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PRINCIPAL INVESTIGATOR: John Wysolmerski

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

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John Wysolmerski

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Yale University School of Medicine
New Haven, CT 06520-8047

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PTHrP was discovered as the tumor product responsible for the syndrome of humoral hypercalcemia of malignancy. Recent experiments have shown that PTHrP has important functions during development. This project is designed to explore the hypothesis that PTHrP is a locally-produced growth factor that regulates epithelial-mesenchymal interactions during mammary development. In the second year we gathered more evidence to support a critical role for PTHrP in the control of mammary stromal cell function during embryonic development. In the absence of PTHrP there is a loss of the androgen-mediated destruction of the mammary buds in male embryos due to a failure of androgen receptor expression by the mesenchymal cells. In addition, there is also a failure of Tenascin C expression in PTHrP and PTH/PTHrP receptor knockout mammary buds. These results demonstrate that PTHrP is necessary for the proper differentiation of the mesenchyme. We have also found that PTHrP appears to regulate mammary stromal cell function during adolescence and pregnancy. Finally, although overexpression of PTHrP inhibits hormonally-induced ductular proliferation during mammary development, it does not appear to inhibit mammary tumor formation, at least in the GR mouse model.

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FOREWORD

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INTRODUCTION

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

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

As summarized in last year's report, in the first year of the project we concentrated on investigating the effects of the loss of the PTHrP gene on mammary development. We found that, in the absence of PTHrP, female mice had no mammary glands. This is the result of a failure of embryonic mammary development at the transition between the mammary bud phase and the first round of branching ductal morphogenesis. In female PTHrP- and PTH/PTHrP receptor-knockout embryos, the mammary bud fails to grow out into the mesenchyme and the mammary epithelial cells degenerate and die before birth. We also found that, during embryonic development, the PTHrP gene is expressed in mammary epithelial cells and that the PTH/PTHrP receptor is

expressed in mesenchymal cells, suggesting that the absence of PTHrP leads to a failure of the mesenchyme's ability to support further development of the mammary epithelial structures. As summarized below, in the second year of this project, we have continued to investigate the consequences of PTHrP gene ablation on the development of the embryonic mammary gland. In addition, we have examined the expression patterns of the PTHrP and the PTH/PTHrP receptor genes during later stages of mammary development and have initiated the experiments aimed at studying the effects of PTHrP on epithelial/mesenchymal interactions during adolescent mammary development. Finally, we have completed the initial round of studies examining the ability of PTHrP to inhibit mammary tumor formation. These findings are presented below, organized by technical objective.

BODY

Technical Objective 1. - Effects of the Loss of PTHrP on Mammary Gland Development

In the second year of the project we have continued to devote a great deal of energy to investigating the consequences of the loss of PTHrP on embryonic mammary development. As summarized in last year's report, we found that ablation of the PTHrP gene leads to a failure of mammary development at the transition of the mammary bud into the initial phase of branching morphogenesis. In the absence of PTHrP, the mammary duct system fails to grow out and the embryonic mammary gland remains bud-like in its appearance until the embryonic mammary epithelial cells degenerate and disappear. The ability to support the initiation of branching morphogenesis is a function of amino-terminal PTHrP acting on the PTH/PTHrP receptor, for ablation of this receptor gene essentially phenocopies the mammary phenotype seen with ablation of the PTHrP gene. Finally, we found that PTHrP is produced by the mammary epithelium while the PTH/PTHrP receptor is present on mammary mesenchyme. Hence, it would appear that PTHrP is an important epithelial signal that regulates mammary mesenchyme function in such a way as to support the initiation of branching morphogenesis. These data were published in the journal *Development* this past spring (4).

An important aspect of embryonic mammary development is the sexual dimorphism that occurs after the formation of the mammary bud (8). In female embryos the mammary bud remains relatively quiescent from its formation on E12 until the initiation of branching morphogenesis on E16. In male embryos, however, the fetal testes begin to make androgens on E13, which leads to the destruction of the mammary buds. In response to androgens, the mammary mesenchyme condenses around the neck of the mammary bud and, by E14, severs its connection with the epidermis (see Fig. 1) (8). In most strains of mice, the mammary epithelial cells in males subsequently degenerate by E15 - E16. This process has been studied in some detail and it is known to rely on a series of epithelial-mesenchymal interactions. First, androgen receptor expression is

induced in the mammary mesenchyme between E12 and E14 under the direction of the mammary epithelial cells (9). The mesenchymal cells are the cells that subsequently respond to fetal androgens, and they in turn sever the epithelial stalk and destroy the mammary epithelial cells (8,10,11). Although the molecular details of this process have not been elucidated, it is clear from the existing literature that epithelial and mesenchymal cells cooperate with one another in the destruction of the mammary bud in response to androgens (8-11).

Because of the total failure of mammary gland development in the absence of PTHrP as described above, we had initially concentrated our studies on female embryos, so that we could define the exact nature of this failure. However, given the similarities between the death of the epithelial cells in the absence of PTHrP and in response to fetal androgens, we have subsequently initiated a careful study of the fate of the mammary buds in PTHrP- and PTH/PTHrP-knockout males. We harvested PTHrP-knockout and PTH/PTHrP receptor knockout embryos at E15, a time point at which the histological destruction of the mammary buds in males should be advanced. Embryos were fixed in paraformaldehyde and genotyped by PCR. They were sexed by the appearance of the internal genitalia and this was confirmed by PCR-based detection of the SRY gene. To our surprise, we found that in the absence of PTHrP or its receptor, there is a loss of the normal sexual dimorphism. That is, in male knockout embryos, the mammary buds do not degenerate on E14-E15. As seen in Fig. 2, by E15 the destruction of the mammary buds in wild-type males is well advanced. One can appreciate that the mammary mesenchyme has condensed around the neck of the bud and the mammary epithelial cells in this region of the bud have degenerated. In addition, it is clear from TUNEL staining (terminal deoxy-transferase labeling) that this androgen-mediated condensation of the mesenchyme is accompanied by a great deal of apoptosis within the mammary mesenchyme as well as within the epithelium. In contrast, in male PTHrP-knockout embryos, at E15, the mammary buds resemble those in wild-type females. There is a lack of mesenchymal condensation, the neck of the mammary bud is well preserved, and by TUNEL staining, there is no apoptosis occurring in either epithelial or mesenchymal cells. As a result, the mammary buds in male and female PTHrP-knockout embryos are indistinguishable. As with the failure of branching morphogenesis in female embryos at E16, PTH/PTHrP receptor knockout embryos demonstrate identical findings. This suggests that it is amino-terminal PTHrP acting via this receptor that participates in the androgen response.

