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BMP4 and PTHrP interact to stimulate ductal outgrowth during embryonic mammary development and to inhibit hair follicle induction

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The mammary glands develop initially as buds arising from the ventral embryonic epidermis. Recent work has shed light on signaling pathways leading to the patterning and formation of the mammary placodes and buds in mouse embryos. Relatively little is known of the signaling pathways that initiate branching morphogenesis and the formation of the ducts from the embryonic buds. Previous studies have shown that parathyroid hormone-related protein (PTHrP; also known as parathyroid hormone-like peptide, Pthlh) is produced by mammary epithelial cells and acts on surrounding mesenchymal cells to promote their differentiation into a mammary-specific dense mesenchyme. As a result of PTHrP signaling, the mammary mesenchyme supports mammary epithelial cell fate, initiates ductal development and patterns the overlying nipple sheath. In this report, we demonstrate that PTHrP acts, in part, by sensitizing mesenchymal cells to BMP signaling. PTHrP upregulates BMP receptor 1A expression in the mammary mesenchyme, enabling it to respond to BMP4, which is expressed within mesenchymal cells underlying the ventral epidermis during mammary bud formation. We demonstrate that BMP signaling is important for outgrowth of normal mammary buds and that BMP4 can rescue outgrowth of PTHrP$^{-/-}$ mammary buds. In addition, the combination of PTHrP and BMP signaling is responsible for upregulating *Msx2* gene expression within the mammary mesenchyme, and disruption of the *Msx2* gene rescues the induction of hair follicles on the ventral surface of mice overexpressing PTHrP in keratinocytes (K14-PTHrP). Our data suggest that PTHrP signaling sensitizes the mammary mesenchyme to the actions of BMP4, triggering outgrowth of the mammary buds and inducing MSX2 expression, which, in turn, leads to lateral inhibition of hair follicle formation within the developing nipple sheath.

KEY WORDS: Mammary gland, Breast, Hair follicle, Epidermal appendages, Branching morphogenesis, Parathyroid hormone-related protein (PTHrP), Bone morphogenic proteins

INTRODUCTION

In mice, mammary gland development begins with the formation of two multilayered ridges of epidermal cells known as the mammary lines, which are located between the fore and hind limb buds on either side of the ventral surface of the embryo. The mammary lines are first discernable on embryonic day 10 (E10) and, between E10.5 and E12.5, cells within these ridges are thought to migrate to ten characteristic locations, where they invaginate into the underlying mesenchyme to form the mammary buds. Between E15 and E16, epithelial cells within the mammary buds begin to divide and give rise to the mammary sprout, which grows out of the initial mesenchyme down through the dermis and into the developing mammary fat pad. Once in this stromal compartment, the nascent mammary duct begins to branch and by birth gives rise to approximately 10-15 primary branches. This rudimentary duct system persists until rising hormone levels at puberty trigger a second round of rapid epithelial proliferation and ductal branching morphogenesis (Hens and Wysolmerski, 2005; Veltmaat et al., 2003; Robinson et al., 1999).

The development of the embryonic mammary gland depends on a series of reciprocal interactions between epithelial and mesenchymal cells, which guide the formation of the placodes and buds, establish mammary cell fates and initiate the three-dimensional morphogenesis necessary for the formation of the primary ductwork (Hens and Wysolmerski, 2005; Veltmaat et al., 2003; Robinson et al., 1999). Our understanding of the molecular events underpinning early mammary development remains rudimentary, but recent work has begun to characterize some of the mediators of these crucial epithelial-mesenchymal interactions. Like in other organs, members of the FGF, hedgehog, WNT and EGF growth factor signaling pathways play important roles in the patterning and formation of the initial mammary placodes (Chu et al., 2004; Davenport et al., 2003; Eblaghie et al., 2004; Hatsell and Cowin, 2006; Howard et al., 2005; Mailleux et al., 2002; Veltmaat et al., 2004; Veltmaat et al., 2006). As discussed below, parathyroid hormone related protein (PTHrP; also known as parathyroid hormone-like peptide, Pthlh) and its receptor are important to the formation of the mammary mesenchyme and outgrowth of the nascent mammary ducts (Hens and Wysolmerski, 2005). However, little else is known of the signaling pathways mediating the initial wave of branching morphogenesis that gives rise to the primary duct system.

PTHrP was originally discovered as the cause of a common paraneoplastic syndrome known as humoral hypercalcemia of malignancy (Wysolmerski and Broadus, 1994). PTHrP is structurally related to parathyroid hormone (PTH), which is the principal regulator of circulating calcium levels in tetrapods (Philbrick et al., 1996). The high degree of homology between the amino-termini of the two proteins allows both PTH and PTHrP to share a common G protein-coupled receptor named the Type 1 PTH/PTHrP receptor (PTH1R, also known as Pthr1) (Juppner et al.,

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1991). Thus, when PTHrP is made by tumors and is secreted into the circulation it mimics the actions of PTH and causes hypercalcemia. However, with the exception of lactation, PTHrP normally does not circulate, but rather is secreted locally to exert autocrine, paracrine and intracrine functions (Philbrick et al., 1996; DeMauro and Wysolmerski, 2005; Dunbar and Wysolmerski, 1999; Dunbar et al., 1998; Wysolmerski et al., 1998; Wysolmerski et al., 1994). PTHrP and the PTH1R are both expressed widely during embryonic development, often in adjacent cell types (Lee et al., 1995). Experiments in genetically manipulated mice have documented important functions for this signaling pathway during bone, tooth, skin, lung and mammary gland development (Philbrick et al., 1996; Wysolmerski and Stewart, 1998; Hastings, 2004; Foley et al., 2001; Lanske et al., 1998). The study of fetuses with loss-of-function mutations in the PTH1R gene has confirmed the importance of PTHrP signaling to proper bone and breast development in humans as well (Wysolmerski et al., 2001).

PTHrP and the PTH1R are both required for normal mammary gland development. Disruption of either gene in mice and loss of PTH1R function in humans results in the complete absence of the mammary epithelium (Wysolmerski et al., 1998; Dunbar and Wysolmerski, 1999; Foley et al., 2001). During murine development, PTHrP is prominently expressed within mammary epithelial cells, beginning on day E11 concurrent with the formation of the mammary placodes. The PTH1R is expressed on immature mesenchymal cells located beneath the entire epidermis. As the mammary bud is invaginating, PTHrP acts on its receptor to induce the differentiation of the surrounding mesenchyme into the specialized condensed mammary mesenchyme. Stimulation by PTHrP is required for this mesenchyme to perform three vital functions: (1) to maintain the mammary fate of the epithelial cells; (2) to trigger the overlying epidermis to form the nipple sheath; and (3) to initiate ductal outgrowth and morphogenesis. In the absence of PTHrP signaling, mammary epithelial cells differentiate into skin cells, no nipple is formed and morphogenesis is interrupted (Wysolmerski et al., 1998; Dunbar et al., 1999; Foley et al., 2001). Conversely, overexpression of PTHrP in the basal keratinocytes of transgenic mice using the keratin 14 promoter (K14-PTHrP mice) leads to the conversion of the subepidermal mesenchyme from dermis into condensed mammary mesenchyme (Foley et al., 2001). This, in turn, suppresses hair follicle development and causes the epidermis to acquire the characteristics of the nipple sheath. However, curiously, the epidermal phenotype of the K14-PTHrP mice is restricted to the ventral surface of the mouse between the borders of the original mammary lines, suggesting that this area represents a specific zone of sensitivity to the effects of PTHrP (Dunbar et al., 1999; Foley et al., 2001).

