H,L,P) Double transgenic mice exposed to tetracycline during gestation but taken off of tetracycline at birth: the transgene was off before birth and was turned on at birth. A-D are stained with Hematoxylin and Eosin, E-H for keratin 14, I-L for keratin 1 and M-P for filaggrin. When double transgenic mice are left off tetracycline (Tg-on, B,F,J,N), hair follicle (HF) development is suppressed and keratinocyte marker expression is altered as in the original K14-PTHrP transgenics. When these mice are reared on tetracycline (Tg-off, A,E,I,M) these changes do not occur. These same nipple-like skin changes occur when PTHrP overexpression occurs before birth, but not after birth (Tg-on/off, C,H,K,O). However, when PTHrP overexpression is initiated after birth (Tg-off/on, D,H,L,P), none of these alterations takes place. Scale bar: 20 μm.
mammary mesenchyme as it is fading away in the mammary epithelium. Second, in the epidermis destined to become the nipple sheath, LEF1 expression becomes undetectable (Fig. 6A). We next examined the expression of LEF1 in mammary buds from PTHR1- and PTHR1-null mice, and in skin from K14-PTHrP transgenic embryos. As seen in Fig. 6B,C, in knockout mice, LEF1 expression was preserved in mammary epithelial cells but was absent in the mammary mesenchyme. In addition, in these mice, LEF1 continued to be expressed in the basal epidermal cells closest to the primary mammary duct. In contrast to these findings, ectopic overexpression of PTHrP in the epidermis of K14-PTHrP mice led to the loss of LEF1 expression in the epidermis and its inappropriate expression in the dermis (Fig. 6D,E). Interestingly, while this was true for the ventral skin of K14-PTHrP embryos, no such changes were observed in skin from the dorsal aspect of the embryos (not shown).

LEF1 has been shown to participate in the Wnt signaling pathway by complexing with nuclear β-catenin to form a binary transcription factor that modulates the expression of Wnt-responsive genes (Behrens et al., 1996; Eastman and Grosschedl, 1999). Given the fact that Wnt signaling affects mammary development (Uyttendaele et al., 1998; Brisken et al., 2000) and given the findings described in the previous paragraph, we also examined β-catenin expression in the embryonic mammary bud. As expected, in the wild-type mammary bud (Fig. 7A,B), we observed the expected pattern of peripheral β-catenin expression within the mammary epithelial cells where it interacts with cadherins at the cell membrane (Miller and Moon, 1996). However, we also saw a patchy and somewhat punctate pattern of what appeared to be membrane-associated β-catenin staining in the mammary mesenchyme. There was also some low-level cytoplasmic staining within these cells. β-Catenin staining was especially prominent in the mesenchymal cells surrounding the neck of the bud, corresponding to the area of maximal LEF1 staining (compare Fig. 7A,B with Fig. 6A). As can be seen in Fig. 7B, there was no evidence for co-localization of β-catenin and a nuclear antigen, suggesting that there was no appreciable nuclear β-catenin in these cells. In contrast to these findings, although epithelial β-catenin expression was unchanged, in PTHrP and PTHR1 knockout buds, the level of β-catenin expression in the mammary mesenchyme was dramatically reduced (Fig. 7C,D).

We also compared β-catenin expression in skin samples taken from K14-PTHrP transgenic embryos with those taken from wild-type littermates. As seen in Fig. 7E,F, in normal embryos, we detected prominent peripheral β-catenin staining in keratinocytes, similar to that seen in mammary epithelial cells. However, we saw little overall β-catenin staining in the normal dermal mesenchyme. In K14-PTHrP transgenic embryos, the pattern in keratinocytes was identical, but there was a clear induction of β-catenin expression in the dermis extending about 4-5 cell layers down from the epidermal basement membrane (Fig. 7G,H). As in the mammary mesenchyme, the staining was patchy and mostly peripheral. As with LEF1 expression, this pattern was not seen in the dorsal skin of the K14-PTHrP embryos, which stained in a pattern indistinguishable from wild-type skin. These data demonstrate that, at least on the ventral surface of the developing embryo, PTHrP-signaling is able to induce LEF1 and β-catenin expression in the mammary and dermal mesenchymes.