Because we know that PTHrP is produced by epithelial cells and acts on stromal cells during embryonic mammary development (4), we interpreted the failure of the androgen response in PTHrP- and PTH/PTHrP receptor-knockouts as further evidence that the mammary mesenchyme requires PTHrP to function appropriately during embryonic development. Our hypothesis is that PTHrP represents a signal from the epithelium to the mesenchyme that contributes to the functional differentiation of the dense mammary mesenchyme. In the absence of PTHrP, mesenchymal cells appear to undergo an initial structural differentiation. They condense, elongate and array themselves radially around the knockout epithelial buds in an apparently normal fashion. However, despite these changes, the cells cannot function as dense mammary mesenchyme cells

and support the proper morphogenesis of the mammary epithelial bud. In males they do not destroy the mammary buds in response to androgens and in females, they cannot support the initiation of branching morphogenesis.

Two molecules whose expression has classically been described as distinguishing the mature, dense mammary mesenchyme from the dermal mesenchyme are tenascin C and the androgen receptor (8). Tenascin C is a heparin-sulfate proteoglycan that has been detected in the extracellular matrix of the condensing mesenchymes associated with several developing organs (12,13). In the embryonic mammary gland it is first expressed within the dense mammary mesenchyme after the bud is fully formed at E14, and continues to be expressed by the mesenchyme surrounding the primary duct throughout embryonic life (14). As noted above, the AR is also first expressed within the dense mammary mesenchyme of the fully formed bud, beginning at E12 (8). Like tenascin, the AR continues to be expressed within the mesenchyme until birth, but only in the area immediately adjacent to the nipple (9). Interestingly, although the AR continues to be expressed, the mesenchyme only responds to androgens until E15-16, implying that androgen response is uncoupled from receptor expression after that point (11). Finally, the expression of both of these molecules within the mammary mesenchyme has been shown to be dependent on epithelial signals (9).

In order to begin to explore the hypothesis that PTHrP is important to the fully differentiated state of the mammary mesenchyme and in order to investigate the possibility that the failure of androgen responsiveness in the knockout embryos might be due to a failure of AR expression, we next examined the expression of AR and tenascin C in PTHrP- and PTH/PTHrP receptor-knockout mammary glands by immunocytochemistry. We obtained a polyclonal rabbit anti-androgen receptor antibody from Dr. Gail Prins (University of Illinois, Chicago) and a polyclonal anti-tenascin antibody from Dr. Teruyo Sakakura (Mei University, Japan). Both antibodies require antigen retrieval for use with paraffin sections. For AR staining this is accomplished by boiling the sections in 0.01M citrate buffer (pH 6) for 30 minutes. For tenascin staining the sections are incubated with 0.1% trypsin for 10 minutes. Primary antibody incubations are performed overnight at 4°C for androgen receptor and for 1 Hour at 37° for tenascin. We routinely use a Vector Elite avidin-biotin detection kit (Vector Laboratories, Burlingame, CA) with DAB as a chromagen, and sections are then counterstained with hematoxylin. As seen in Fig. 3, in wild-type controls there is strong staining for both tenascin C and AR that is limited to the dense mammary mesenchyme. However, in knockout embryos, the mammary mesenchyme does not stain for either androgen receptor or for tenascin C. This is true both for buds in female knockout embryos and for the inappropriately persisting buds in the male knockout embryos. Furthermore, the reliance of tenascin C and androgen receptor expression on PTHrP is not a generalized phenomenon in the knockout embryos, for we have detected androgen receptor expression in the testes and seminiferous tubules of PTHrP-null animals, and we have detected tenascin C in the developing bones of PTHrP-knockout mice. These result are interesting on two levels. First, they lend support to our hypothesis that PTHrP contributes to the differentiation of the dense mammary mesenchyme, as both classical markers of this phenotype are absent in these cells in the absence of PTHrP. Second, these results provide a

mechanistic explanation for the failure of the androgen -mediated destruction of the knockout mammary buds. That is, PTHrP appears to be the epithelial signal that induces androgen receptor expression within the mesenchymal cells, and without PTHrP the mesenchymal cells fail to express this receptor and become deaf to the androgen signal.

These experiments were not part of the original proposal as they followed from data that was unknown at the time of its submission. As a result, they are not a part of the original Statement of Work.

Technical Objective 2. - The effects of PTHrP on the branching morphogenesis of mammary epithelial cells.

The central hypothesis underlying this technical objective is that PTHrP regulates epithelial stromal interactions that support proper morphogenesis. As in Technical Objective 1, as the project has evolved over the last year, we have performed several experiments that support this hypothesis, but were not described in the original proposal. First, we examined the expression patterns of PTHrP and the PTH/PTHrP receptor by in situ hybridization histochemistry. We examined the expression of these mRNA's at E12 through E18 in embryos, and during adolescent and early pregnancy, time points which encompass phases of active ductal branching morphogenesis. As shown in Figs. 4 and 5, throughout mammary development, PTHrP was expressed in epithelial cells, while the PTH/PTHrP receptor was expressed in stromal cells. Interestingly, during adolescent development, PTHrP is expressed by cap cells within terminal end buds, but not by epithelial cells within mature ducts. Similarly, although there is a low level of receptor expression generally within the stromal cells of the mammary fat pad, there is a clear augmentation of PTH/PTHrP receptor expression within the stromal cells surrounding the terminal end buds. Therefore, in all phases of mammary development in which there is active ductal morphogenesis PTHrP is expressed in epithelial cells and its receptor is expressed in stromal cells. These findings are consistent with the hypothesis that PTHrP exerts its effects on ductal morphogenesis by regulating mammary stromal cell function.

