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Award Number: DAMD17-98-1-8140

TITLE: Potential Risk of Growth Promoter in Beef for Breast Cancer Growth

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REPORT DATE: July 2000

TYPE OF REPORT: Annual

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 |
|---|---|---|
| | July 2000 | Annual (1 Jul 99 - 30 Jun 00) |

**4. TITLE AND SUBTITLE**
Potential Risk of Growth Promoter in Beef for Breast Cancer Growth

**5. FUNDING NUMBERS**
DAMD17-98-1-8140

**6. AUTHOR(S)**
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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
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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**
This report contains colored photos

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

**14. SUBJECT TERMS**
Breast Cancer

**15. NUMBER OF PAGES**
14

**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 |

## Table of Contents

Anabolic agents increase weight gain in meat-producing farm animals by enhancing protein deposition and improving feed conversion, as well as increasing the muscle-to-fat ratio. By promoting growth, reducing feed costs and improving the quality of meat produced, the use of such agents has become an important practice in the meat-producing industry. These anabolic agents have biological activities in common with natural estrogenic, androgenic and progestogenic steroid hormones that have potential effects on protein metabolism. The liver easily degrades trace amounts of natural steroid hormones, such as testosterone and estradiol-17β, in foodstuffs consumed by humans, therefore the concern that these natural compounds may pose a health risk to humans is considered negligible.

Our research project has focused on the anabolic compound, zeranol (Ralgro®) which is a non-steroidal, synthetic agent possessing estrogenic action similar to the natural estrogens. Zeranol has been approved by the FDA as a growth promotant for use in the beef, veal, and lamb industries in the U.S. The FDA-approved route and dosage of administration is the subcutaneous implantation in the ear of a pellet containing 36 mg of zeranol per beef heifer followed by a second pellet 30 days later. Although concerns regarding the potential health risk of zeranol residues in the edible tissues (such as muscle, fat, liver and kidney) of zeranol-implanted beef cattle exist, no scientific evidence has demonstrated in a convincing manner the presence of such a risk to federal regulatory agencies such as the FDA and the USDA or to the U.S. consumers at-large. Our research focus has been the potential health risk posed by the consumption of meat from zeranol-implanted beef cattle for human breast cancer growth.

To date, we have investigated the effects of zeranol residues present in the serum and extracts of edible tissues of zeranol-implanted beef cattle on the proliferation of primary cultured normal and cancerous human breast cells and human breast cancer cell lines *in vitro*. These results were described in the last Annual Report. Recently, our research has led us to explore the involvement of a candidate tumor suppressor gene, protein tyrosine phosphatase-gamma (PTP-γ), in mammary tumorigenesis and the ability of estradiol-17β and zeranol to regulate its expression in breast tissues and cells. The expression of PTP-γ has been shown to be reduced in human ovarian and lung tumors (van Niekerk and Poels, 1999) and in diethylstilbestrol (DES)-induced kidney tumors in Syrian hamsters (Lin et al., 1994). Recently, our laboratory demonstrated the expression of PTP-γ mRNA in human breast cells, tissues and breast cancer cell lines and the down-regulation of PTP-γ mRNA expression by estradiol-17β via an estrogen receptor-mediated mechanism (Zheng et al., 2000). These findings are the basis of our contention that suppression of PTP-γ mRNA levels by natural estrogens or estrogenically active agents (xenoestrogens/endocrine disruptors) like zeranol, plays a role in mammary tumorigenesis. Down-regulation of PTP-γ by estrogenically active compounds may be intimately related to the transformation of normal breast cells to preneoplastic or neoplastic cells and may serve as a molecular biomarker for breast cancer. This report describes our recent findings on the regulation of PTP-γ expression in human breast tissues and cells by estradiol-17β and zeranol, and the importance of epithelial-stromal interactions in this regulation. These experiments utilized both normal and cancerous human breast tissue specimens obtained from human patients through the Tissue Procurement Program of the NCI-funded Cooperative Human Tissue Network at The Ohio State University Hospital and Comprehensive Cancer Center. Tissue cultures and primary cultured breast epithelial and stromal cells were used to determine the effects of treatment with estradiol-17β and zeranol on PTP-γ expression levels. A dual chamber co-culture system was used to investigate the role of epithelial-stromal cell interactions on PTP-γ expression. Expression of PTP-γ mRNA and protein were evaluated by semiquantitative RT-PCR and immunohistochemistry, respectively.