Bone morphogenetic proteins (BMPs) have been shown to be important to the dorsoventral patterning of early embryos (Reversade et al., 2005; Pyati et al., 2005; De Robertis and Kuroda, 2004). In addition, Zhang and colleagues have reported the BMP4 gene to be strongly expressed within the developing ventral epidermis at E13.5 (Zhang et al., 2002). BMPs constitute a large family of secreted growth factors that are involved in many aspects of development and that have been implicated as classical morphogens because of their ability to alter cell fate in a concentration-dependent fashion (Gurdon and Bourillot, 2001; O'Connor et al., 2006; Rosen, 2006). BMPs signal through a heteromeric complex of type I and II receptor serine/threonine kinases (Massague, 1996). Binding of BMPs to their cognate receptors induces phosphorylation of members of the receptor-regulated SMAD family (rSMADs). Once phosphorylated, rSMADs associate with SMAD4, translocate into the nucleus and regulate the transcription of specific genes (Massague and Chen, 2000; von Bubnoff and Cho, 2001). In addition, BMP signaling is modulated by a complex series of interacting factors such as secreted inhibitors, specific inhibitory Smads and interactions between Smads and other transcription factors such as β-catenin and LEF1 (Labbe et al., 2000; Massague and Chen, 2000; von Bubnoff and Cho, 2001; Rosen, 2006; Hussein et al., 2003; Sakai et al., 2005).

MSX1 and MSX2 are BMP-responsive homeodomain-containing transcription factors that have been shown to participate in the relay of signals between epithelium and mesenchyme during development (Phippard et al., 1996; Satoh et al., 2004; Satokata et al., 2000). Both are particularly important to the normal development of epidermal appendages, including the mammary gland. MSX1 and MSX2 are each expressed in the epithelial cells of the forming mammary bud, and in mice with both genes deleted, mammary development fails at the placode stage (Satokata et al., 2000). MSX2, but not MSX1, is also expressed within the dense mammary mesenchyme surrounding the mammary epithelial cells, and after E14.5 its expression becomes restricted to the mesenchymal compartment (Phippard et al., 1996; Satokata et al., 2000). Similar to the phenotype of PTHrP$^{-/-}$ mice, mammary buds are reported to form in MSX2$^{-/-}$ mice, but their development arrests at E16.5 and no ductal outgrowth is formed (Satokata et al., 2000). Interestingly, activation of the PTH1R modulates *Msx2* gene expression in aortic adventitial cells and in osteoblasts (Shao et al., 2003; Shao et al., 2005; Bidder et al., 1998; Dodig et al., 1999; Towler et al., 2006).

In this study, we examined potential interactions between PTHrP and BMP signaling during early embryonic mammary gland development. We demonstrate that PTHrP signaling is permissive for BMP signaling in the mammary mesenchyme. This interaction, in turn, activates *Msx2* gene expression within the mesenchymal cells, which enables them to suppress hair follicle formation in the overlying nipple skin. PTHrP and BMP signaling also cooperate to enable the mesenchyme to initiate outgrowth of the mammary epithelial buds.

MATERIALS AND METHODS

Animals

Animal experiments were approved by the Yale University IACUC. MSX2$^{+/-}$ (tm1R1m) mice were obtained through the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis and MSX2$^{-/-}$ mice were identified as previously described (Satokata et al., 2000). *Bmp4-lacZ^{neo}*, K14-PTHrP, PTHrP$^{-/-}$ and PTH1R$^{-/-}$ mice were identified as described previously (Wysolmerski et al., 1998; Foley et al., 2001; Zhang et al., 2002).

Histology and immunohistochemistry

Histology and immunohistochemistry were performed using standard techniques. Skin samples were fixed overnight in 4% paraformaldehyde or Bouin's and were embedded in paraffin. Antigen retrieval was performed by heating sections in 7 mM citrate under pressure, after which incubation with primary antibody was carried out for 12 hours at 4°C. Antibodies to filaggrin, K14 and tenascin C (Covance, Berkeley CA) were detected with the Vector Elite ABC kits (Vector Laboratories, Burlingame. CA) and 3,3′ diaminobenzidine as a chromagen. β-Catenin antibodies (Transduction Laboratories, Lexington KY) were detected with a goat anti-mouse Alexa546-conjugated secondary antibody (Molecular Probes, Eugene, OR).

Western blotting

C3H10T1/2 cells (a gift from Dr Mark Horowitz, New Haven, CT) and C2C12 cells (from ATCC) were cultured in 10% fetal bovine serum (FBS), 0.2 mM L-glutamine on 100 mm Falcon culture dishes (Becton-Dickinson, Franklin Lakes, NJ). For BMP2 or BMP4 stimulation, media was changed to 0.1% FBS the day before treatment. Cells were treated with 0, 10, 50 and 100 ng/ml recombinant BMP4 or BMP2 (R&D Systems, Minneapolis, MN)

with or without 10^{-7} M PTHrP (Sigma-Aldrich, St Louis, MO) and were harvested after 18-20 hours' exposure. For protein detection, cells were homogenized in PBS containing a cocktail of protease inhibitors (Complete protease inhibitor cocktail tablets, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, Calbiochem, La Jolla CA). Lysates (50 μg) were run on an 8% SDS-PAGE reducing gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 3% dried milk in PBS, and then were incubated in the same solution with the following antibodies: rabbit anti-phospho-SMAD 1,5,8 (Cell Signaling Technology, Danvers, MA), mouse anti-MSX2 (4G1) (Developmental Studies Hybridoma Bank), and mouse anti-actin (Sigma-Aldrich). Goat anti-rabbit and goat anti-mouse secondary antibodies (Sigma-Aldrich) were used at 1/5000 dilution. Proteins were detected using chemiluminescence (Supersignal, Pierce, Rockford, IL).

In situ hybridization

In situ hybridization was performed on 5 μm paraffin sections as described previously (Dunbar et al., 1998). The probe for MSX2 was a kind gift of Dr Richard Maas and has been previously described (Satokata et al., 2000). BMP receptor probes were a generous gift of Dr Vicki Rosen (Boston, MA). Sense and antisense probes were generated from linearized fragments using an in vitro transcription kit (Promega, Madison WI) in the presence of [^{35}S]UTP (1000 Ci/mmol, Amersham, Life Science, Arlington Heights, IL).

RNA isolation and RT-PCR

Total RNA was isolated from cells or tissue using Trizol reagent (Gibco, Gaithersville, MD) and samples were treated with DNase as described by the manufacturer (GenHunter Corp., Nashville, TN). Quantitative RT-PCR (qRT-PCR) was performed by standard methods using the OpticonII DNA engine (JM Research, Waltham, MA). The mouse gene expression assay (Applied Biosystems, Foster City, CA) was used for MSX2 (Mm00442992_m1). We generated the following primer sets for SYBR-Green-based qRT-PCR: BMPr1a forward primer 5′-GGTATCTGGGTCAAAGCTGTTC-3′ and reverse primer 5′-CCTGCTGTCTCACTGGTGTAAG-3′, which spans nucleotides 87-244 of the BMPr1a coding sequence (NM_009758). Probe-based qRT-PCR was performed with Brilliant qRT-PCR master mix (Stratagene, La Jolla, CA) and SYBR-Green-based qRT-PCR was performed with Brilliant SYBR-Green qRT-PCR master mix (Stratagene). Samples were normalized for relative quantification of expression by the $2^{-\Delta\Delta}$CT method (Applied Biosystems 1997). Relative quantitation of gene expression: ABI Prism 7700 sequence detection system, user bulletin 2, revision B. Samples were run in duplicate. cDNA was prepared using the ABI PRISM as per the manufacturer's instructions.