In order to test the above hypothesis directly, we performed a series of tissue recombination and transplantation experiments using mammary epithelial buds and mammary mesenchyme from wild-type and PTH/PTHrP receptor-knockout embryos. In these experiments, knockout and wild-type mammary epithelial buds and mammary mesenchyme were recombined in all possible combinations (see Table 1) and grown under the kidney capsule of recipient females. We reasoned that if our hypothesis was valid, and PTHrP and the PTH/PTHrP receptor do represent an epithelial-mesenchymal signaling circuit , the PTH/PTHrP receptor-null phenotype would be expected to segregate with mesenchymal tissue. That is, receptor knockout mesenchyme should be unable to support the outgrowth of either receptor knockout or normal epithelial buds, but receptor knockout epithelium should be able to form ducts when combined with normal mesenchyme.

Table 1 and Fig. 6 summarize the results of these experiments. As expected, wild-type epithelial buds paired with wild-type mammary

mesenchyme (wt-MGE + wt-MGM) consistently gave rise to a series of branched epithelial ducts contained within a fatty stroma (see Fig. 6A). In contrast, ductal outgrowth was never detected when PTH/PTHrP receptor-knockout epithelial buds were paired with PTH/PTHrP receptor-knockout mesenchyme (KO-MGE + KO-MGM, see Fig. 6B). These transplants gave rise to a fatty stroma that was devoid of mammary epithelial cells, reproducing the phenotype of the PTH/PTHrP receptor-knockout embryos. Recombinations consisting of PTH/PTHrP receptor-knockout epithelium paired with wild-type mesenchyme (KO-MGE+wt-MGM) uniformly gave rise to branched epithelial ducts within a fatty stroma (Fig. 6C). However, although the receptor-knockout epithelial buds consistently grew out and formed a rudimentary branching ductal structure, the growth of the resulting ducts appeared stunted compared with the ducts produced by wild-type epithelial buds paired with wild-type mesenchyme. Nonetheless, the PTH/PTHrP receptor-knockout epithelial cells survived and had the capacity to initiate branching morphogenesis when paired with normal mesenchyme. As expected, similar to the results seen with knockout buds paired with knockout mesenchyme, all recombinants composed of wild-type epithelial buds paired with receptor-knockout mesenchyme (wt-MGE+KO-MGM) lacked any evidence of epithelial ductal outgrowth and consisted of fatty stroma alone (see Fig. 6D), suggesting that PTH/PTHrP receptor-knockout mesenchyme was unable to support the survival or morphogenesis of normal epithelial cells in this transplant system. These results demonstrate that the defects in mammary epithelial cell morphogenesis and survival seen in the PTH/PTHrP receptor-null mice segregate with mesenchymal tissue and demonstrate conclusively that the mesenchyme is a critical target for the actions of PTHrP during mammary ductal morphogenesis.

Now that we have conclusive proof that PTHrP participates in the epithelial-mesenchymal interactions that govern the regulation of branching morphogenesis, we are concentrating on developing systems in vitro with which we can study the mechanisms underlying PTHrP's effects. As noted in last years summary, we had attempted to study the effects of PTHrP on the ability of mammary epithelial cells to form branching colonies when co-cultured with mammary stromal cells in collagen gels. Our initial studies suggested that mammary epithelial cells harvested from K14-PTHrP overexpressing mice formed colonies with a 60-70% reduction in both their overall size and branching complexity. This mirrored the in vivo findings in the K14-PTHrP mammary glands. However, when we attempted to reproduce these findings by adding exogenous PTHrP to normal mammary epithelial cells co-cultured with stromal cells in collagen, we saw no such reduction. These results called the results with the transgenic cells into question. However, an alternative explanation is that the effects seen on the epithelial growth using the transgenic cells represented an intracellular effect of PTHrP directly on the epithelial cells, rather than an effect mediated by the stromal cells. This was an attractive hypothesis, since PTHrP has been shown to exert intracellular effects in several other systems. However, interpreting these results was made difficult by the degree of variability in the extent of branching growth shown by primary cultures of mammary epithelial cells in this system. Therefore, in order to pursue this question further we have decided to switch from the use of primary cultures of mammary epithelial cells to a cell line. We obtained the EpH4K6 cell line from

the Birchmeir laboratory in Germany. They have shown that these cells respond to HGF/SF and form branching tubules when grown in collagen gels (15). These cells do not express the PTH/PTHrP receptor and we therefore plan to grow them in co-culture with mammary stromal cells in the presence or absence of 10-7M PTHrP. If there is no effect of exogenous PTHrP on the ability of these cells to grow in collagen gels, then it would suggest that PTHrP does not effect branching morphogenesis in this system by interacting with mammary stromal cells. We also plan to create a cell line overexpressing PTHrP in order to see if intracellular PTHrP overexpression can interfere with the ability of these cells to exhibit branching morphogenesis in culture.

If PTHrP affects epithelial ductal branching morphogenesis by regulating stromal cell function, then there must be an effector signaling limb though which the stromal cells, in turn, act back on the epithelial cells. We have begun to investigate the effects of PTHrP on the stromal cell production of several stromally-derived growth factors that might serve as downstream agents of PTHrP's actions. In our original proposal, we had proposed that PTHrP might regulate the stroma's production of HGF/SF. Therefore, we began by examining the ability of PTHrP to regulate HGF mRNA levels in cultured mammary stromal cells by RNase Protection analysis. We were successful in assaying HGF mRNA in our cultured stromal cells, but we did not find any evidence that PTHrP treatment altered HGF message levels in these cells (Fig. 7). As outlined in the original proposal our next step will be to examine the effects of PTHrP on HGF signaling in myoepithelial cells. In addition, we also plan to examine PTHrP's effects on the expression of several other growth factors, such as KGF, IGF-1, and CSF-1, in cultured mammary stromal cells.

We are generally on time with respect to our Statement of Work as far as this technical objective is concerned. We performed several experiments that were not part of the original proposal this past year. We performed the collagen gel co-culture experiments as outlined in the original proposal, with the exception of examining the growth of PTHrP-null cells. Since the K14-PTHrP/PTHrP-null mice have no mammary glands, there is no source of PTHrP-null cells for these experiments. We will continue to pursue these experiments in the next year, using the EpH4K6 cell line as noted above. Finally, we have examined mammary stromal cells for changes in HGF expression in response to PTHrP as described in the original statement of work.

Technical Objective 3 - Examination of the effects of PTHrP on hormonally-induced proliferation in mammary epithelial cells.

