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Award Number: DAMD17-01-1-0201

TITLE: The Influence of Stromal Transforming Growth Factor-β Receptor Signaling on Mouse Mammary Neoplasia

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REPORT DATE: August 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual Summary (1 Aug 01 - 31 Jul 04)

4. TITLE AND SUBTITLE
The Influence of Stromal Transforming Growth Factor-β Receptor Signaling on Mouse Mammary Neoplasia

5. FUNDING NUMBERS
DAMD17-01-1-0201

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this proposal was to determine if loss of TGF-β signaling in the stroma affects tumor development and to identify TGF-β regulated genes via custom gene chip microarrays. Our laboratory had developed mice transgenic for a dominant-negative TGF-β type II receptor driven by the metallothionine promoter whose expression was restricted to the mammary stroma. Transgenic and wild type mice were given pituitary isografts, zinc water and either left untreated or treated with 7,12-dimethylbenz-(a)-anthracene (DMBA). Mice treated with carcinogen did not differ in latency, tumors per mouse, growth rate, or tumor free survival. They did differ with respect to histology, however. Mice not treated with carcinogen had a significantly shorter tumor free survival in addition to differences in tumor type. Mammary gland specific custom gene chip microarrays were produced and screened for genes regulated by TGF-β. Several genes were identified following microarray analysis yet only approximately 15% were verified to be regulated by TGF-β. One gene, the platelet derived growth factor receptor alpha (PDGFRα), was analyzed in detail. The PDGFRα was up-regulated in the mammary glands while down-regulated in the mammary fibroblasts treated with TGF-β. PDGFRα did not regulate branching morphogenesis as PDGF pellets implanted into the mammary gland had no effect.

14. SUBJECT TERMS
Mammary gland, tumorigenesis, microarray, gene chips, mouse

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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The transforming growth factor betas (TGF-β1, -β2, and -β3) are members of a family of peptide growth factors that include inhibins, bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs) (1-3). The TGF-βs signal through a family of hetereodimeric serine threonine receptor kinases that ultimately result in changes in gene expression. The TGF-βs and their receptors are expressed throughout the mammary gland where they have a negative effect on the growth of the ductal epithelium and lobuloalveolar development depending on the timing of TGF-β expression. Utilizing antibodies that can discriminate between the active and latent forms of TGF-β1 Ewan et al., (2002) showed intense active TGF-β1 staining in the TEBs with more moderate levels in the subtending ducts. The latent form of TGF-β1 was observed at high levels throughout the epithelium and the fibrous stroma (4). TGF-β signaling in the stroma has been shown to be necessary for proper ductal development in the mammary gland (5). Expression of a kinase-deficient dominant-negative TGF-β type II receptor (DNIIR) in the mammary epithelium and in stromal fibroblasts resulted in precocious lobuloalveolar development and increased lateral branching, respectively, compared to wild type littermates (5,6). In mice, several studies support TGF-β signaling as a tumor suppressor for the mammary gland. TGF-β expression inhibited tumor formation when mice expressing an epithelial TGF-β transgene were challenged with 7,12-dimethybenz (a) anthracene (DMBA) (7). In a separate experiment, DMBA treatment of mice expressing the DNIIR transgene in the mammary epithelium, eliminating TGF-β signaling, increased the incidence and number of tumors (8). More recently, expression of a DNIIR transgene directed to the mammary epithelium resulted in spontaneous tumor development, albeit after and extended latency (27.5 months) (9). Moreover, targeted elimination of the TGF-β type II receptor in the mouse prostate stromal fibroblasts resulted in the appearance of intraepithelial neoplasia (10). These results suggest that TGF-β signaling in the stroma and epithelium is necessary for normal homeostasis.

The purpose of this proposal was to test the overall hypothesis that development of mammary tumors will be altered in the absence of TGF-β receptor signaling in the mammary stroma. It was also hypothesized that there were changes in gene expression resulting from the lack of TGF-β signaling in the mammary stroma that would ultimately influence tumor development. To test these hypotheses Serra and colleagues (1999) have engineered two transgenic mouse lines (MTR4 and MTR28) that expressed a kinase-defective dominant-negative TGF-β type II receptor driven by a metallothionine promoter (MT-DNIIR), which allows for regulation of the transgene by heavy metals (e.g. zinc sulfate) (6). Assessing what role, if any, the lack of TGF-β receptor signaling in the mammary stroma affects mammary neoplasia will help to identify novel diagnostic or therapeutic strategies heretofore unexplored.

Previously, Gorska et al., (1999) showed through in situ hybridization experiments that expression of the DNIIR transgene under the regulation of the metallothionine gene promoter was restricted to the mammary stroma. Expression in the mammary stroma resulted in an increase in ductal side branching in non-parous animals. To further test the importance of stromal TGF-β signaling in the normal development of the mammary gland we performed reciprocal transplantation experiments. Epithelium from wild type mice was transplanted into the cleared fat pads of transgenic mice (both MTR4 and MTR28) and epithelium from transgenic mice transplanted into the cleared fat pads of wild type mice. Following the transplantations all the mice were placed on zinc to stimulate the transgene, for the transgenic

mice, or to control for the effects of zinc sulfate on the mammary gland, for control mice. The epithelium from the wild type mice transplanted into the transgenic fat pad resulted in excess ductal branching, similar to that of the transgenic mice. Epithelium from transgenic mice transplanted into the wild type fat pad did not develop excess branching while on zinc sulfate. These results suggest that the excess branching seen in the non-parous mammary gland in the transgenic mice was a result of lack of TGF-β signaling in the mammary stroma and not from epithelial expression of the transgene (Fig. 1). Next, we tested the theory that epithelial TGF-β was necessary for proper ductal development during puberty. It has been suggested that TGF-β regulates pubertal mammary development through the epithelium and that stromal TGF-β signaling was not required (11). MTR4, MTR28 and wild type mice were placed on zinc sulfate at 3 weeks of age and their mammary glands removed and analyzed at 5 weeks and 7 weeks of age. The mammary ductal tree from the transgenic mice had progressed to fill approximately 60% of the mammary fat pad by 5 weeks and 100% of the fat pad by 7 weeks. In contrast the ductal epithelium from the wild type mice filled roughly 25% of the fat pad at 5 weeks and only 60% by 7 weeks (Fig. 2 and Table 1). These results, in addition to the transplantation studies, suggest that TGF-β signaling through the mammary stroma was required for regulation of ductal development both during puberty and in the adult.