Embryo and mammary bud cultures

Freshly harvested E13 embryos were decapitated and placed on PET track-etched membrane cell culture inserts containing 0.4 μm pores (Becton Dickinson) in six-well plates. Embryos were cultured in F12/DMEM media containing 10% FBS and antibiotics. Embryos were treated for 12-16 hours with or without 10^{-7} M PTHrP (Sigma) after which ventral and/or dorsal epidermis was dissected and used to make RNA.

In order to prepare bud cultures, individual mammary buds were microdissected from E13 wild-type and PTHrP$^{-/-}$ embryos and placed on Whatman 13 mm nuclepore Track-etched membranes (8 μm pore size; Thomas Scientific, Swedesboro, NJ) on top of a tuft of ventral mesenchyme. All dissections were performed in DMEM at 4°C. The filters were cultured on EC587-40 mesh screen grills (Thomas Scientific, Swedesboro, NJ) in six-well plates containing 10% FBS in DMEM/F12 media with antibiotics. Media was changed every other day for the length of the experiment. Bud cultures were fixed in acid alcohol and stained in carmine alum. Stained tissue was then dehydrated and mounted in Permount (Fisher Scientific, NJ) for viewing.

RESULTS

PTHrP modulates BMP signaling in the embryonic mammary mesenchyme

Overexpression of PTHrP in the basal keratinocytes of transgenic mice (K14-PTHrP mice) causes dermal mesenchyme to become mammary-specific mesenchyme, which, in turn, causes epidermis

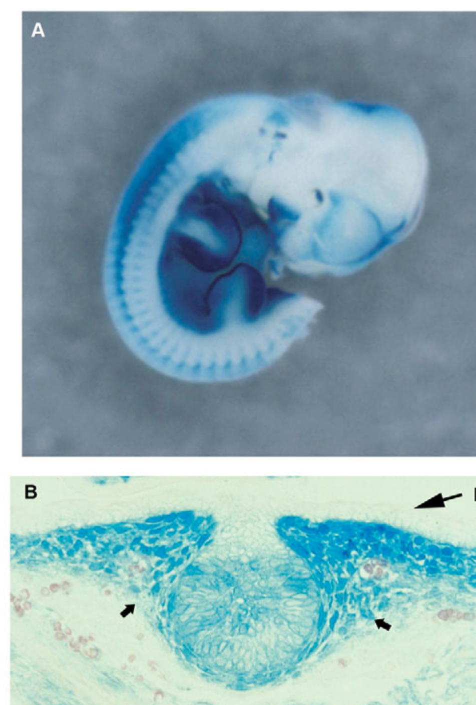

Fig. 1. BMP4 is expressed in the ventral mesenchyme during embryonic mammary gland development. (**A**) β-galactosidase staining of a *Bmp4-lacZ^{neo}* embryo on E12.5. In these embryos β-galatosidase is expressed under the control of the BMP4 promoter. At this stage of development, BMP4 is normally prominently expressed on the ventral surface of the embryo. (**B**) Cross-section of an E13.5 *Bmp4-lacZ^{neo}* mammary bud. BMP4 is expressed in both the mammary epithelium and the mammary mesenchyme, but more prominently within the mesenchme (arrows). Note the absence of expression in the epidermis (E).

to become specialized nipple skin (Foley et al., 2001). These changes in cell fate are restricted to the ventral surface of the embryo, despite the fact that both the K14-PTHrP transgene and the PTH/PTHrP receptor are expressed within both the dorsal and ventral surfaces of the embryo. Therefore, we hypothesized that the effects of PTHrP on mammary mesenchyme and skin differentiation depend on the cooperation of some other, ventrally restricted, signaling pathway. Recently, using a mouse in which the *lacZ* gene was knocked into the *Bmp4* gene locus, Zhang and colleagues reported that BMP4 expression was strongly expressed within the ventral surface of E13.5 embryos (Zhang et al., 2002). Using the same model, we examined the expression of BMP4 during mammary bud formation. As shown in Fig. 1, BMP4 was expressed in mesenchymal cells underlying the ventral epidermis between the four limb buds. BMP4 was also expressed in epithelial cells of the mammary bud, but was not expressed within keratinocytes. This pattern of expression was present between E11.5 and E14.5, after which the *lacZ* expression became primarily associated with developing hair follicles.

Because the pattern of BMP4 expression in the subepidermal mesenchyme was essentially identical to the pattern of ectopic mammary mesenchyme in K14-PTHrP embryos (Foley et al., 2001), we next examined the possibility that PTHrP and BMP4 signaling interact during the formation of mammary buds. BMP signaling is complex, but BMP4 is typically thought to induce the phosphorylation of rSMADs, which includes SMADs 1, 5 and 8 (Massague and Chen,

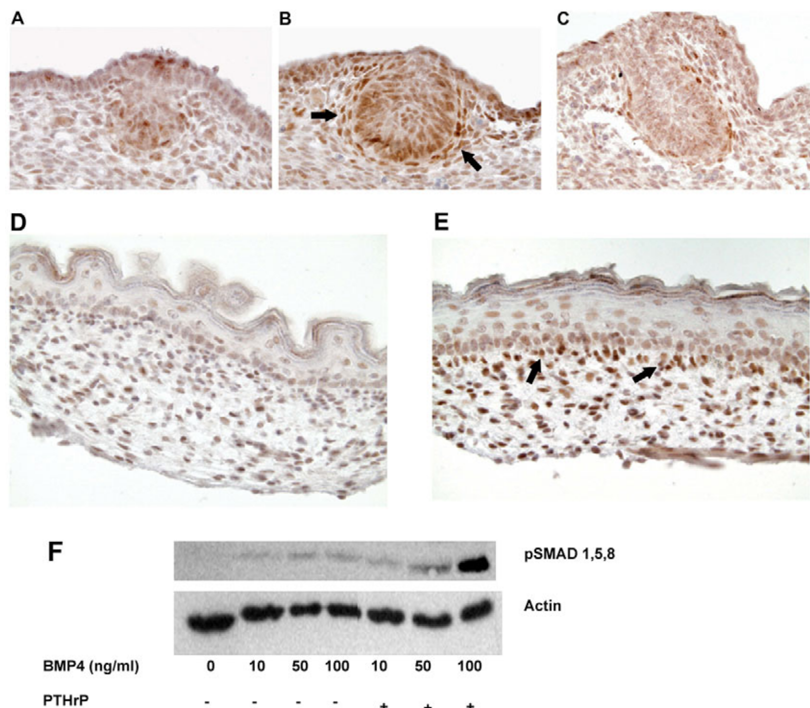

Fig. 2. PTHrP augments BMP signaling in vivo and in vitro. (**A-E**) Immunohistochemistry for phospho-SMAD 1, 5, 8 in sections through a PTHrP$^{-/-}$ mammary bud at E15.5 (A), a wild-type bud at E15.5 (B), a PTH1R$^{-/-}$ bud at E15.5 (C), wild-type ventral skin at E18.5 (D) and K14-PTHrP ventral skin at E18.5 (E). Note the nuclear staining for phospho-SMADs in the wild-type mammary mesenchyme (arrows in B) and its absence in the mammary mesenchyme of PTHrP$^{-/-}$ and PTH1R$^{-/-}$ buds (A,C). Also note the induction of nuclear phospho-SMAD staining in the mesenchyme of the ventral skin in K14-PTHrP embryos (arrows in E). (**F**) Western blot for phospho-SMAD 1, 5, 8 of whole cell lysates from C3H10T1/2 cells treated overnight with graded BMP4 concentrations (0, 10, 50, 100 ng/ml BMP4) with or without 10^{-7} M PTHrP. The addition of PTHrP augments phospho-SMAD expression (representative blot of five experiments).