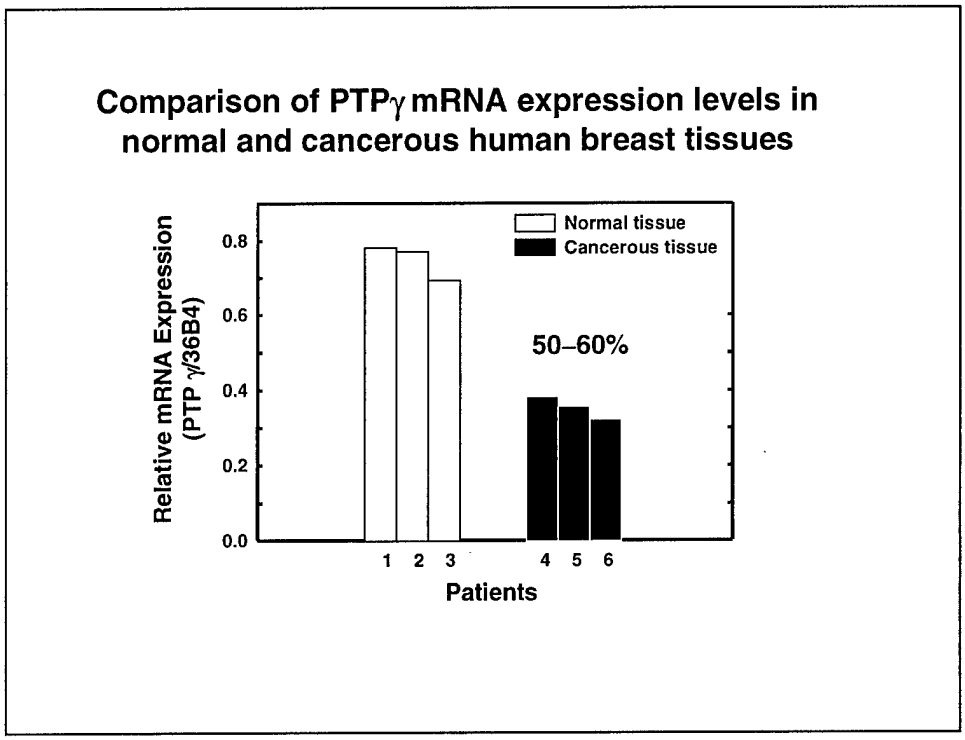


**Figure 1.** Noncancerous human breast tissues from 3 different reduction mastectomy patients and cancerous breast tissues from 3 different breast cancer patients were obtained through the Tissue Procurement Program of the NCI-funded Cooperative Human Tissue Network at The Ohio State University Hospital and Comprehensive Cancer Center. Total RNA was isolated and RT-PCR used to determine mRNA levels of PTPγ in the various tissues (expressed as the relative mRNA expression ratio of PTPγ to 36B4).

**Results:** The PTPγ mRNA levels in 3 different breast cancer tissue specimens were 50 – 60% less than that measured in 3 different normal breast tissue specimens.

**Significance:** These results confirm our previous findings in an animal model of estrogen-induced carcinogenesis. DES-induced kidney tumors in Syrian hamsters possessed lower levels of PTPγ mRNA than kidneys of untreated hamsters (Lin et al., 1994). Reduced expression of PTPγ has also been reported in human ovarian and lung tumors (van Niekerk and Poels, 1999). Thus, the findings are consistent with a potential role of PTPγ as a cancer suppressor in multiple cancers, including breast cancer.