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

Because the PTHR1 is expressed in the dermis but not on keratinocytes, our working hypothesis is that the nipple-like skin changes in the K14-PTHrP transgenic mice are the result of paracrine interactions. We believe that PTHrP acts on the dermis, which, in turn, alters the differentiation of the epidermis. However, PTHrP has also been shown to act via a nuclear pathway (Henderson et al., 1995; Massfelder et al., 1997; Lam et al., 1999; Aarts et al., 1999), and it has been shown to induce LEF1 expression in the epidermis and its inappropriate expression in the dermis (Fig. 6D,E). Interestingly, while this was true for the ventral skin of K14-PTHrP embryos, no such changes were observed in skin from the dorsal aspect of the embryos (not shown).

**Fig. 6.** Immunohistochemistry for LEF1 in mammary buds and ventral skin at E15. (A) Wild-type mammary bud. Note the intense nuclear staining for LEF1 in the mesenchymal cells surrounding the epithelial bud (EB). LEF1 is also expressed in the mammary epithelial cells, but not in the basal keratinocytes near the mammary bud. (B) PTHR1 knockout bud. (C) PTHrP knockout bud. In the knockout LEF1 expression in the mammary mesenchyme is lost, but it is retained within the basal keratinocytes near the buds. (D) Wild-type ventral skin at E15. LEF1 is normally expressed in basal keratinocytes, but not within the dermis. (E) Ventral skin from a K14-PTHrP transgenic embryo at E15. LEF1 expression is induced in the transgenic dermis but is lost in the keratinocytes, reproducing the pattern normally seen in the developing nipple. Scale bar: 10 μm.
suggested that there are PTHrP receptors other than the PTHR1 that exist on squamous cells (Orloff et al., 1995). Therefore, it is possible that the skin phenotype of the K14-PTHrP mice is caused by an autocrine effect of PTHrP. In order to distinguish between these possibilities, we generated mice that were hemizygous for the K14-PTHrP transgene and homozygous for the disruption of the gene for PTH/PTHrP receptor (K14-PTHrP/PTHR1<sup>-/-</sup> mice), so that we could overexpress PTHrP in the absence of type I receptors. These mice died shortly after birth, owing to skeletal complications resulting from disruption of the PTHR1 gene. As expected, there was a failure of mammary development in these mice identical to that seen in PTHrP and PTHR1 knockout mice. Disruption of the gene for PTHR1 also prevented the induction of mammary mesenchyme markers in the ventral dermis in K14-PTHrP/PTHR1<sup>-/-</sup> mice. Fig. 8 demonstrates that, despite bearing the K14-PTHrP transgene, these embryos failed to express androgen receptor, tenascin C, LEF1 or β-catenin in the dermal mesenchyme. Finally, there was no evidence of nipple-like changes in the ventral epidermis of the K14-PTHrP/PTHR1<sup>-/-</sup> embryos. LEF1 expression was retained in the basal keratinocytes of the ventral surface and developing hair follicles were found throughout the ventral epidermis. In addition, the K14-PTHrP/PTHR1<sup>-/-</sup> epidermis lacked the alterations in epidermal marker expression seen in the ventral epidermis of K14-PTHrP neonates (Fig. 8G,H,K,L). At this age, the K14-PTHrP transgenic epidermis is only slightly thicker than wild-type epidermis, and the layer of cells staining for K14 appear slightly elongated compared with those cells staining for K14 in the wild type. However, comparing Fig. 8A with Fig. 8B and Fig. 8K with Fig. 8L, one can see that these changes do not occur in a PTHR1-null background. Therefore, the changes in the ventral dermis and epidermis that result from overexpression of PTHrP in basal keratinocytes depend on the presence of the PTHR1. Furthermore, because the PTHR1 is expressed in the developing dermis but not in the epidermis, the nipple-like changes in the epidermis of the K14-PTHrP transgenic mice would appear to be mediated by the effects of PTHrP on the dermis.