2000). Therefore, we examined the pattern of SMAD phosphorylation in wild-type mammary buds and in genetic models of loss and gain of PTHrP function using an antibody specific for the phosphorylated forms of these three SMADs. As demonstrated in Fig. 2, we performed immunohistochemistry on mammary buds from wild-type, PTHrP$^{-/-}$ and PTH1R$^{-/-}$ mammary buds harvested at E15.5. Wild-type buds were positive for nuclear phospho-SMAD 1, 5, 8 in both the epithelial and mesenchymal compartments (Fig. 2B). Furthermore, only the mammary mesenchyme and not the general dermal mesenchyme stained. By contrast, in PTHrP$^{-/-}$ and PTH1R$^{-/-}$ buds, phospho-SMAD 1, 5, 8 staining was significantly reduced in the mammary mesenchyme compared with the wild-type buds (Fig. 2A,C). We also compared phospho-SMAD 1, 5, 8 staining in the ventral skin of K14-PTHrP mice to that in wild-type ventral skin. As shown in Fig. 2D, there was little phospho-SMAD staining in the dermis of wild-type mice at E18.5, but there was prominent staining in the ectopic mammary mesenchyme beneath the ventral skin of K14-PTHrP littermates (Fig. 2E). Dorsal skin from wild-type and K14-PTHrP mice showed phospho-SMAD staining associated with developing hair follicles and their associated mesenchyme, but there was no significant staining in the interfollicular dermis of either genotype (data not shown). These results demonstrate that alterations of PTHrP signaling in embryonic mammary buds and skin lead to changes in SMAD phosphorylation within mesenchymal cells, but only within the ventral zone of BMP4 expression in vivo, suggesting that PTHrP signaling may interact with BMP signaling in these cells.

PTHrP sensitizes mesenchymal cell lines to the effects of BMP

In order to see if there was a direct interaction between PTHrP and BMP signaling, we treated two well-characterized mesenchymal cell lines with both PTHrP and BMP2 or 4. We first confirmed that C3H10T1/2 and C2C12 cells expressed the PTH1R (data not shown). Next we assayed SMAD phosphorylation in both cell lines treated for 18-20 hours with varying doses (0-100 ng/ml) of BMP4 or BMP2 in the presence or absence of 10^{-7} M PTHrP 1-34. Fig. 2F shows the results for C3H10T1/2 cells treated with BMP4. Similar results were obtained with BMP2 and BMP4 in C2C12 cells (data not shown). In the absence of PTHrP, increasing doses of BMP resulted in a progressive increase in SMAD phosphorylation. The addition of PTHrP sensitized these cells to BMP treatment such that the levels of phospho-SMAD 1, 5, 8 were increased at each BMP concentration used (Fig. 2F). These data suggest that PTHrP signaling interacts directly with BMP signaling within mesenchymal cells.

PTHrP augments BMP signaling through regulation of BMPR1A expression

We next attempted to define a mechanism by which PTHrP might sensitize the mammary mesenchyme to the actions of BMP. A previous report had suggested that PTHrP increased the expression of the BMPR1A in C3H10T1/2 cells as assessed by Northern blot (Chan et al., 2003). We confirmed this observation by performing qRT-PCR on RNA prepared from C3H10T1/2 cells treated with 10^{-7} M PTHrP for 18-20 hours. As seen in Fig. 3A, this resulted in an approximate doubling of BMPr1a mRNA levels. We next asked if PTHrP was able to increase the expression of BMPr1a mRNA in mammary mesenchyme in vivo. First, we performed in situ hybridization to examine BMPr1a expression in embryonic mammary buds. Fig. 3E,F demonstrates the results for wild-type buds at E15.5. As one can see, BMPr1a mRNA was expressed at low levels throughout the entire subepidermal mesenchyme, including the dense mammary mesenchyme. It was difficult to determine if there was specific expression within the epidermis, but the gene was not expressed within mammary epithelial cells at this stage of development. We did not detect clear differences in expression of BMPr1a within the mammary mesenchyme of PTHrP$^{-/-}$ buds or within the ectopic mammary mesenchyme beneath the ventral epidermis of K14-PTHrP mice by in situ hybridization (data not shown). Because this receptor appeared to be expressed at low levels in the mesenchyme and because in situ hybridization is not a sensitive

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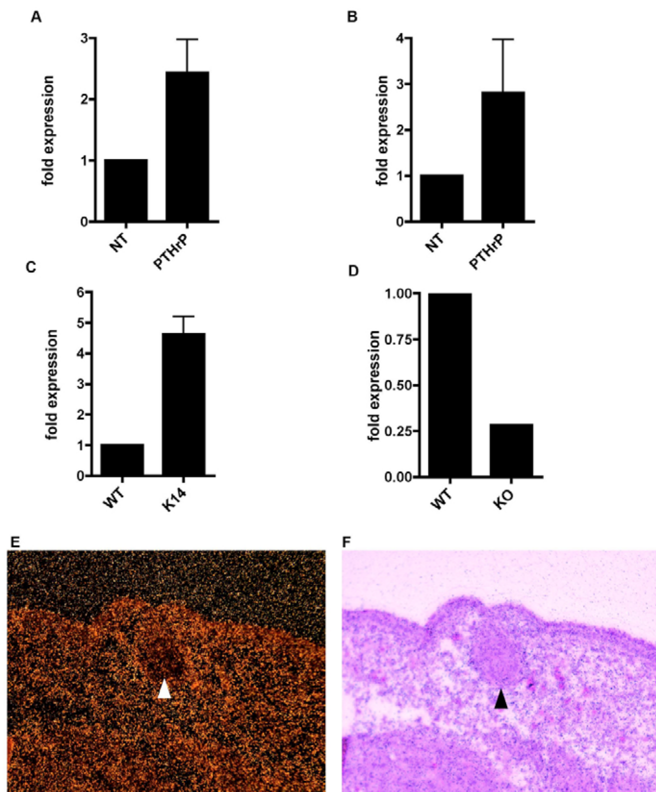

Fig. 3. PTHrP induces BMPr1a mRNA expression in vitro and in vivo. (**A**) BMPr1a mRNA detected by qRT-PCR in C3H10T1/2 cells treated with or without 10^{-7} M PTHrP overnight (*n*=3 experiments). (**B**) qRT-PCR for BMPr1a mRNA in ventral skin from cultured wild-type E13.5 embryos treated with or without 10^{-7} M PTHrP overnight (three experiments with two to three embryos per treatment). Note that PTHrP increases BMPr1a expression. (**C**) qRT-PCR for BMPr1a mRNA in the ventral skin from E18.5 K14-PTHrP transgenic and wild-type control mice. BMPr1a expression is higher in K14-PTHrP epidermis (*n*=4 experiments). (**D**) qRT-PCR for BMPr1a mRNA in mammary buds microdissected from either wild-type or PTHrP$^{-/-}$ embryos on E15.5. Expression is reduced in the PTHrP knockout buds compared with wild-type controls. RNA was from pooled buds (50-100 buds per sample). (**E**,**F**) Dark-field (E) and corresponding light-field (F) images of in situ hybridization for BMPr1a expression in an E15.5 wild-type mammary bud. Arrowhead points to the epithelial bud. The BMPr1a gene is expressed at a low level throughout the mesenchyme, but appears not to be expressed within the mammary epithelium.