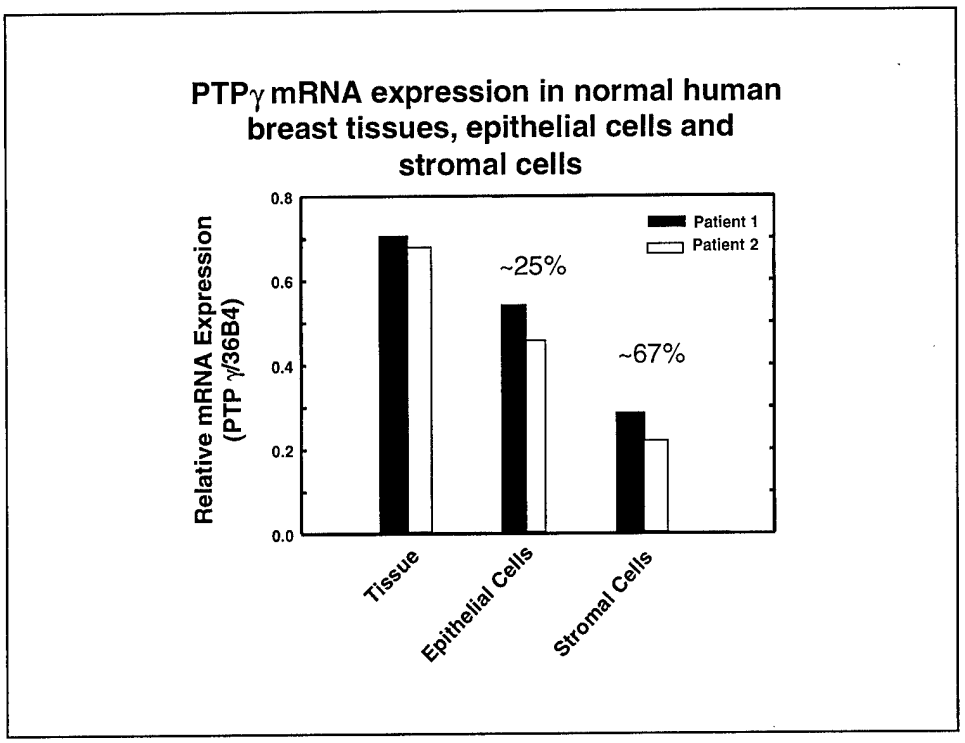


**Figure 2.** Human breast epithelial and stromal cells were isolated and cultured from 2 different normal breast tissue specimens. Total RNA was isolated from these specific cell types, as well as the corresponding tissue specimens from which they were derived. Levels of PTPγ mRNA were determined by RT-PCR and expressed as relative expression ratios of PTPγ to 36B4.
**Results:** The results show that breast epithelial cells express higher levels of PTPγ mRNA than stromal cells that were isolated from normal breast tissue specimens from 2 different reduction mastectomy patients.
**Significance:** These findings show that expression of PTPγ mRNA is predominantly localized to the epithelial component of the human breast. These findings are confirmed by immunohistochemical localization of PTPγ in cultured breast tissues (see Figure 5).