As stated in Task 1 of the Statement of Work (S.O.W.), four groups of mice were established for tumor studies. The groups consisted of wild type mice given pituitary isografts and zinc-water, wild type mice with pituitary isografts, zinc-water and carcinogen, MTR28 transgenic mice with pituitary isografts and zinc-water and MTR28 transgenics with pituitary isografts, zinc-water and carcinogen. When female mice were 5 weeks of age pituitaries from sibling male mice were removed and implanted under the kidney capsule of the recipient female. The mice were placed on zinc-water at 6 weeks of age to induce the transgene or to control for the effects of zinc, if any, on gene expression. The mice were then either treated weekly for 4 weeks with 1mg per week of 7,12-dimethybenz (a) anthracene (DMBA) beginning at 8 weeks of age or not treated with the carcinogen. As stated previously, there were no statistically significant differences between the transgenic group and the wild type group with respect to latency, tumor growth, average number of tumors per mouse and tumor free survival. However, the tumors did differ in histological type with adenocarcinoma the most common tumor found in wild type mice (6 of 14 tumors) while differentiated squamous carcinoma was the most common tumor in the transgenic mice (7 of 17) (See Table 2). Interestingly, there were no adenomyoepitheliomas detected in the transgenic group treated with DMBA yet 3 of 14 tumors in the wild type group were adenomyoepitheliomas (Table 3). In situ hybridization analysis of the tumors for transgene expression showed that in the transgenic mice on zinc the DNIIR transgene was expressed in both the stromal compartment and in the tumor epithelium (Fig 3). It was unclear if transgene expression in the tumors occurred before, during or after tumorigenesis.

In addition to carcinogen treatment, wild type (n=59) and transgenic mice (n=32) were given pituitary isografts under the kidney capsule and zinc sulfate only. Unlike the carcinogen treated group, there was a statistically significant difference in tumor free survival between wild type and transgenic mice. Specifically, the transgenic mice had a decreased tumor free survival time compared to the wild type mice (Fig. 4). The tumors did not differ in latency, tumor growth or number of tumors per mouse. Histological analysis revealed the wild type tumors were all squamous metaplasia while the tumors from the transgenic mice

were all papillary adenocarcinoma with areas of necrosis (Fig.5). These data show that while TGF-β signaling in the mammary stroma does not affect development of the tumors, it does influence the type of tumors that develop. Molecular analysis of the tumors is currently under way. For example, proteins isolated from the various DMBA induced tumors were analyzed for increased c-met phoshorylation as seen in prostate tumors (10). We observed no difference in the induction of c-met phosphorylation between the tumors from wild type and transgenic mice (data not shown).

Task 2 of the statement of work outlined the production and screening of mammary gland specific gene chips to identify genes that were regulated between the wild type and transgenic mice. The mammary gland specific gene chips were hybridized in triplicate with cDNA synthesized using RNA isolated from wild type and DNIIR transgenic mammary glands treated with zinc sulfate for 1 week. In total, 1,921 duplicate spots were recognized on the gene chips with approximately 1,000 spots listed as present on all 3 experiments. The standard error for each spot was calculated based on replicates within the array and two types of normalization were used to standardize expression levels. After normalization, 399 spots were determined to be regulated with ratios either 2 fold above the mean or 0.5 fold below the mean. Since it's not possible to study the regulation of 400 genes in detail we chose several for an initial verification to further refine our list of genes to analyze. Currently we have analyzed 34 genes for expression in the mammary gland using semiquantitative RT-PCR and real time RT-PCR. The sequences assayed represented a number of diverse biological pathways including signal transduction (PDGFR, ErbB3 receptor, Wnt5a), RNA splicing (SnrpE, SnrpG), DNA polymerization (PCNA, DNA Polymerase-δ), cell cycle regulation (cyclin D) and several others (data not shown). Of the many genes regulated in the mammary gland as determined by real-time PCR we analyzed the possible role of the PDGFRα in branching morphogenesis in further detail. The PDGFRα has been found expressed in stromal fibroblasts while production of its ligand, PDGF-AA, was epithelial (12). Mammary glands isolated from transgenic mice treated with zinc sulfate had a 2.1 fold increase in PDGFRα mRNA compared to wild type mice (Table 4). In contrast, when primary mammary fibroblasts were treated with TGF-β in culture there was a 2.4 fold reduction in PDGFRα levels (Table 4). The changes in gene expression observed in the mammary glands and the cell cultures were also seen in levels of protein expression by western analysis (Fig. 6A). Immunohistochemical analysis of the PDGFRα protein in the mammary gland revealed expression in the stromal compartment with an increase in expression observed in mammary glands from transgenic mice compared to wild type mice (Fig. 6B). To assess what role, if any, of PDGFR signaling in the mammary stroma has on branching morphogenesis we implanted PDGF-AA containing Elvax pellets into one number 4 inguinal mammary gland of wild type control mice and BSA impregnated pellets into the contralateral gland as a control. Mammary glands exposed to PDGF-AA and BSA were removed and analyzed for histology and smooth muscle actin immunofluorescence after 6 days (n=3) and whole mount analysis after 12 days (n=3). Ductal development was similar between the mammary glands with the BSA implants compared to the PDGF-AA implants, suggesting PDGF was not affecting branching morphogenesis (Fig. 7A and B). Previously, we found an increase in the amount of stromal matrix by Masson's trichrome stain in the mammary glands from transgenic mice when compared to control mice. To test if PDGF has an effect on the increase in collagen fibers seen in the transgenic mice, mammary glands with PDGF-AA and BSA implants were stained with Masson's trichrome. There was no difference in trichrome staining between

mammary glands with BSA pellets compared to mammary glands with PDGF pellets (Fig 7C and D). Staining of the mammary glands with an anti-smooth muscle actin antibody showed a possible increase in vasculature around the implanted PDGF pellets compare to the control pellets (Fig. 7E and F). These results suggest that TGF-β regulated PDGFRα at the transcriptional level but PDGF signaling had little, if any, discernable affect on branching morphogenesis in the adult mammary gland.