We have not started these experiments yet and are thus slightly behind schedule. We plan to initiate these experiments this coming year.

Technical Objective 4 - The Effects of PTHrP on Mammary Tumor Formation in GR Mice.

Because PTHrP appears to inhibit the hormonally-stimulated proliferation of mammary epithelial ducts during normal development, we had hypothesized

that overexpression of PTHrP might inhibit the formation of hormone-dependent mammary tumors in mice. To investigate this possibility, as outlined in the original proposal, we crossed our K14-PTHrP transgenic mice to GR mice (16), and then examined the incidence of tumor formation after multiple pregnancies. We analyzed 11 GR/K14-PTHrP transgenic mice and 13 plain GR mice. After 3-5 pregnancies 9/11 (82%) GR/K14-PTHrP mice developed 19 tumors and 10/13 (77%) developed 24 tumors. Thus it appeared that overexpression of PTHrP did not inhibit tumor formation. In order to confirm that these tumors actually did overexpress PTHrP we analyzed tumors harvested from either GR/K14-PTHrP or plain GR mice for the presence of transgene mRNA by RNase protection analysis. The transgene contains human growth hormone sequences that are transcribed but not translated and which serve as a useful tag for transgene expression. As seen in Fig. 8, tumors from the GR/K14-PTHrP mice clearly are expressing the transgene. In addition, we cultured cells from either GR/K14-PTHrP or plain GR tumors and assayed for PTHrP production in conditioned media. Conditioned media from the GR/K14-PTHrP tumor cells contained 180 pM of intact PTHrP 1-74, while conditioned media from plain GR cells contained 10pM of intact PTHrP 1-74. Therefore, the tumors arising from GR/K14-PTHrP mice both expressed the transgene and overproduced PTHrP. We conclude then that overexpression of PTHrP does not inhibit tumor formation, at least in the GR mouse tumor model.

In addition to the simple generation of tumors we also analyzed these mice to see if PTHrP overexpression led to any differences in tumor histology. For this analysis, we examined 9 GR/K14-PTHrP tumors and 11 plain GR tumors. H&E-stained sections of each tumor were analyzed by a pathologist in a blinded fashion and classified based on the Dunn criteria (17). There was no difference in the degree of differentiation or histological grade of the tumors arising from GR/K14-PTHrP mice as compared to those arising in plain GR mice. We also examined these mice for evidence of bony metastases, as PTHrP expression has been implicated in the ability of human breast cancer to metastasize to the skeleton (18). Each mouse underwent whole body radiograph using the facilities of the Department of Pathology here at Yale. There was no evidence of bony metastases in any mice, nor was there evidence of macroscopic soft tissue metastases to lung, liver or brain.

Because there was no evidence of the inhibition of tumor formation in the first round of GR/K14-PTHrP mice, we have terminated these experiments. Therefore, we are on time in completing this technical objective, although the results were disappointing. However, we have decided to examine the effects of PTHrP overexpression on another mammary tumor system, treatment with DMBA. In collaboration with Dr. Archibald Perkins in the Department of Pathology at Yale we will be treating K14-PTHrP and normal littermate female mice with DMBA and observing them for the development of tumors. In normal mice, this system leads to the development of tumors that metastasize to soft tissues and thus may also allow us to investigate the effects of PTHrP overexpression on the development of bone metastases.

CONCLUSIONS

Our most exciting findings this past year again come from the study of the consequences of PTHrP gene ablation on embryonic mammary development. It appears that PTHrP is the epithelial signal that is responsible for inducing androgen receptor and tenascin C expression within the dense mammary mesenchyme. These results are interesting for they provide a molecular explanation for the long-standing observation that the epithelial bud was responsible for inducing mesenchymal androgen receptor expression. In addition, and perhaps more significantly, these data suggest that PTHrP is an important signal from the mammary bud involved in the cell fate decisions that lead ventral mesenchyme cells to become differentiated dense mammary mesenchyme cells, with the ability to direct proper epithelial morphogenesis.

We have also provided strong supporting evidence for the notion that PTHrP affects mammary stromal function in a way that affects epithelial branching morphogenesis both in embryos as well as during later stages of ductal development. First, we have shown that PTHrP is expressed in mammary epithelial cells and its receptor is expressed in stromal cells throughout mammary development. Interestingly, during adolescence, both genes are primarily expressed in terminal endbuds, the epicenters of epithelial proliferation and morphogenesis. Second in a series of tissue recombination and transplantation studies, we have demonstrated that mammary stromal cells must express the PTH/PTHrP receptor in order to support the outgrowth and morphogenesis of the mammary epithelium. These later observations prove that PTHrP acts to modulate stromal function in a way that is necessary for these cells to support branching morphogenesis. Over the next year the task will be to begin to define the mechanisms through which PTHrP enables the stroma to support epithelial morphogenesis.

Finally, we have tested the hypothesis that PTHrP acts as a growth inhibitor that might deter mammary tumor formation. This appears not to be true, at least with regards to the GR mouse model of mammary tumorogenesis. We are now looking at the ability of PTHrP to impact on chemical carcinogenesis. However, now that we know the results from the PTHrP-knockout studies, our original hypothesis was probably too simple. PTHrP clearly plays a role in the epithelial-mesenchymal interactions that regulate epithelial proliferation, survival and morphogenesis. However, it does so in a way in which both its underexpression and overexpression lead to growth inhibition. Therefore, although PTHrP most likely also plays a role in tumor-stromal interactions, it may not directly affect tumorigenesis or tumor cell proliferation.