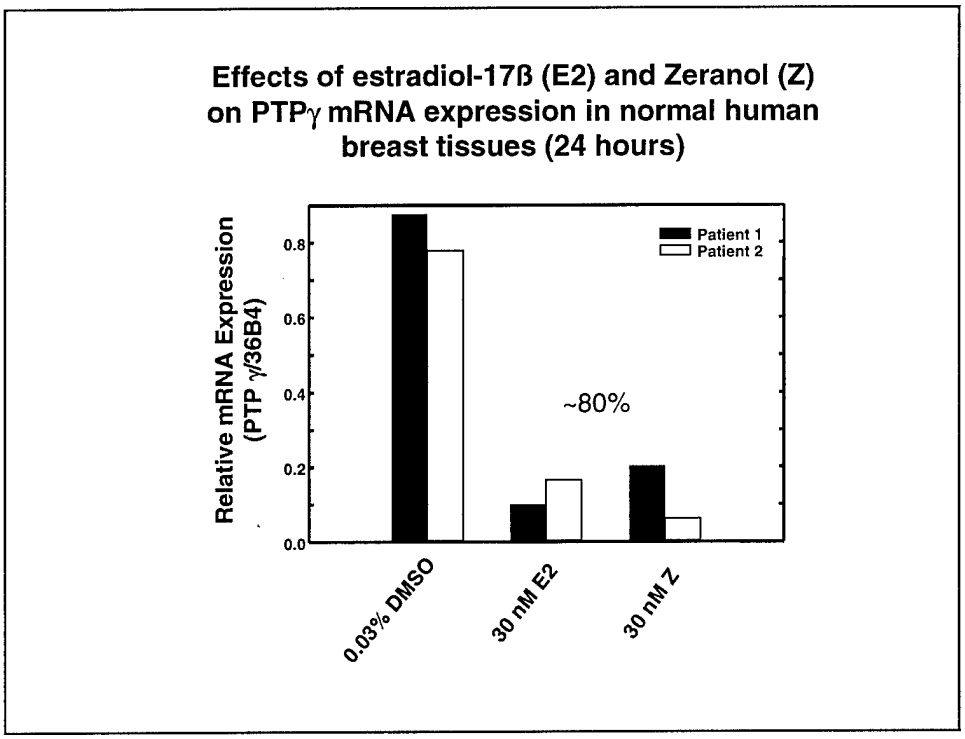


**Figure 3.** Noncancerous breast tissues from 2 different reduction mastectomy patients were divided into approximately 10 mg pieces and cultured on collagen sponge supports. Cultured tissues were treated for 24 hours with 30 nM E2 or Z (5 – 6 tissue pieces per treatment). Total RNA was isolated and RT-PCR was performed to determine PTPγ mRNA expression levels. **Results:** Consistent with our previously published results in which E2 reduced PTPγ mRNA levels in cultured breast cells (Zheng et al., 2000), E2 dramatically suppressed PTPγ mRNA levels in cultured human breast tissues. Interestingly, Z induced an approximately equivalent level of suppression of PTPγ mRNA.
**Significance:** This finding is significant in light of our contention that E2-induced suppression of PTPγ expression plays a role in mammary tumorigenesis. If this hypothesis is true, and equimolar amounts Z and E2 can suppress PTPγ expression in human breast to similar degrees, then long-term, low-dose exposure to Z through the diet may induce molecular level changes in mammary tissues that are associated with E2-induced carcinogenesis.

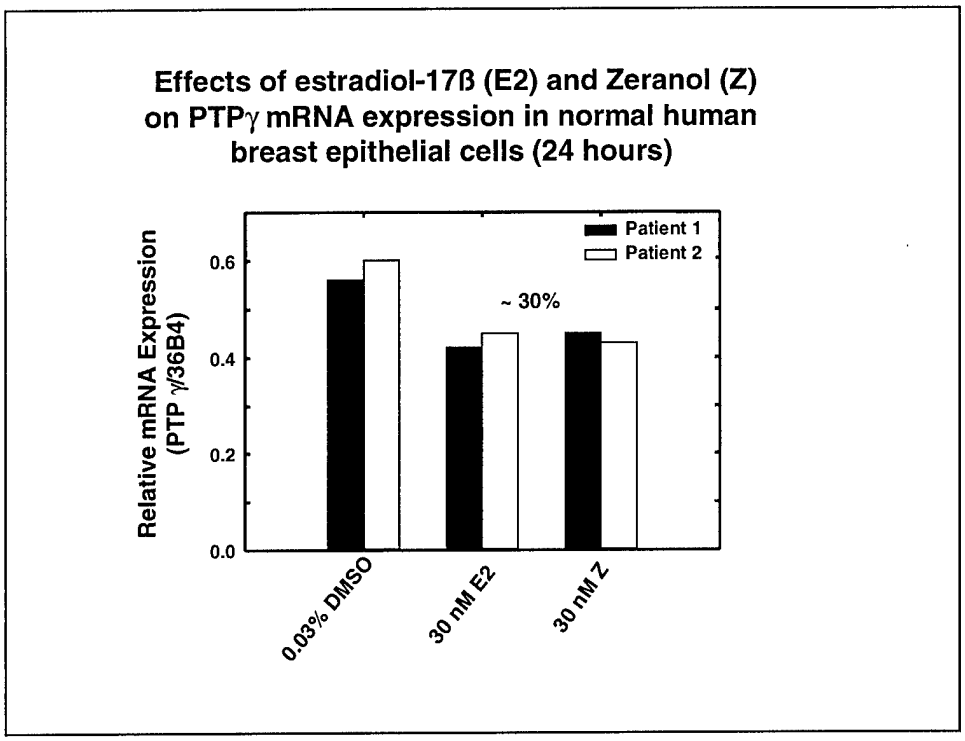


**Figure 4.** Epithelial cells were isolated and cultured from noncancerous breast tissues from 2 different reduction mastectomy patients. The cells were treated for 24 hours with 30 nM E2 or Z. Total RNA was isolated and RT-PCR was performed to determine PTPγ mRNA expression levels.

**Results:** Both E2 and Z reduced PTPγ mRNA levels in epithelial cells isolated from 2 different noncancerous breast tissue specimens. The degree of reduction was similar for both agents (approximately 30%), but was much less than that observed in identically treated cultured human breast tissues (approximately 80%; Figure 3).

**Significance:** Like the results shown in Figure 3, Z induced a suppressive response in PTPγ mRNA expression that was equivalent to that induced by E2. Thus, in the human breast, Z may induce effects nearly identical to those resulting from E2 exposure. Furthermore, the substantially greater response to E2 and Z observed in cultured breast tissue sections compared to isolated breast epithelial cells suggests that interaction of the epithelium with other tissue components, such as the stromal compartment, is required for full suppression of PTPγ in response to estrogenic stimuli.