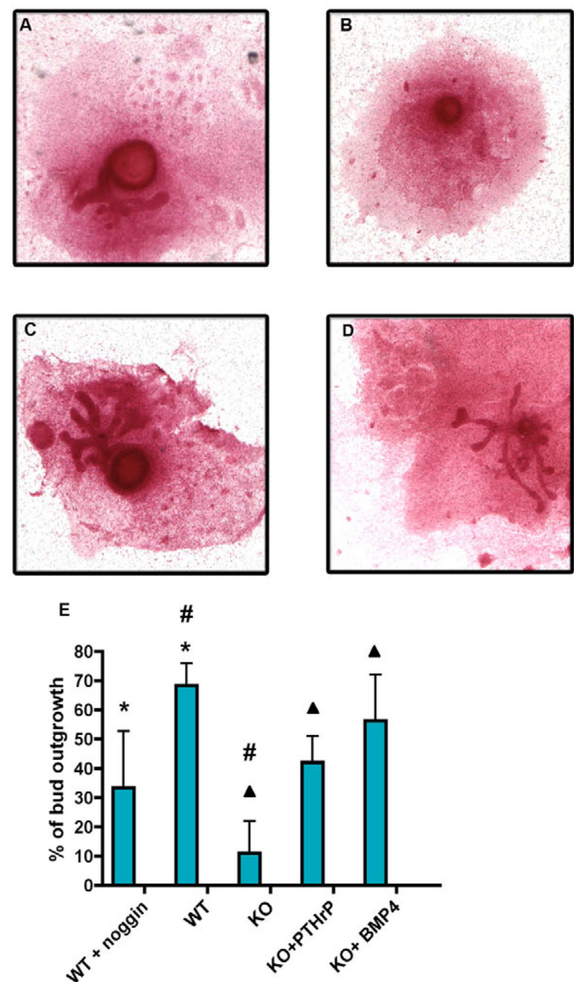

Fig. 4. BMP4 rescues outgrowth of PTHrP$^{-/-}$ mammary buds in organ culture. (**A-D**) Representative examples of bud outgrowths after 7 days in organ culture. (A) Wild-type mammary bud. (B) PTHrP$^{-/-}$ bud. (C) PTHrP$^{-/-}$ bud treated with 10^{-7} M PTHrP. (D) PTHrP$^{-/-}$ bud treated with 10 ng/ml BMP4. (**E**) Quantification of the frequency of bud outgrowth under different conditions as noted on the *x*-axis. Each experiment was performed on 15 buds over three separate experiments, except for the wild-type buds, which represent a total of 40 buds over eight separate experiments. *, $P<0.05$ for WT versus WT+noggin; #, $P<0.05$ for WT versus KO; triangle, $P<0.05$ for KO versus KO+PTHrP and KO versus KO+BMP4.

quantitative technique, we also addressed this question by performing qRT-PCR on samples of skin and mammary buds microdissected from pharmacologically and genetically manipulated embryos. Wild-type E13.5 embryos were cultured for 18-20 hours in the presence or absence of 10^{-7} M PTHrP. The ventral epidermis and its associated mesenchyme were then removed and assayed for *BMPr1a* mRNA expression by qRT-PCR. As seen in Fig. 3B, PTHrP treatment resulted in an approximate doubling of *BMPr1a* gene expression in the ventral epidermis, a result similar to its effects on *BMPr1a* gene expression in C3H10T1/2 cells. *BMPr1a* mRNA expression also was increased in ventral skin isolated from K14-PTHrP embryos at day E18 (Fig. 3C). Finally, we examined the relative levels of *BMPr1a* mRNA in freshly isolated mammary buds from E15.5 wild-type and PTHrP$^{-/-}$ embryos. As seen in Fig. 3D, *BMPr1a* mRNA levels were reduced by 75% in the PTHrP$^{-/-}$ buds compared with wild-type buds. As the *Pth1r* and *BMPr1a* genes are expressed on mesenchymal cells in embryonic skin and mammary buds, these data suggest that PTHrP secreted by the embryonic mammary buds regulates expression of the *BMPr1a* gene in mammary mesenchyme.

BMP4 can rescue outgrowth of mammary buds in PTHrP$^{-/-}$ embryos

Our next question concerned the physiological relevance of the ability of PTHrP to augment BMP signaling in the embryonic mammary bud. We reasoned that if BMP4 acted in a pathway downstream of PTHrP in the mammary bud, then supplying it to PTHrP$^{-/-}$ buds might rescue their developmental arrest. In order to test this possibility, we developed a mammary bud culture system, which allowed us to recapitulate the PTHrP$^{-/-}$ bud phenotype. As adapted from Robinson et al. (Robinson et al., 2000), mammary buds were isolated from wild-type and

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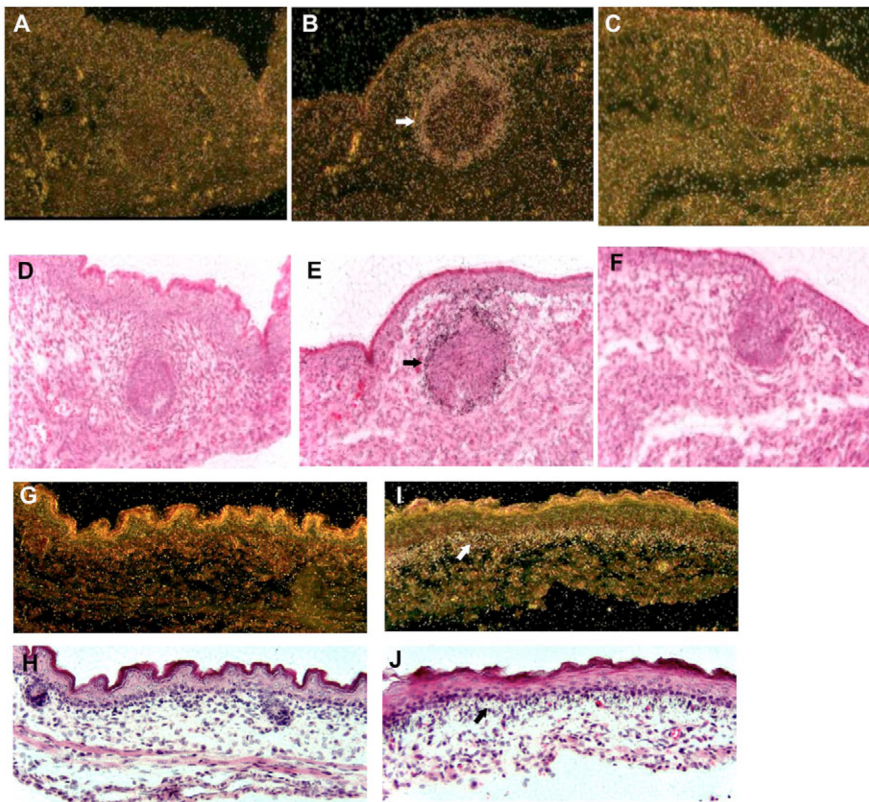

Fig. 5. PTHrP regulates *Msx2* gene expression in mammary mesenchyme. In situ hybridization for *Msx2* mRNA. (**A**,**D**) PTHrP$^{-/-}$ mammary buds at E15.5; (**B**,**E**) wild-type mammary buds at E15.5; (**C**,**F**) PTH1R$^{-/-}$ buds at E15.5. (A-C) Light-field images; (D-F) corresponding dark-field images. Arrows in B,E point to the mammary mesenchyme. (**G**,**H**) Wild-type ventral skin from an E18.5 embryo. (**I**,**J**) K14-PTHrP ventral skin from an E18.5 embryo. (G,I) Dark-field images; (H,J) corresponding bright-field images. Arrows in I,J point to the underlying dermis that is induced to express *Msx2* in response to overexpression of PTHrP in the overlying keratinocytes.