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Table 1- Summary of Tissue Recombination Experiments

Mesenchyme	Epithelium	n	Stroma only	Ducts
WT	WT	4	0	4
Receptor-KO	Receptor-KO	4	4	0
WT	Receptor-KO	2	0	2
Receptor-KO	WT	3	3	0

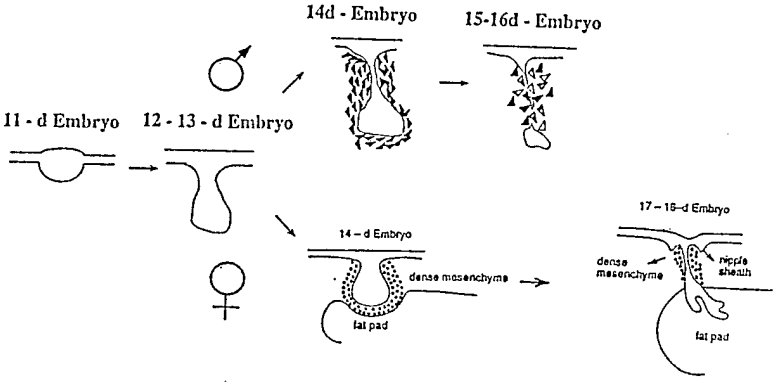


Figure 1. Outline of mammary development. See text for details. Triangles represent mesenchymal cells responding to androgens in male outline; circles represent mesenchymal cells in female outline.

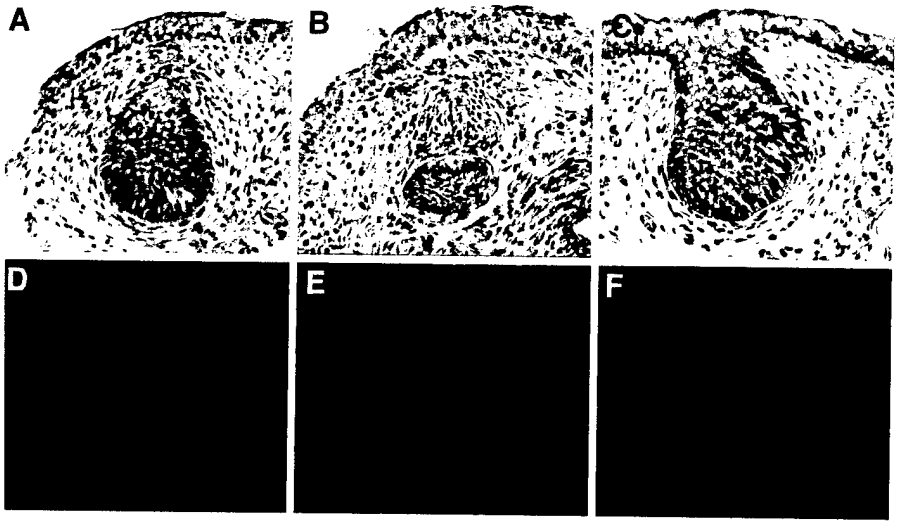
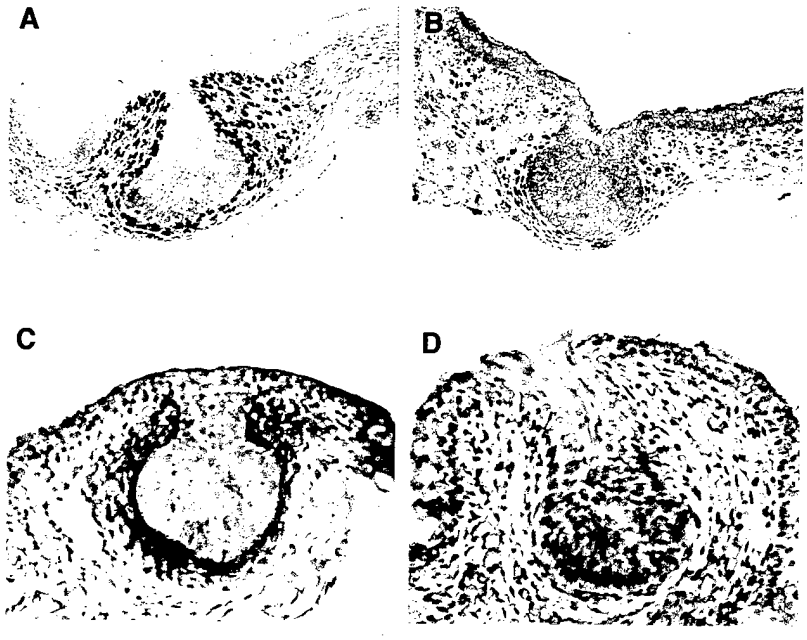


Figure 2. Histology of mammary buds in male wild-type and PTHrP knockout embryos at E15. A-C are H&E-stained sections. D-F are results of TUNEL assay for apoptotic cells (bright green nuclei). A&D are wild-type female buds, B&E are wild-type male buds, and C&F are PTHrP knockout male buds. Note that in the wild-type male buds the mesenchyme has condensed around the epithelial stalk, severing the connection to the epidermis (B). In addition, there is a great deal of apoptosis in the normal male mesenchyme and epithelium (E). In contrast, in the PTHrP knockout male buds, there is no condensation of the mesenchyme and there is no apoptosis (C&F). The knockout male buds are indistinguishable from normal female buds (compare A&D with C&F).

Figure 3. Expression of androgen receptor (A&B) and tenascin C (C&D) in wild-type (A&C) and PTHrP knockout (B&D) mammary buds at E15 by immunohistochemistry. Note that the androgen receptor is expressed in the nuclei of the dense mammary mesenchyme surrounding wild-type buds (A), but that there is virtually no androgen receptor staining within the mesenchymal cells surrounding the knockout buds (B). Likewise, tenascin is located within the extracellular matrix of the mesenchyme in the wild-type (C) bud, but not within the matrix surrounding the knockout bud (D).