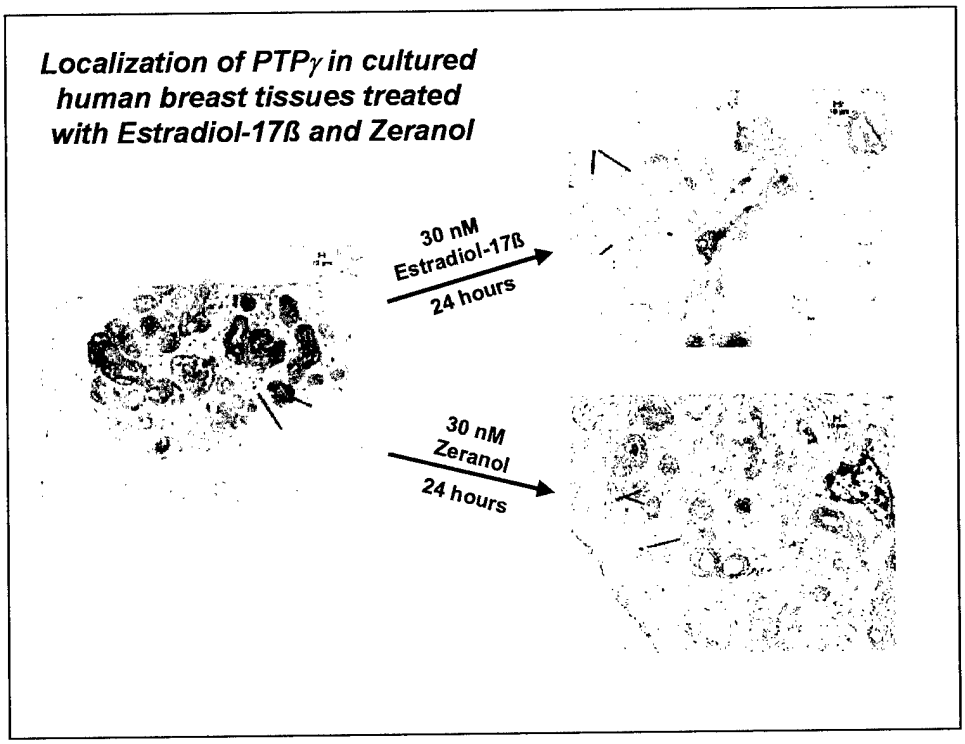


**Figure 5.** Noncancerous breast tissues from reduction mastectomy patients were divided into approximately 10 mg pieces and cultured on collagen sponge supports. Cultured tissues were treated for 24 hours with 30 nM E2 or Z. Immunohistochemical localization of PTPγ in paraffin-embedded tissues using a commercial antibody (Santa Cruz Biotech, CA) was performed.
**Results:** PTPγ was localized to the glandular epithelium (epithelial cells, EC) both before and after treatment. After treatment with E2 or Z, immunopositive staining was still present in the epithelium but was observably reduced.
**Significance:** These findings are consistent with the results shown in Figure 2 which revealed higher PTPγ mRNA levels in cultured breast epithelial cells than in stromal cells. Furthermore, the reductions in PTPγ immunostaining observed after treatment with E2 or Z support the results reported in Figure 3 in which E2 and Z dramatically reduced PTPγ mRNA levels in cultured breast tissues. Taken together, these results show, at both the mRNA and protein levels, that PTPγ is localized to the breast epithelium and is suppressed in human breast tissues by exposure to E2 or Z.