PTHrP$^{-/-}$ embryos and were cultured with extra tufts of ventral mesenchyme on nucleopore filters. After 7 days in the presence or absence of 10^{-7} M PTHrP, or 5 ng/ml or 10 ng/ml BMP4, cultures were scored for bud outgrowth. A bud was considered to have grown out normally if the length of the initial sprout was more then the length of the bud itself and if at least one branch point had formed. Fig. 4A-D demonstrate the typical appearance of the cultures, and Fig. 4E shows the quantification of outgrowth from multiple bud cultures. As can be seen, after 7 days in culture, wild-type buds produced an elongated sprout with several primary branches in approximately 70% of the cultures (Fig. 4A,E). The degree of branching of the outgrowths was variable but reminiscent of the normal embryonic duct system at E18.5 (Hens and Wysolmerski, 2005). By contrast, as depicted in Fig. 4B, the majority of PTHrP$^{-/-}$ buds failed to sprout, with only 10% of the buds showing outgrowth (Fig. 4E). Growth of PTHrP$^{-/-}$ buds was restored by the addition of PTHrP to the media (45% of cultures sprouted, Fig. 4C,E). In addition, treatment of PTHrP$^{-/-}$ bud cultures with BMP4 also rescued bud outgrowth so that 56% of buds met our sprouting criteria (Fig. 4D,E). The addition of equivalent concentrations of PTHrP or BMP4 to wild-type buds had no effect on bud outgrowths (data not shown). Finally, we examined the effects of inhibiting BMP signaling on the growth of wild-type buds by using the soluble BMP inhibitor, noggin (Rosen, 2006; Botchkarev et al., 1999). As shown in Fig. 4E, the addition of noggin, a secreted BMP inhibitor, to wild-type buds reduced the likelihood of outgrowth by half so that only 33% of noggin-treated wild-type buds sprouted compared with the 68% of control buds that sprouted. These data demonstrate that both PTHrP and BMP signaling are important to the initiation of normal bud outgrowth. Furthermore, the ability of BMP4 to complement the loss of PTHrP in mammary bud cultures strongly suggests that BMP4 signaling acts downstream of PTHrP to initiate ductal branching morphogenesis from embryonic mammary buds.

PTHrP regulates MSX2 expression in mammary mesenchyme

MSX2 is a homeodomain-containing transcription factor known to be involved in epithelial-mesenchymal interactions during development. It is thought to have important functions during the formation of epidermal appendages, as disruption of the *Msx2* gene results in abnormal tooth, hair follicle and mammary gland development (Satokata et al., 2000). In fact, mammary development in MSX2$^{-/-}$ mice has been reported to arrest after the formation of an apparently normal bud, a phenotype remarkably similar to that seen in PTHrP$^{-/-}$ embryos (Satokata et al., 2000). Furthermore, in vascular cells, MSX2 expression is regulated by both BMPs and parathyroid hormone (Shao et al., 2003; Shao et al., 2005). For these reasons, we hypothesized that MSX2 might mediate the effects of combined PTHrP and BMP signaling in the mammary bud.

In order to explore this hypothesis, we first examined Msx2 expression in wild-type, PTHrP$^{-/-}$ and PTH1R$^{-/-}$ mammary buds in vivo by in situ hybridization. As previously described, we found the *Msx2* gene to be expressed within the mammary mesenchyme in wild-type buds at E15.5 (Fig. 5B,E) (Satokata et al., 2000; Phippard et al., 1996). Interruption of PTHrP signaling through disruption of either the *Pthrp* or *Pth1r* genes resulted in a significantly reduced level of *Msx2* mRNA in the mammary mesenchyme (Fig. 5A,D,C,F). Furthermore, *Msx2* gene expression was prominently and ectopically induced in the ventral dermis by expression of PTHrP in the basal keratinocytes of K14-PTHrP transgenic mice (compare Fig. 5G,H with 5I,J). Thus, in vivo, MSX2 expression correlates with alterations in PTHrP signaling as well as the parallel alterations of BMP signaling demonstrated in Fig. 2.

We next examined changes in *Msx2* mRNA levels in response to PTHrP and BMP signaling in C3H10T1/2 cells in order to test directly if PTHrP and BMP signaling interact to regulate *Msx2* expression. As

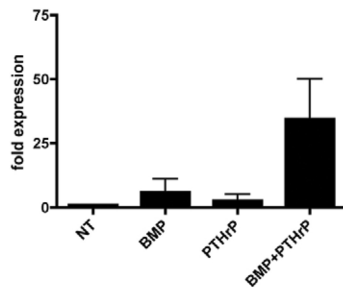

Fig. 6. PTHrP and BMP4 interact to regulate *Msx2* gene expression in C3H10T1/2 cell. qRT-PCR for *Msx2* mRNA in C3H10T1/2 cells treated for 7 days with 10^{-7} M PTHrP, 50 ng/ml BMP4, or both together. Shown is the average level of expression represented as the fold induction over no treatment (NT). Each bar represents the average±s.e.m. of three individual experiments.

shown in Fig. 6, treatment of the cells with either PTHrP or BMP4 alone modestly stimulated *Msx2* mRNA expression. However, the combination of PTHrP and BMP4 augmented *Msx2* expression to a much greater extent. These data again demonstrate that PTHrP and BMP signaling interact and identify the *Msx2* gene as a target of the cooperative interaction between the two.

MSX2 mediates the effects of PTHrP on hair follicle development in vivo

In order to determine if MSX2 mediated the effects of the combined actions of PTHrP and BMP on the mammary mesenchyme, we bred the K14-PTHrP transgene on to an MSX2$^{-/-}$ background. Our reasoning was that if MSX2 were important to the functions of PTHrP, then removing MSX2 should prevent the effects of PTHrP overexpression. As shown in Fig. 7B, overexpression of PTHrP in basal keratinocytes converts the epidermis into nipple skin and the ventral dermis into condensed mammary mesenchyme (Foley et al., 2001). As a result, the ventral epidermis of K14-PTHrP mice is thickened, lacks hair follicles and displays characteristic alterations in keratin and filaggrin expression. In addition, the dermis in these mice is hypercellular and expresses markers usually restricted to the mammary mesenchyme (Foley et al., 2001). As is evident from Fig. 7, disruption of the *Msx2* gene mitigated the K14-PTHrP phenotype but did not completely reverse it. The most striking finding was the recovery of hair follicle development in the K14-PTHrP/MSX2$^{-/-}$ embryos (Fig. 7C). Although deletion of MSX2 has been reported to cause abnormal hair cycling, the initial development of follicles in the embryo is normal (Satokata et al., 2000; Ma et al., 2003). As one can see, removing MSX2 from the K14-PTHrP epidermis led to the recovery of hair follicle induction in the ventral skin. In addition, there was some resolution of the epidermal thickening and the dermis was less compact in K14-PTHrP/MSX2$^{-/-}$ embryos compared with K14-PTHrP controls. However, there was no change in epidermal expression of keratin 14 or filaggrin in the ventral skin of K14-PTHrP/MSX2$^{-/-}$ embryos compared with the changes in the expression of these markers previously documented in the ventral mesenchyme of K14-PTHrP embryos (data not shown). Furthermore, K14-PTHrP/MSX2$^{-/-}$ embryos continued to express ectopic markers of the mammary mesenchyme such as LEF1, β-catenin, androgen receptor and tenascin C in the ventral dermis, despite the clear morphological changes noted above (data not shown). These data identify MSX2 as a crucial mesenchymal factor that allows PTHrP and BMP4 to suppress hair follicle induction in the vicinity of the developing mammary bud and nipple.