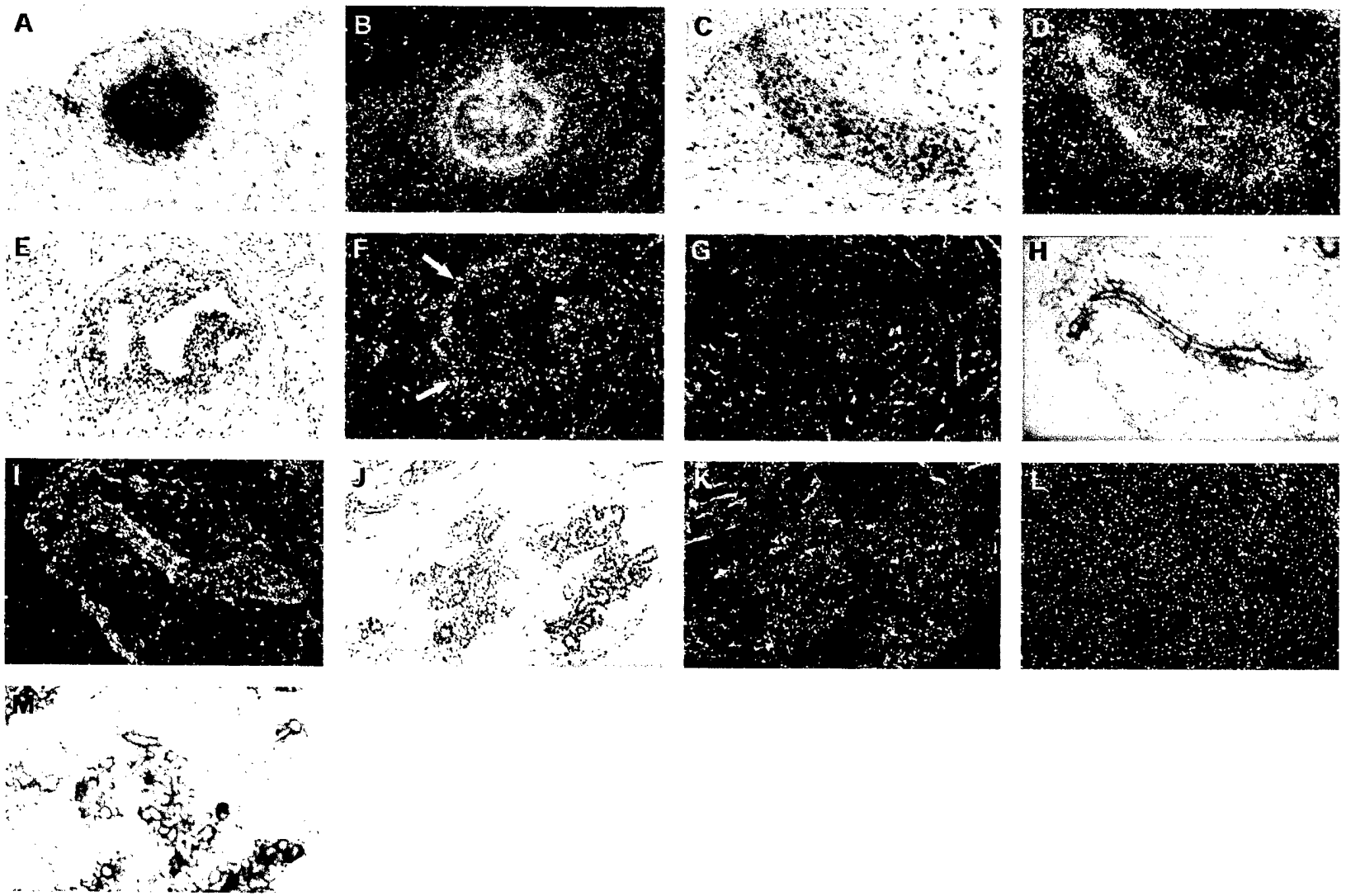


Figure 4. Localization of PTHrP mRNA expression in mammary glands of embryonic, adolescent, and pregnant mice. A-D. In situ hybridization for PTHrP mRNA in embryonic mammary rudiments at E12 (A&B) and E18 (C&D). A&C represent brightfield images and B&D represent darkfield images, respectively, of the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