Currently, we are analyzing several more genes for their regulation in the DNIIR expressing mammary glands compared to wild type. Many of the genes analyzed from the micro arrays have not verified upon further inspection suggesting expression of many genes has to be examined to identify those that are regulated by TGF-β. Indeed, less than 15% (5 of 37) of the genes assayed were found to be regulated in the mammary glands from the transgenic mice compared to the wild type mice. This process has been slow and cumbersome, however it has recently been simplified by the use of reliable quantitative real time PCR. A more direct and pertinent method of examining the differences between mammary glands from wild type and transgenic mice or cells in culture may be two dimensional gel electrophoresis with proteins isolated from the different tissues. Alterations in protein expression and the modification of proteins, i.e. phosphorylation (which may be more important than overall protein levels) could be analyzed directly. In the future 2D gel experiments may be more direct and possibly reduce the false positive rate experienced with the use of gene chip microarrays.

List of Key Accomplishments

Task 1. Induce mammary tumors in MT-DNIIR (MTR28) and wild type mice. Months 1-24.

- Mice given pituitary isografts, zinc and carcinogen did not have differences in:
  - Tumor free survival
  - Tumor frequency
  - Tumor size
  - Tumor latency
- Tumors from this group did vary with respect to histology
  - Adenosquamous carcinoma most common in wild type mice
  - Differentiated squamous carcinoma most common in transgenic mice.
- Mice give pituitary isografts and zinc only did have a difference in:
  - Tumor free survival
  - Tumor frequency
- Mice in this group did not have differences in:
  - Tumor size
  - Tumor latency
- Tumors from this group had histological differences.
  - Squamous metaplasia was dominant in wild type mice.
  - Papillary adenocarcinoma was dominant in the transgenic mice.

Task 2. Produce and screen tissue specific microarrays. Months 18-36.

- Expression of approximately 30% of the genes identified through filter based arrays and commercial gene chips have been analyzed.
- Mammary gland specific microarrays have been constructed and screened.
- Approximately 40% of the genes were regulated
- Several of these genes have been assessed for expression in the mammary gland and in mammary fibroblasts in culture.
- This screening identified a potentially important signaling pathway, the PDGFR pathway.
- PDGFRα was found regulated by TGF-β yet it had no discernable effect on branching morphogenesis in the mammary gland.
- Several other genes have been analyzed by real time RT-PCR and have been found regulated in MT-DNIIR expressing mammary glands.

List of Reportable Outcomes.

M. Crowley, D. Bowtell and R. Serra, 2004. TGF-β, Cbl and PDGFR-alpha in the mammary stroma. Developmental Biology, Submitted.

R. Serra and M. Crowley. 2004. TGF-β in mammary gland development and breast cancer. Breast Disease 18:61-73

Presented abstract "Stromal Regulation of Ductal Side Branching in the Mammary Gland: The TGF-β-Cbl Connection" at the 2003 Gordon Conference on Mammary Gland Biology.

Presented abstract "Transforming Growth Factor-β in the Stroma Influences Gene Expression and Development in the Mouse Mammary Gland" at the 2003 Gordon Conference on Mammary Gland Biology

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APPENDIX

Table 1. The percentage of fat pad filled by the epithelium in wild type, DNIIR transgenic, Cbl-null and Cbl+/- mice.

Mouse	Epithelium cm	Fat Pad cm	Percent Filled
WT 5wks[a]	0.48 ± 0.13	1.9 ± 0.14	25.3
DNIIR 5wks[b]	1.06 ± 0.2	1.7 ± 0.37	62.4
Cbl$^{-/-}$ 5wks[c]	0.99 ± 0.17	2.04 ± 0.33	48.5
Cbl$^{+/-}$ 5wks[d]	0.92 ± 0.27	2.06 ± 0.30	45.1
WT 7wks[e]	1.42 ± 0.32	2.2 ± 0.06	64.5
DNIIR 7wks[f]	2.1 ± 0.10	2.1 ± 0.01	100.0

a. n=8
b. n=7
c. n=5
d. n=10
e. n=3
f. n=3

Table 2. DMBA-induced mammary tumors in wild type mice.

Mouse no.	Diagnosis
1	Squamous carcinoma
2	Adenosquamous carcinoma
2F	Intraductal squamous metaplasia
3	Squamous carcinoma
4a	Adenosquamous carcinoma
4b	Squamous cyst
5	Squamous carcinoma
6a	Adenocarcinoma, solid
6b	Adenomyoepithelial tumor, invasive
7	Squamous carcinoma
8	Adenosquamous carcinoma
9	Intraductal squamous metaplasia
10a	Adenocarcinoma, acinar
10aF2	Adenomyoepithelial tumor, invasive
10aF3	Intraductal squamous metaplasia
10b	Adenomyoepithelial tumor, invasive
10c	Intraductal squamous metaplasia
10d	Squamous carcinoma
11a	Adenomyoepithelial tumor, invasive
11b	Adenomyoepithelial tumor, invasive
11c	Intraductal squamous metaplasia
12	Squamous carcinoma
13	Adenosquamous carcinoma
14a	Adenosquamous carcinoma
14b	Adenosquamous carcinoma
14bF2	Adenosquamous carcinoma
14bF3	Intraductal squamous metaplasia
14bF4	Squamous carcinoma

Fourteen mice developed 21 tumors. Four tumors had multiple foci.
F= foci, a,b,c,d= separate tumors in one mouse.