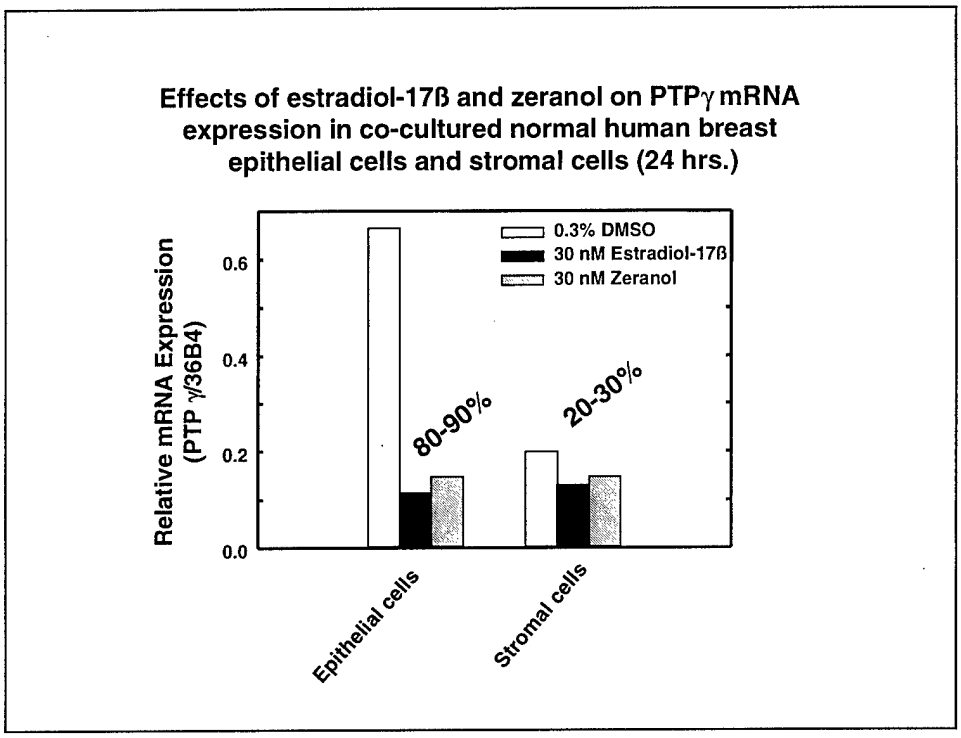


**Figure 6.** Figures 3 and 4 indicated the potential importance of human breast epithelial-stromal interactions in the suppression of PTPγ expression by estrogenically active agents. Epithelial and stromal cells isolated from noncancerous breast tissues from reduction mastectomy patients were treated for 24 hours with 30 nM E2 or Z during culture in a dual chamber co-culture system. Total RNA was isolated and RT-PCR was performed to determine PTPγ mRNA expression levels.

**Results:** First, vehicle-treated epithelial cells contained greater levels of PTPγ mRNA than the corresponding stromal cells. This finding is similar to that shown in Figure 2 and reinforces the conclusion that PTPγ is predominantly localized to the epithelium. Secondly, in both cell types, Z induced reductions in PTPγ mRNA expression that were similar in magnitude to those induced by E2. Finally, epithelial cells exhibited a drastically greater reduction in PTPγ mRNA expression level in response to E2 or Z than the stromal cells (80-90% vs 20-30%, respectively).

**Significance:** The primary significance of this result is that the degree of E2- or Z-induced suppression of PTPγ mRNA expression in co-cultured epithelial cells shown above is much greater (80-90%) than the level of suppression induced in epithelial cells cultured alone (30%; Figure 4). Thus the result indicates the importance of epithelial-stromal cell interaction(s) in the response of breast cells to estrogenically active agents, including Z, specifically as it relates to the expression of PTPγ.