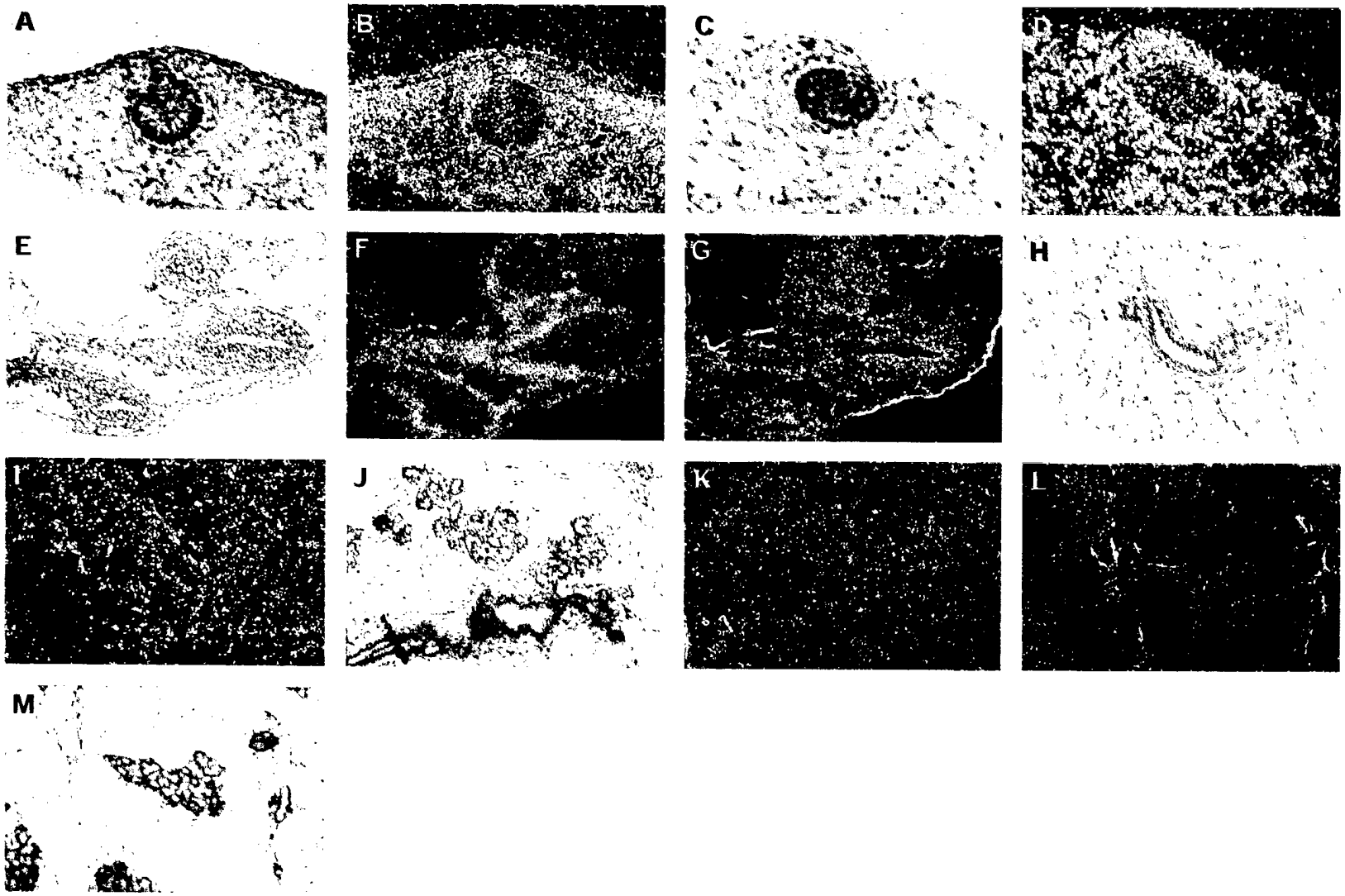


Figure 5. Localization of PTH/PTHrP receptor mRNA during mammary development. In situ hybridization for PTH/PTHrP receptor mRNA in embryonic mammary rudiments at E12 (A&B) and E18 (C&D). A&C represent brightfield images and B&D represent darkfield images of the same sections hybridized with antisense probe. E-I. In situ hybridization for PTH/PTHrP receptor mRNA in the adolescent mammary gland. E&F represent brightfield and darkfield images, respectively, of a section through an end bud of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H&I represent brightfield and darkfield images, respectively, of a section through a mature duct of a mammary gland from an adolescent (4 week old virgin) mouse hybridized with an antisense probe. J-M. In situ hybridization for PTH/PTHrP receptor mRNA in the pregnant mammary gland. J&K represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days post coitus) mouse hybridized with an antisense probe. L&M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

Figure 6. Mesenchymal PTH/PTHrP receptor is necessary for the initiation of epithelial outgrowth.