Table 3. DMBA-induced mammary tumors in MT-DNIIR-28 mice.

Mouse no.	Diagnosis
1	Squamous carcinoma
2	Intraductal squamous metaplasia
3	Adenosquamous carcinoma
4a	Squamous carcinoma
4b	Squamous carcinoma
5	Squamous cyst
6	Sarcoma, bone metastasis
7a	Adenosquamous carcinoma
7b	Squamous carcinoma
8a	Squamous carcinoma
8aF	Squamous carcinoma
8b	Squamous carcinoma
9a	Adenosquamous carcinoma
9b	Intraductal squamous metaplasia
9c	Squamous carcinoma
10	Squamous carcinoma
11	Squamous cyst
12	Intraductal squamous metaplasia
13	Adenocarcinoma, papilary
14	Adenocarcinoma, papilary
15	Adenocarcinoma, papilary
16	Adenosquamous carcinoma
17	Squamous cyst

Seventeen mice developed 22 tumors. One tumor was mutifocal.
F=foci, a,b,c= separate tumors in one mouse.

Table 4. Real time RT-PCR measurements of PDGFRα mRNA.

	Fold Difference	**P Value**
MTR4 Mammary Gland (3 wk on Zn)	+2.36*	0.001
MTR4 Mammary Gland (2 wk on Zn)	+1.77*	0.001
Fibroblast (Treated with TGF-β1)	-2.40**	0.001

* relative to WT, ** relative to untreated.

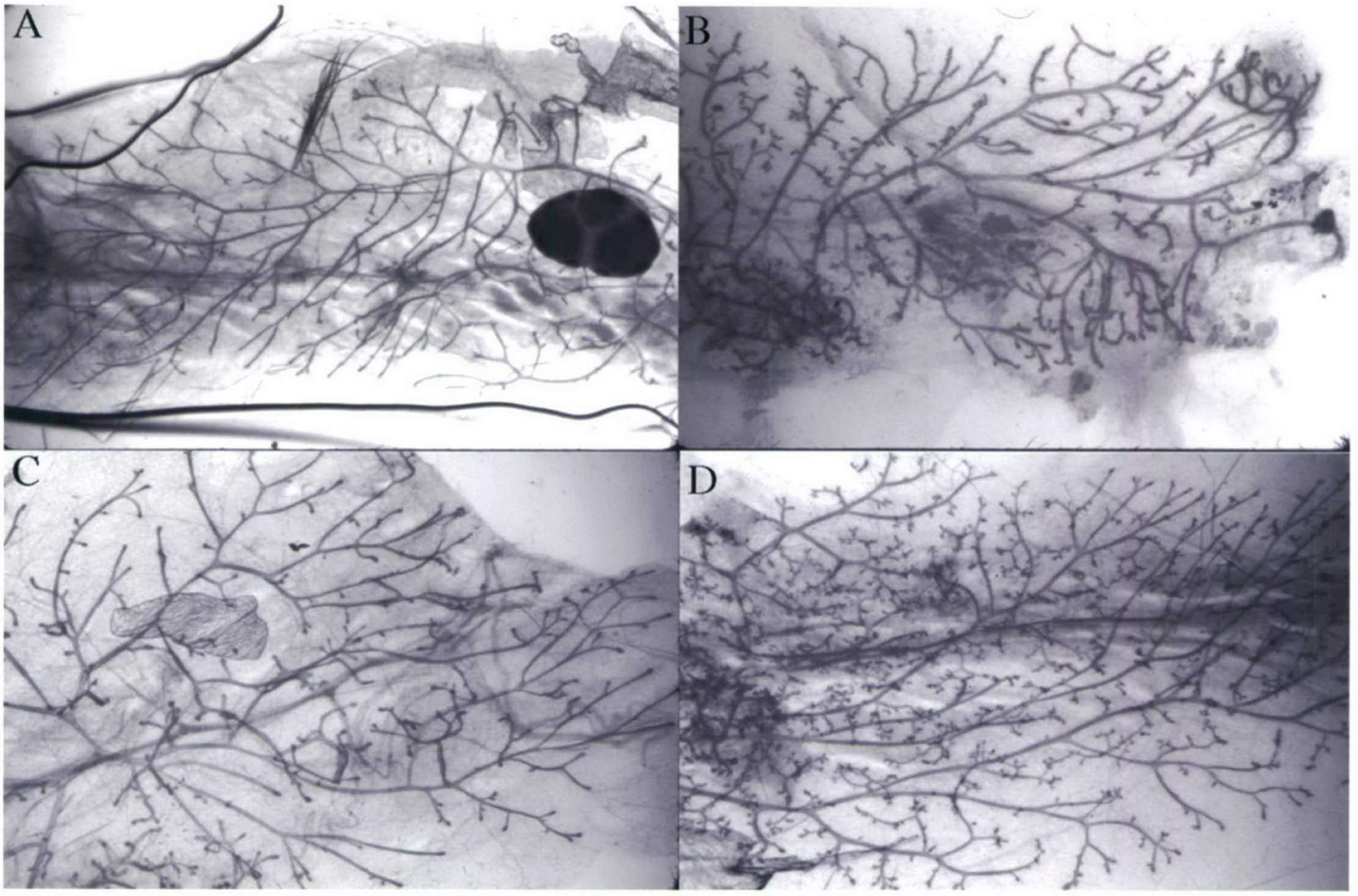


Figure 1. Recipricol mammary gland transplantions in MTR4 and wile type mice. A. Mammary epithelium from a wild type donor was placed into the cleared fat pad of a wild type recipient as a control for the transplantation techinque. B. Epithelium from a MTR4 donor mouse was transplanted into the cleared fat pad of a MTR4 recipient as a control. C. Epithelium from a MTR4 donor mouse was transplanted into the cleared fat pad of a wild type recipient mouse. Notice the normal development of the ductal tree (compare to D). D. Mammary epithelium from a wild type mouse was transplanted into the cleared fat pad of a MTR4 recipient. This resulted in an increase in ductal side branching. All mice were placed on zinc sulfate for 4 weeks post surgery (n=4).