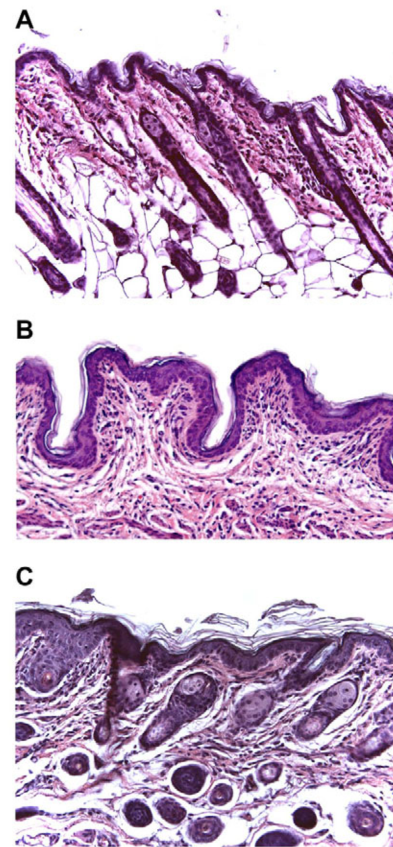

Fig. 7. Loss of *Msx2* rescues hair follicle induction in K14-PTHrP mice. Hematoxylin and Eosin-stained sections of wild-type ventral skin (**A**), K14-PTHrP transgenic ventral skin (**B**) and K14-PTHrP/Msx2$^{-/-}$ ventral skin (**C**). Sections were cut at 5 μm. Notice the recovery of hair follicles in the K14-PTHrP/Msx2$^{-/-}$ transgenic mice.

DISCUSSION

Our data document interactions between PTHrP and BMP signaling during embryonic mammary gland development. Previous studies had shown that PTHrP regulates a series of cell fate decisions necessary for the differentiation of the mammary mesenchyme, the formation of the nipple and the initiation of branching morphogenesis from the mammary bud (Wysolmerski et al., 1998; Dunbar et al., 1998; Foley et al., 2001). The current study suggests that PTHrP acts, at least in part, by modulating BMP signaling in mammary mesenchyme cells. We show that during the formation of the mammary buds, BMP4 is expressed by mesenchymal cells on the ventral surface of the embryo. PTHrP signaling sensitizes mesenchymal cells to BMP in vitro and in vivo, by increasing the expression of the BMP1A receptor. The interaction between PTHrP and BMP4 is important for mammary bud sprouting and BMP4 treatment can rescue outgrowth of PTHrP$^{-/-}$ mammary buds in organ culture. Finally, we demonstrate that the combination of PTHrP and BMP signaling increases the expression of the homeobox gene, MSX2, in the mammary mesenchyme, which, in turn, allows PTHrP to suppress hair follicle formation in the vicinity of the developing mammary bud and nipple.

Our data showing that PTHrP signaling upregulates the expression of the BMPR1A in the mammary mesenchyme are similar to those of Chan and colleagues (Chan et al., 2003), who demonstrated an increase in the expression of this receptor in response to PTHrP in the pluripotent mesenchymal cell line, C3H10T1/2. Using qRT-PCR, we showed an approximate doubling of *Bmpr1a* expression in these same

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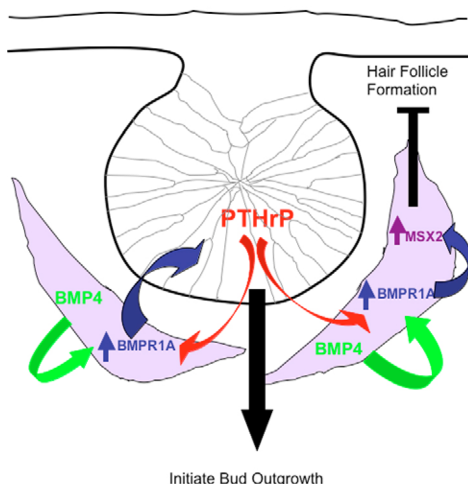

Fig. 8. PTHrP and BMP signaling interact to initiate mammary bud outgrowth and nipple formation. PTHrP is secreted from mammary epithelial cells and increases BMPR1A expression in the mammary mesenchyme. This increases the sensitivity of these cells to BMPs and allows them to respond to BMP4 in a paracrine and/or autocrine fashion. BMP4 signaling in the mesenchyme, in turn, triggers epithelial outgrowth and augments MSX2 expression, which causes the mammary mesenchyme to inhibit hair follicle formation within the nipple sheath.

cells when they were treated with PTHrP. In addition, we saw a doubling of *Bmpr1a* mRNA expression in the skin of embryos treated with PTHrP ex vivo as well as in the epidermis of K14-PTHrP embryos overexpressing PTHrP in basal keratinocytes. We also noted a 75% reduction in the level of *Bmpr1a* mRNA in mammary buds microdissected from PTHrP$^{-/-}$ embryos compared with buds derived from wild-type littermates, demonstrating that PTHrP expression by mammary epithelial cells is important to the native level of BMPR1A expression surrounding the buds. Although we could not detect a difference in *Bmpr1a* gene expression by in situ hybridization, these experiments did demonstrate that the receptor is expressed in mesenchymal cells and not in mammary epithelial cells during early bud development, suggesting that the alterations in expression noted above are likely to be in the mammary mesenchyme surrounding the developing bud and in the ectopic mammary mesenchyme that forms under the epidermis in K14-PTHrP embryos. This conclusion is also consistent with the localization of PTH1R expression in mesenchymal cells at this stage of development (Dunbar et al., 1998). Finally, results from a model of transgenic overexpression of PTHrP in developing lung also demonstrate upregulation of *Bmpr1a* gene expression in response to PTHrP (W. Philbrick, personal communication). Thus, PTHrP may regulate BMP receptor expression in several organs and modulation of BMP signaling may be a more general feature of the actions of PTHrP during development.