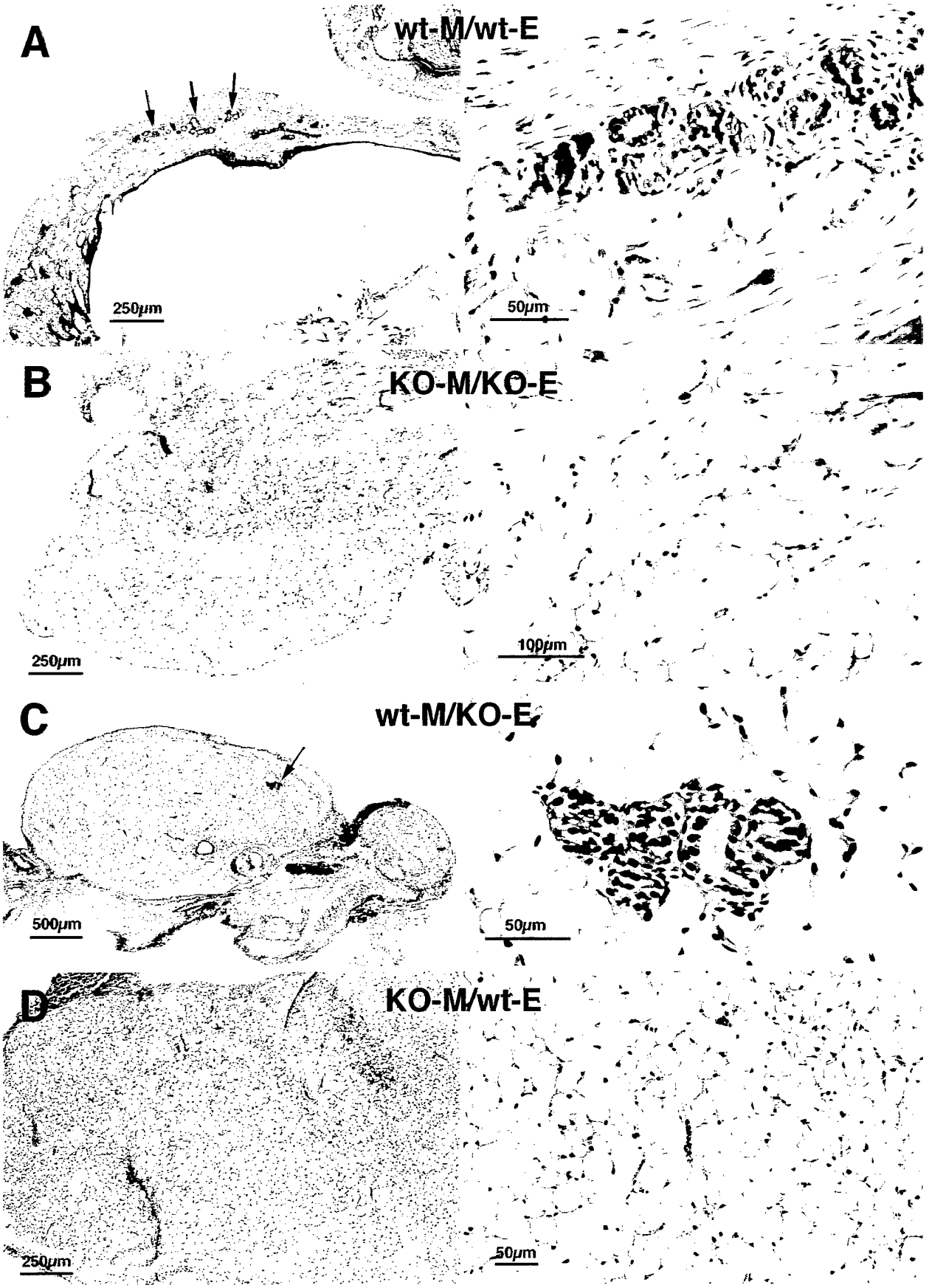


Figure 6. Mesenchymal PTH/PTHrP receptor is necessary for the initiation of epithelial outgrowth. Mammary buds were dissected from wild-type and PTH/PTHrP receptor knockout embryos at E13, and the epithelium and mesenchyme were separated and then recombined in the four possible combinations and grown under the kidney capsule of recipient female mice for one month. Shown here are H&E-stained sections through the resultant transplants after they were removed from beneath the kidney capsule. The left side of the figure represents low-power views and the right side represents high-power magnifications of the same sections displayed on the left. Each transplant consists of a fragment of connective tissue containing varying amounts of fatty stroma, fibrous stroma, epidermal structures and mammary epithelium. A. Representative transplant resulting from wild-type epithelium recombined with wild-type mesenchyme (wt-MGE + wt-MGM). Note the mammary epithelial ducts (higher power view on right) located within a mixture of fibrous and fatty stroma. The structures at the lower left in the left-sided panel are hair follicles. B. Representative transplant resulting from PTH/PTHrP receptor-knockout epithelium (KO-MGE) paired with PTH/PTHrP receptor-knockout mesenchyme (KO-MGM). Note that there are no epithelial ducts in this section, only fatty stroma. C. Representative transplant resulting from wild-type mesenchyme (wt-MGM) paired with PTH/PTHrP receptor-knockout epithelium (KO-MGE). Note that epithelial ducts are present within the stroma (higher magnification on right), but that there are fewer ducts than in A. D. Representative transplant resulting from knockout mesenchyme (KO-MGM) paired with wild-type epithelium (wt-MGE). As in B, note the complete absence of epithelial ducts.

The arrows in A & C indicate mammary epithelial ducts. The scale bars in each panel demonstrate magnification as labeled.

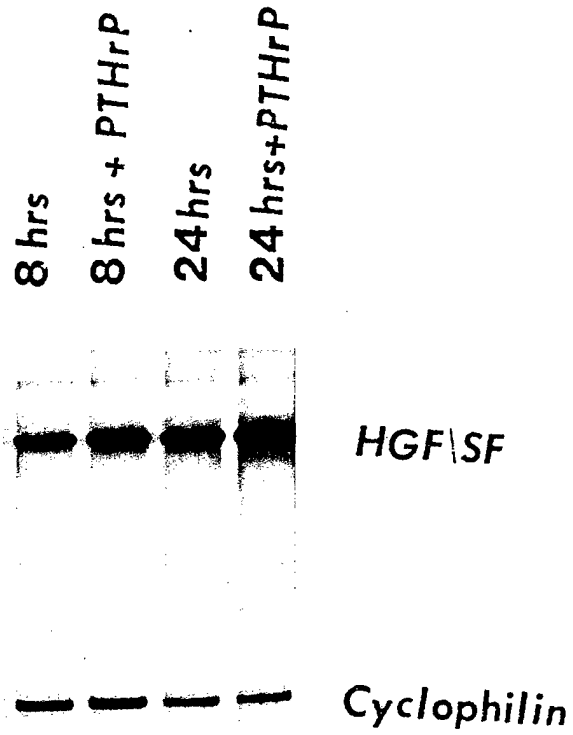


Figure 7. Effects of PTHrP on the expression of HGF/SF mRNA in mammary stromal cells. Mammary stromal cells were grown in phenol red free media containing 10% charcoal stripped serum for 5 days. The cells were then treated with either serum free media or serum free media containing 10^{-7} M PTHrP for 8 or 24 hours. 20 µg of total cellular RNA was then assayed for HGF/SF mRNA by RNase protection. Note that HGF/SF mRNA levels do not significantly change in response to PTHrP in mammary stromal cells.

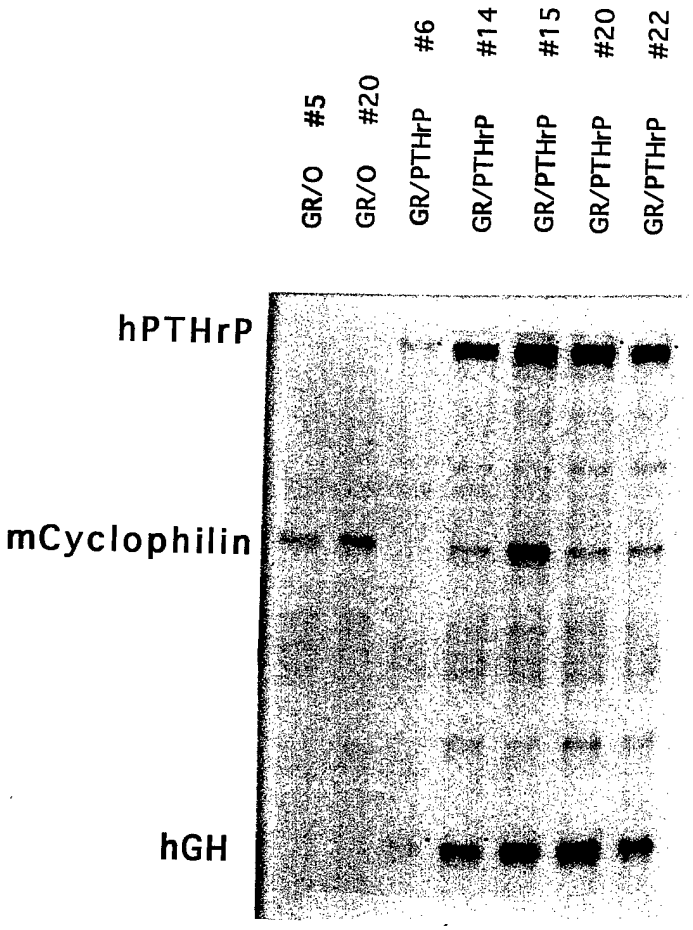


Figure 8. K14-PTHrP Transgene Expression in Mammary Tumors. RNase Protection analysis of 20 mcg of total cellular RNA prepared from 2 nontransgenic GR mice (GR/O) and 5 K14-PTHrP transgenic GR mice (GR/PTHrP) as indicated above the lanes. In this experiment three separate probes were used simultaneously: a.) a human PTHrP probe (hPTHrP), b.) a mouse cyclophilin probe (mCyclophilin), and c.) a human growth hormone probe (hGH). Cyclophilin serves as a loading control, while the human PTHrP and human growth hormone probes are specific indicators of K14-PTHrP transgene expression. Note that in all 5 K14-PTHrP transgenic GR mice, the transgene is expressed at high levels within the tumor tissue. In contrast, as expected, the transgene is not expressed in tumors from the non-transgenic GR mice. Therefore, the K14 promoter reliably targets PTHrP overexpression to mammary tumors.