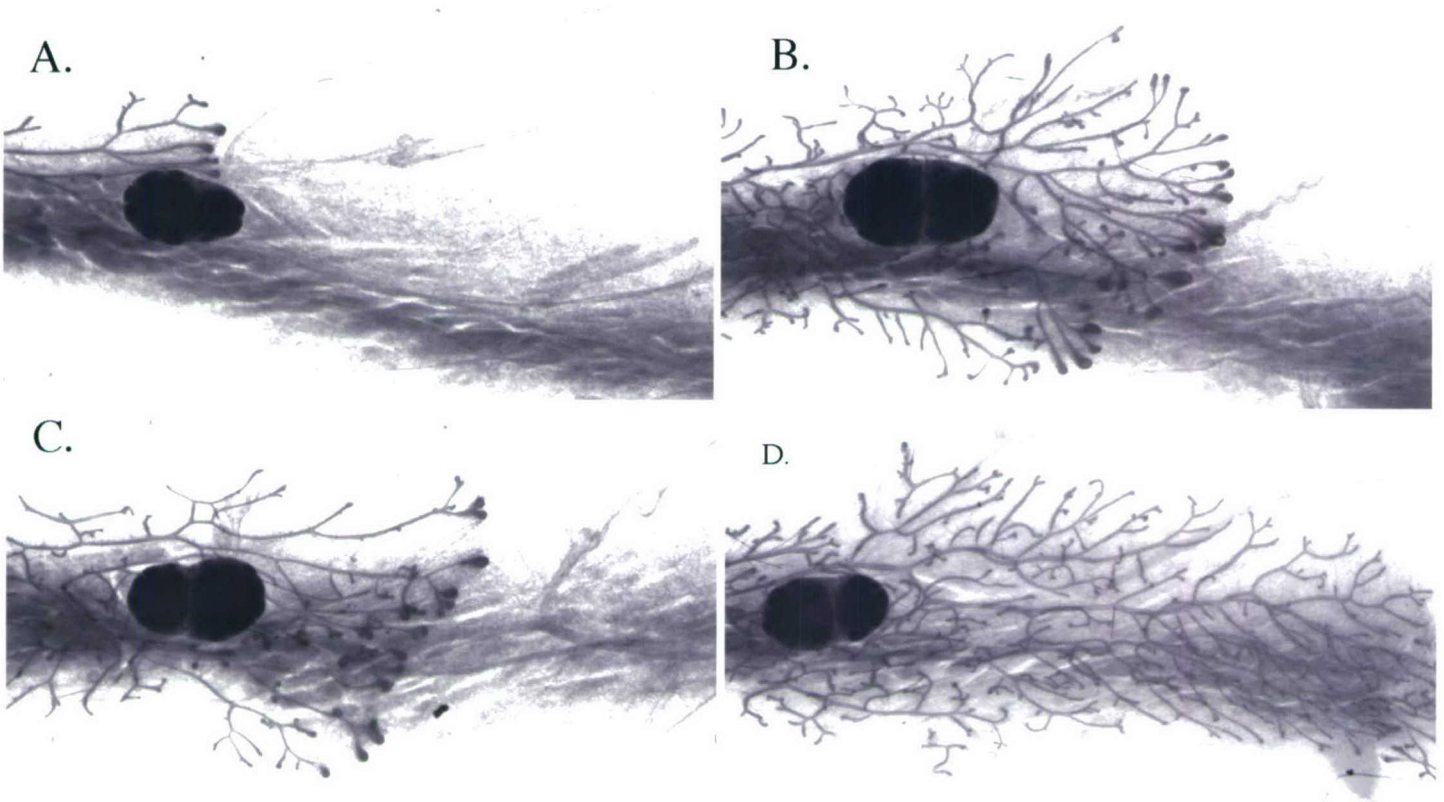


Figure 2. Pubertal development of the mammary gland in wild type and transgenic mice. Mammary glands from 5 week old wild type (A) and MTR4 transgenic (B) mice treated with zinc sulfate for two weeks. Mammary glands from 7 week old wild type (C) and MTR4 transgenic mice (D) treated with zinc sulfate for 4 weeks (n=3 for each condition)