**DISCUSSION**

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

**Fig. 8.** Comparison of K14-PTHrP transgenic mice and K14-PTHrP/PTHR1−/− mice. (A,C,E,G,I,K) Sections of ventral skin from a newborn K14-PTHrP female mouse. (B,D,F,H,J,L) Sections of skin from a newborn K14-PTHrP/PTHR1−/− mouse. (A,B) Hematoxylin and Eosin staining. (C,D) Tenascin staining. (E,F) Androgen receptor staining. (G,H) LEF1 staining. (I,J) β-catenin staining. (K,L) Keratin 14 staining. In the absence of the PTHR1 (D,F,H,J), the dermis fails to express mammary mesenchyme markers normally induced in K14-PTHrP transgenic skin (C,E,G,I); hair follicle growth returns (arrows in B,L) and the epidermis reverts back to a normal thickness (compare B with A). In neonates, there are only subtle differences in keratinocyte marker expression in the K14 mice when compared with normal. Note the elongated appearance of the basal keratinocytes and the frequent suprabasal cells expressing keratin 14 in K. However, in the absence of the PTHR1, the basal keratinocytes are flatter and only rare suprabasal cells express keratin 14 (L). Scale bar: 16 μm in A-H,K,L; 10 μm in I,J.
mesenchyme and nipple differentiation is restricted to the ventral surface even though the dermal mesenchyme on the dorsum also expresses PTHrP receptors. Many vertebrates have distinct skin appendages on their dorsal versus ventral surfaces, including the presence of mammary glands on the ventrum of placental mammals. It is likely that these patterning differences, at least in part, reflect underlying differences in the mesenchymal tissue beneath the epidermis. In fact, the dorsal and ventral dermal mesenchymes have distinct embryological origins, with the ventral cells arising from the somatopleural mesoderm and the dorsal cells arising from the dermatomyotome (Sengel, 1976). Whether it is these differences in mesenchymal lineage or other differences in growth or transcription factor expression that explain the differential response to PTHrP, it is clear that only the ventral surface is competent to generate mammary mesenchyme in response to PTHrP signaling.

Our data demonstrate a correlation between the changes in mesenchymal and epidermal cell fate resulting from manipulation of PTHrP signaling and changes in the patterns of mesenchymal and/or epidermal LEF1 and β-catenin expression. LEF1 and β-catenin expression are normally induced in the mammary mesenchyme, and LEF1 expression is normally lost in the keratinocytes destined to give rise to the nipple sheath. When PTHrP-signaling is disrupted, LEF1 and β-catenin are no longer expressed in the mesenchymal cells around the mammary bud, and LEF1 continues to be expressed in the keratinocytes that should give rise to the nipple. Conversely, when PTHrP is ectopically overexpressed in the epidermis, LEF1 and β-catenin are inappropriately expressed in the ventral dermis and, in the ventral epidermis, LEF1 expression is lost. In the classical Wnt signaling cascade, β-catenin and LEF1 interact to form a transcription factor complex that is responsible for stimulating the expression of Wnt-responsive genes (Behrens et al., 1996; Eastman and Grosschedl, 1999). Both have been found to regulate skin patterning, keratinocyte proliferation, and hair follicle

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\begin{align*}
\text{PTHrP and PTHR KO} & \quad \text{K14 - PTHrP Transgensics} \\
\text{Localized PTHrP Signaling} & \quad \text{Diffuse Ventrail Nipple Formation} \\
\text{Mammary Mesenchyme Differentiation} & \quad \text{Diffuse PTHrP Signaling} \\
\text{Mammary and Nipple Morphogenesis} & \quad \text{Inappropriate Mammary Mesenchyme Differentiation} \\
\text{No PTHrP Signaling} & \quad \text{Failure of Mammary Mesenchyme Differentiation} \\
\text{No Nipple or Mammary Morphogenesis} & \quad \text{No PTHrP} \\
\end{align*}
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Fig. 9. Model for the regulation of cell fate by PTHrP-signaling during mammary gland and nipple development. The different colors represent different cell fates: red circles represent mammary epithelial cells, yellow squares represent mammary mesenchyme, green ovals represent dermal mesenchyme, blue ovals represent typical epidermal cells and purple squares represent nipple cells. (A) Normally, the mammary epithelial cells express PTHrP after the bud starts to form. PTHrP signals to the dermal mesenchyme near the developing bud and, as a result, these cells become mammary mesenchyme. The mammary mesenchyme maintains the mammary fate of the epithelial cells, triggers their morphogenesis and induces the overlying epidermis to become the nipple. (B) In the absence of PTHrP signaling, no mammary mesenchyme is formed. Therefore, the mammary epithelial cells revert to an epidermal fate, no morphogenesis occurs and the nipple does not form. (C) In the presence of diffuse PTHrP signaling, the entire ventral dermis becomes mammary mesenchyme and the ventral epidermis becomes nipple sheath.