Overexpression of PTHrP in basal keratinocytes in K14-PTHrP mice leads to a transformation of dermal mesenchyme into mammary mesenchyme, which, in turn, suppresses the formation of hair follicles and alters the differentiation of keratinocytes (Foley et al., 2001). Interestingly, these changes occur only on the ventral surface of the embryo, despite the fact that the K14-PTHrP transgene is expressed in both ventral and dorsal keratinocytes and that the PTH1R is expressed on mesenchymal cells in the dermis on both surfaces. In addition, it has been shown that dermal mesenchyme harvested from the ventral surface of the embryo responds to the mammary epithelial bud by upregulating expression of the androgen receptor (a marker of the mammary mesenchyme), whereas mesenchyme from the dorsal skin does not (Heuberger et al., 1982) (G.W.R. and K. Kratochwil, unpublished). Our findings offer a potential explanation for these observations. We propose that PTHrP acts to promote mammary mesenchyme differentiation and bud outgrowth by modulating mesenchymal cell responsiveness to BMPs. Therefore, the presence of a specific BMP is needed for the full expression of the effects of PTHrP. In essence, the pattern of BMP4 expression creates a ventrally restricted zone of responsiveness to the effects of PTHrP. In the case of normal mammary development, the expression of BMP4 in the ventral mesenchyme coincides with the formation of the mammary buds, which express PTHrP and allows both signaling pathways to interact to form the mammary-specific mesenchyme. In the setting of the K14-PTHrP transgene, ectopic formation of mammary mesenchyme is restricted to the ventral area of BMP 4 expression.

Deletion of the PTHrP gene in mice and humans leads to a failure of the mammary bud to initiate branching morphogenesis (Wysolmerski et al., 1998; Wysolmerski et al., 2001). Culture of PTHrP$^{-/-}$ mammary buds ex vivo recapitulates the failure of bud outgrowth in vivo. While 70% of wild-type mammary buds initiated branching growth in culture, only 10% of PTHrP$^{-/-}$ buds did so, a defect rescued by the addition of PTHrP to the culture media. Significantly, the addition of BMP4 to the bud cultures was able to complement the loss of PTHrP and also rescue outgrowth of PTHrP$^{-/-}$ buds. Furthermore, noggin treatment was able to inhibit the growth of wild-type buds. These experiments suggest that BMP4 acts downstream of PTHrP to initiate outgrowth of the mammary buds. BMP4 has been shown to regulate branching morphogenesis in several other organs including the lung, submandibular gland, prostate, kidney and ureter (Eblaghie et al., 2006; Shao et al., 2005; Martinez et al., 2002; Miyazaki et al., 2000; Bellusci et al., 1996; Bragg et al., 2001; Weaver et al., 2000; Lamm et al., 2001; Dean et al., 2004; Shi et al., 2001). Its role has been best studied in the lung, where it appears either to stimulate or inhibit branching, depending on the experimental context (Eblaghie et al., 2006; Shao et al., 2005; Bellusci et al., 1996; Bragg et al., 2001; Weaver et al., 2000; Shi et al., 2001). Some of these conflicting effects may be related to the level of BMP signaling or to differing effects on epithelial cells versus mesenchymal cells. In the mammary bud, our results suggest that PTHrP-mediated upregulation of BMPR1A expression allows for spatially restricted, autocrine or paracrine BMP signaling within the mesenchyme. We believe that this BMP signal, in turn, enables mammary mesenchyme cells to trigger and/or support outgrowth of the bud epithelium.

In addition to promoting bud outgrowth, the mammary-specific mesenchyme instructs the overlying epidermis to form the nipple sheath, an activity that is also dependent on PTHrP signaling. A prominent feature of the nipple sheath is its lack of hair, which is probably the result of lateral inhibition of hair follicle formation by PTHrP secreted by the epithelial bud. In the absence of PTHrP or the PTH1R, hair follicles develop too close to the mammary bud, and in the presence of PTHrP misexpression by basal keratinocytes, hair follicle development is suppressed throughout the entire ventral epidermis (Foley et al., 2001; Wysolmerski et al., 1994). We now find that the ability of PTHrP to suppress hair follicle development depends on the actions of the homeobox transcription factor MSX2. Confirming previous reports, using in situ hybridization we found that MSX2 was expressed within the mammary mesenchyme (Phippard et al., 1996; Satokata et al., 2000). Furthermore, mesenchymal expression of MSX2 requires PTHrP signaling, because MSX2 levels were reduced around PTHrP$^{-/-}$ buds and MSX2 expression was induced within the ectopic mammary mesenchyme formed beneath the ventral epidermis in K14-PTHrP transgenic mice. The ventral

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restriction of MSX2 induction in these mice suggests that this transcription factor is a specific target of the interaction between PTHrP and BMP signaling discussed previously. This is not surprising, given the fact that MSX2 is known to be regulated by BMPs in several sites during development (Kratochwil et al., 1996; Andl et al., 2004; Towler et al., 2006; Hussein et al., 2003). It is also consistent with our data in vitro showing that while PTHrP or BMP4 alone have only a modest effect on MSX2 expression in C3H10T1/2 cells, the combination has a robust inductive effect on its expression. Given these results, it will be interesting to determine whether PTHrP might potentiate the effects of BMPs on MSX2 expression in other developing organs as well.

BMPs are known to suppress hair follicle formation, and antagonism of BMP signaling in the mesenchyme by secreted BMP inhibitors is thought to be important for the induction of at least some classes of hair follicles (Kobielak et al., 2003; Botchkarev et al., 2002; Andl et al., 2004; Botchkarev et al., 1999). Furthermore, BMP signaling is thought to be important for lateral inhibition and spacing of hair follicles and feathers (Jung et al., 1998; Noramly et al., 1999; Mou et al., 2006). Therefore, the sensitization of mesenchymal cells to BMP4 signaling by PTHrP might be expected to suppress hair follicle formation, as happens on the ventral surface of K14-PTHrP mice and around the mammary buds and nipple. Because crossing the K14-PTHrP transgene onto an $MSX2^{-/-}$ background led to the recovery of hair follicle formation in the ventral surface of K14-PTHrP/$MSX2^{-/-}$ mice, it would also appear that PTHrP and BMP4 interact to suppress hair development by inducing Msx2 in the mammary mesenchyme. Like mammary glands, hair follicle induction requires reciprocal interactions between epithelial and mesenchymal cells. Our findings suggest that MSX2 is able to interfere with the ability of the mesenchyme to induce the formation of hair placodes. While MSX2 has previously been reported to be necessary for the outgrowth of the mammary bud in mice, in our hands $MSX2^{-/-}$ mice are able to form normal mammary ducts (Satokata et al., 2000). Furthermore, the expression of several markers of mammary mesenchyme differentiation was also normal in $MSX2^{-/-}$ mammary buds (J.R.H. and J.W., unpublished). Thus, MSX2 appears to be relatively specific for mediating the hair-suppressing effects of PTHrP.

In closing, the experiments detailed in this report demonstrate an important interaction between PTHrP and BMP4 during the development of the embryonic mammary bud. As illustrated in Fig. 8, PTHrP is secreted by the mammary epithelial cells in the bud and interacts with its receptor on surrounding mesenchymal cells. In response, these cells upregulate BMPR1A expression and become able to respond in an autocrine and/or paracrine fashion to mesenchymal BMP4 found within the ventral epidermis. As a result of this cooperation between PTHrP and BMP signaling, the mammary-specific mesenchyme forms and exerts its actions on promoting outgrowth of the primary mammary ducts and instructing nipple sheath development. The suppression of hair follicle formation in the epidermis immediately surrounding the nipple is mediated by the induction of MSX2 expression in mesenchymal cells. It is likely that other growth factors and/or transcription factors will also be regulated by these pathways in the mammary mesenchyme, and it will be particularly interesting to define which factor(s) allows the mesenchyme to initiate outgrowth of the epithelial bud.

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