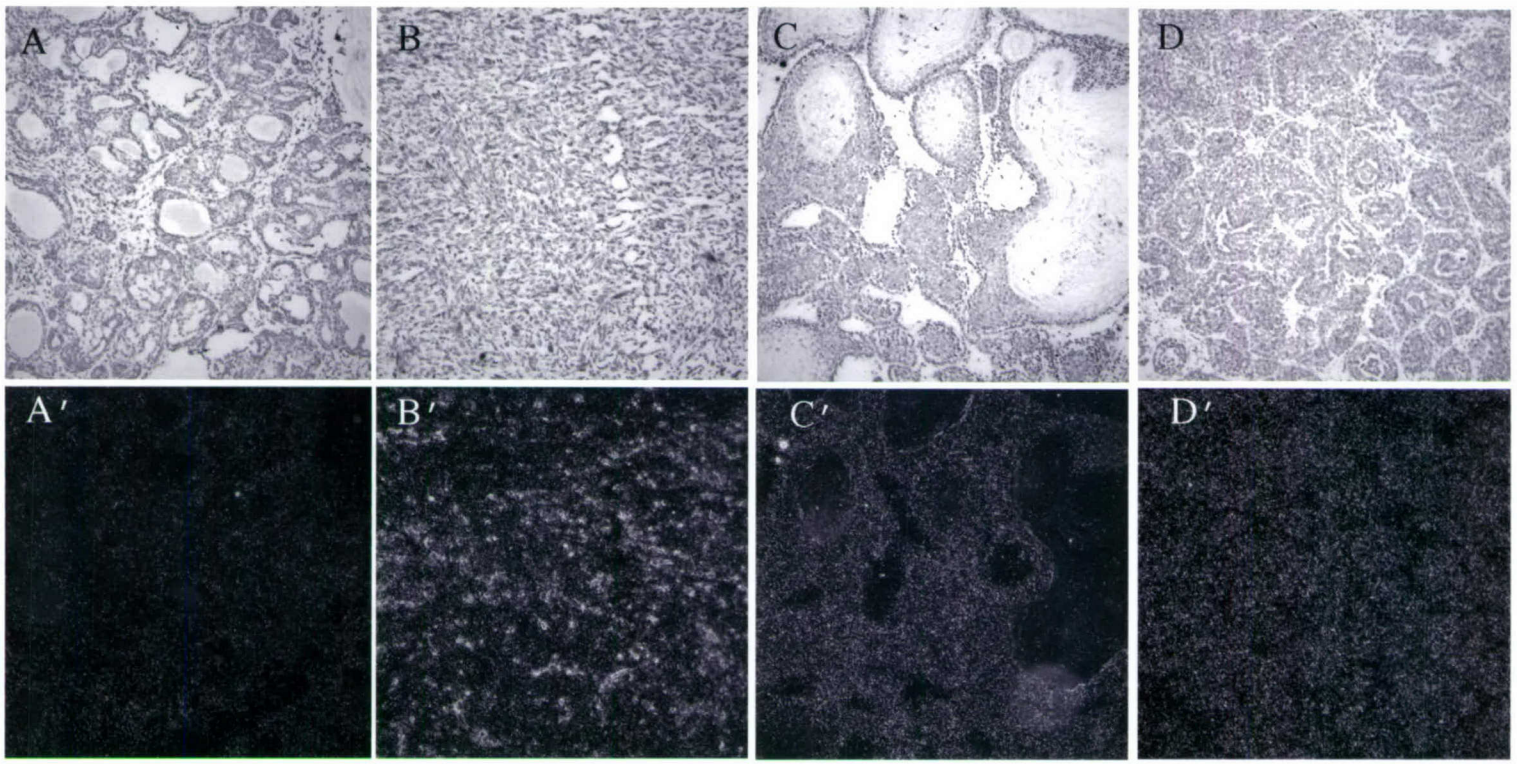
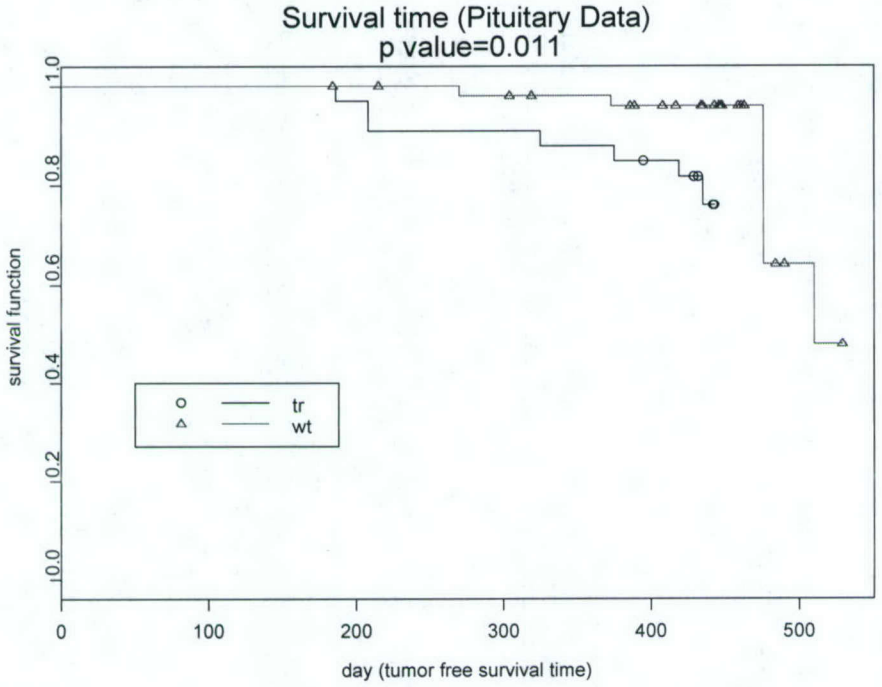


Figure 3. In situ hybridization for expression of the dominant-negative TGF-β type II receptor in mammary tumors . A-D. Bright field images of mammary tumors that arose in wild type mice (A) and transgenic mice (B-D) treated with carcinogen. A'-D'. Dark field images of the same tumors showing background levels (A') and expression of the DNIIR transgene (B'-D') in both the stroma and epithelial compartments of the mammary tumors. The tumor in A represents an adenosquamous carcinoma from the wild type mice treated with carcinogen, B represents a sarcoma, C shows an adenosquamous carcinoma and D depicts a representitive squamous carcinoma. Magnification was 10X.

Figure 4. The survival analysis shows statistically significant for the tumor free survival time between treated and wild type groups. Specifically, the wild type group has a longer survival time.