One of the most intriguing aspects of the K14-PTHrP transgenic phenotype is the abrupt loss of virtually all nipple-like aspects of the transgenic skin at the transition from the ventral to the dorsolateral aspects of the trunk. This line of demarcation is almost identical to the position of the nipple, or milk lines, thickened ridges of epidermis that exist between the limb buds from E10-E11 and along which the mammary buds form (Sakakura, 1987). With the exception of the developing mammary gland, we have not detected differences in native PTHrP, PTHR1 or K14-PTHrP transgene expression between the dorsal and ventral skin of mouse embryos (Dunbar et al., 1999). Therefore, the ability of PTHrP to induce mammary mesenchyme and nipple differentiation is restricted to the ventral surface.
development (Zhou et al., 1995; Gat et al., 1998; Zhu and Watt, 1999; Widelitz et al., 2000). In this regard, it is likely that the loss of LEF1 expression in the developing nipple skin is important in suppressing hair follicle development, as LEF1-deficient mice are hairless and forced expression of LEF1 induces hair-follicle development in normally hairless squamous epithelia (vanGenderen et al., 1994; Zhou et al., 1995).

LEF1 is also critical for the formation of the mammary gland. As in PTHrP or PTHR1 knockout mice, mammary development in LEF1-deficient mice does not proceed past the bud stage. At E14-E15, we have found that, in addition to being expressed in the mammary epithelium, LEF1 is prominently expressed in the primary mammary mesenchyme. Our data also indicate that, in vivo, PTHrP, acting through the PTHR1, is both necessary for the induction of mesenchymal LEF1 expression, and, at least on the ventral surface of the embryo, sufficient for mesenchymal LEF1 expression. Given these findings and the similarities between the mammary phenotype upon disruption of the genes for PTHrP, PTHR1 and LEF1, it is possible that LEF1 acts downstream of PTHrP signaling in the mammary mesenchyme. Furthermore, since changes in β-catenin expression upon manipulation of PTHrP-signaling parallel the changes in LEF1 expression, it is tempting to speculate that PTHrP signaling might interact directly with Wnt signaling in these cells. Wnt10b is expressed in the developing mammary bud, and mesenchymal cells have been shown to be targets of Wnt signaling during hair follicle morphogenesis (Christiansen et al., 1995; DasGupta and Fuchs, 1999; Kishimoto et al., 2000). Therefore, it is possible that the mammary mesenchyme might also be a target of Wnt signaling.

In conclusion, in this report we demonstrate that PTHrP regulates a series of cell fate decisions central to the embryonic development of the murine mammary gland. PTHrP acts as an epithelial signal that induces the mesenchyme around the epithelial bud to become mammary specific. As a result, the mammary mesenchyme acts on the epithelial bud to maintain the mammary identity of the epithelium and to support ductal morphogenesis. It also acts upon the epidermis around the mammary bud to suppress hair follicle formation and trigger nipple sheath formation. Only the mesenchymal cells underlying the ventral epidermis are competent to respond to the mammary mesenchyme-inducing functions of PTHrP. Finally, these changes in cell fate correlate with changes in β-catenin and LEF1 expression within the mesenchyme and epidermis, implicating the involvement of the Wnt signaling cascade. We hope that future work aimed at unraveling the mechanisms by which PTHrP alters these cell fates will lead to a better understanding of mammary development and epidermal patterning.

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