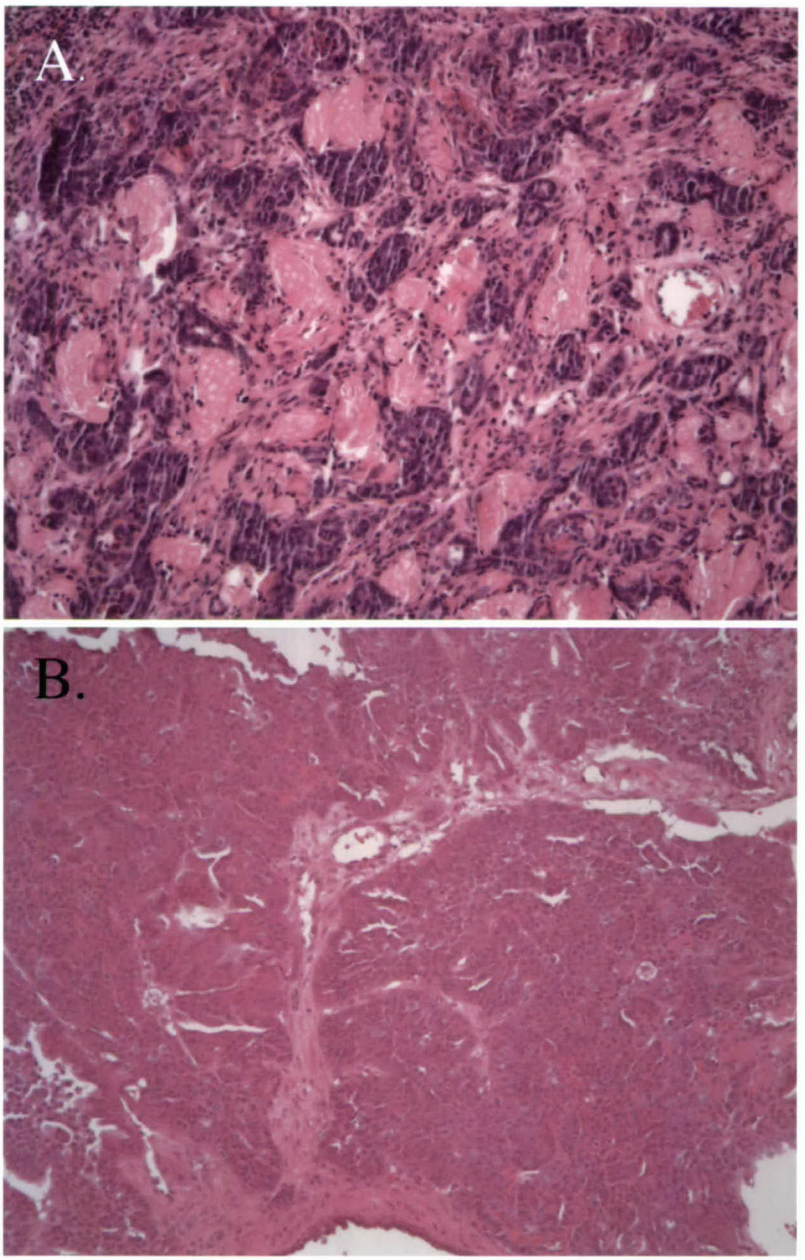
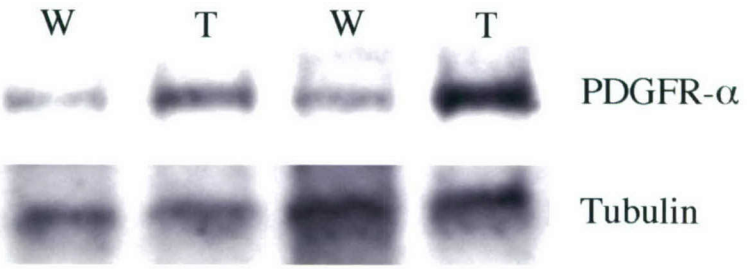


Figure 5. Histological analysis of mammary tumors from wild type and transgenic mice without carcinogen treatment. A. A representitive section of the squamous metaplasia observed in the wild type mice treated with pituitary isograft and zinc sulfate only. B. A representitive section of pappilary adenocarcinoma that arose in the transgenic mice with a pituitary isograft and zinc sulfate. Magnification of both tumors was 20X.

A.



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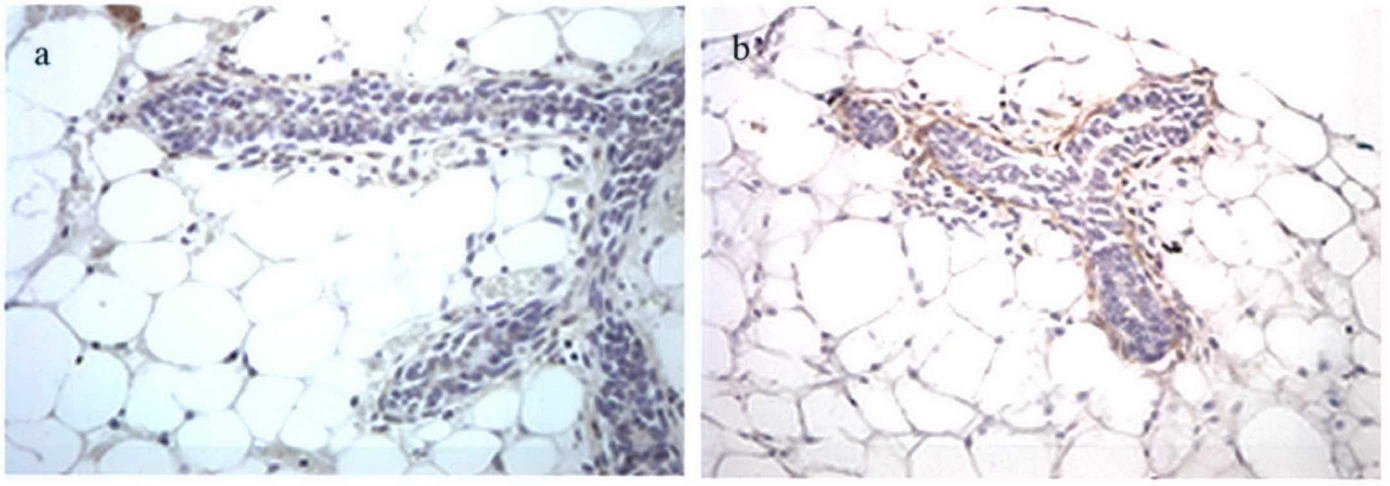

Figure 6. PDGFR-alpha expression in the mammary gland. A. Western analysis of PDGFRα in mammary glands of wild type (W) and transgenic (T) mice . Tubulin was used as a loading control. PDGFRα expression was increased in the mammary glands from the transgenic mice compared to the wild type controls. B. Immunohistochemistry of the PDGFRα in mammary glands from wild type (a) and MTR4 transgenic (b) mice on zinc sulfate for 1 week were stained for the presence of PDGFRα (brown stain). Mammary glands from wild type mice showed little PDGFR staining (a), while mammary glands from transgenic mice had strong staining in the mammary stroma (b).

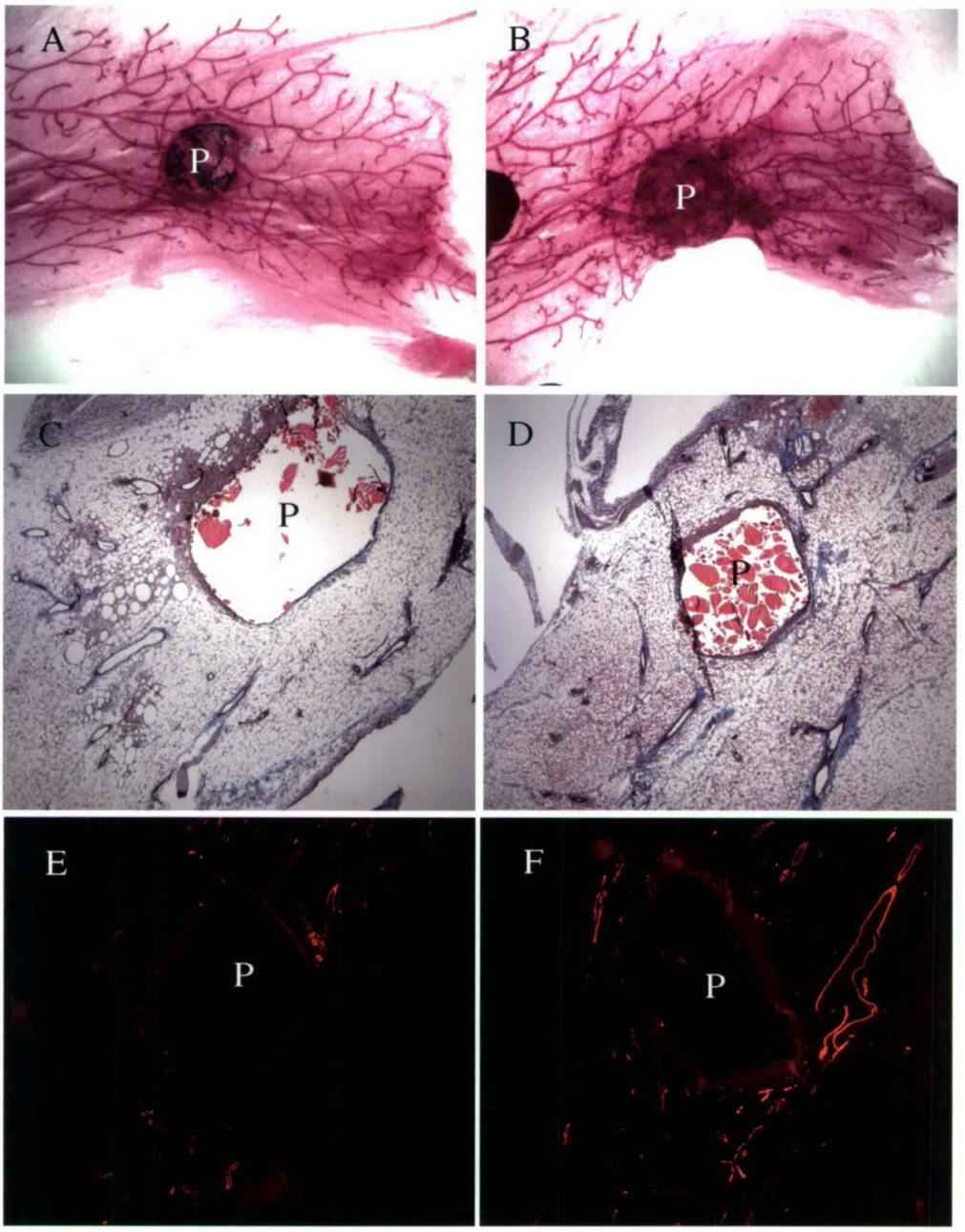

Figure 7. The effects of PDGF-AA on branching morphogenesis in the mammary gland. Mammary glands with BSA pellets (A,C,E) and PDGF-AA pellets (B,D,F) were processed for whole mount and histological analysis. Whole mount analysis of mammary glands from wild type mice implanted with a BSA containing pellet (A) or a PDGF-AA containing pellet (B). No increase in branching was observed. Mammary glands were stained with Masson's trichrome to assess the amount of collagen deposition in BSA containing pellets (C) and PDGF-AA containg pellets (D). Mammary glands were stained with a Cy3 conjugated anti-smooth muscle α-actin antibody. There appeared to be an increase in vascular SMA staining in the PDGF-AA (F) mammary gland compared to the BSA (E) containing mammary gland.