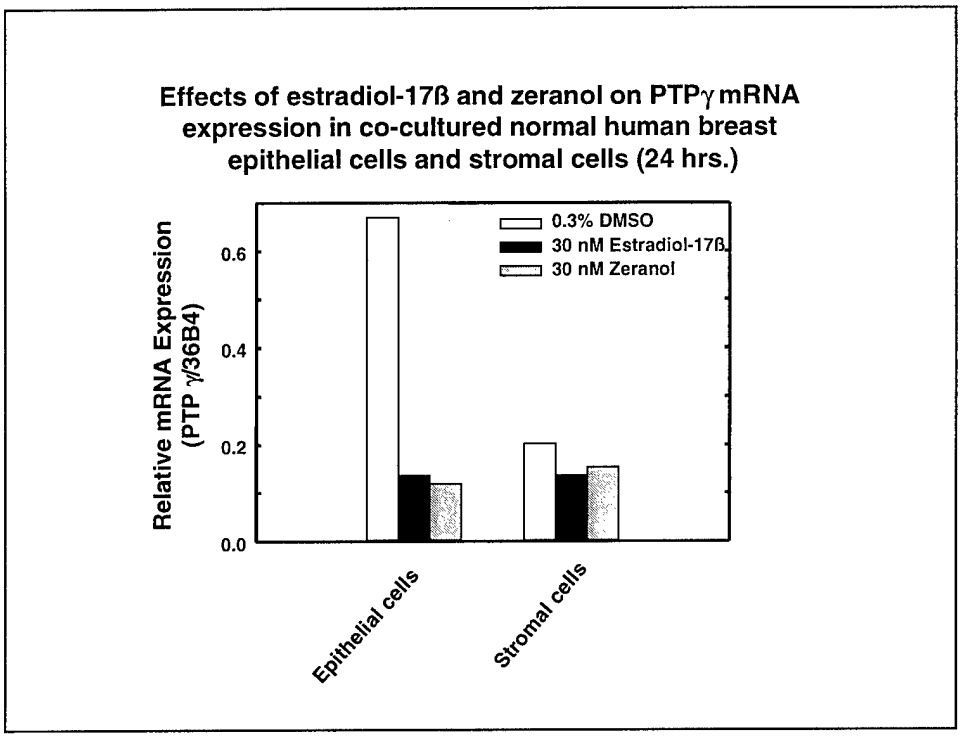


**Figure 7.** This figure shows the results of an experiment identical to that described in Figure 6, but using cells isolated from the breast tissue of a different patient. Nearly identical results were obtained.

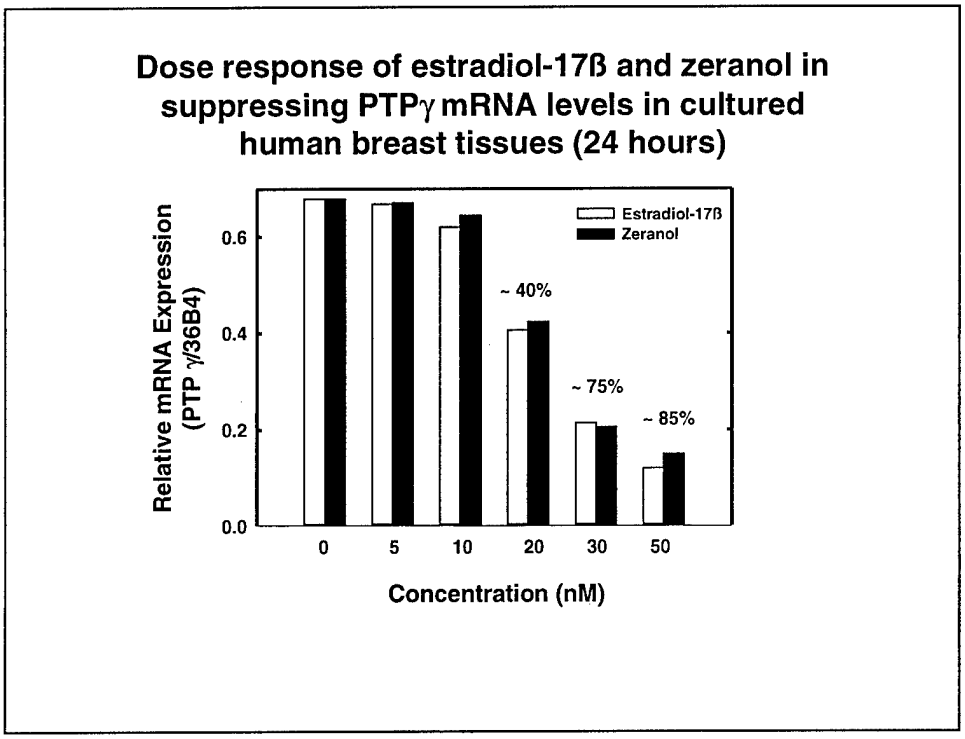


**Figure 8.** Noncancerous breast tissues from reduction mastectomy patients were divided into approximately 10 mg pieces and cultured on collagen sponge supports. Cultured tissues were treated for 24 hours with E2 or Z at various concentrations (0 – 50 nM). Total RNA was isolated and RT-PCR was performed to determine PTPγ mRNA expression levels.
**Results:** At concentrations less than 20 nM, neither E2 nor Z affected PTPγ mRNA expression levels. Over the concentrations of 20, 30 and 50 nM, both E2 and Z induced dose-dependent reductions in PTPγ mRNA expression levels.
**Significance:** At all concentrations tested, the degree of PTPγ mRNA suppression caused by Z is nearly identical to that induced by E2. This results suggests that exposure of breast tissues to Z can result in estrogenic responses that may lead to tumor formation.

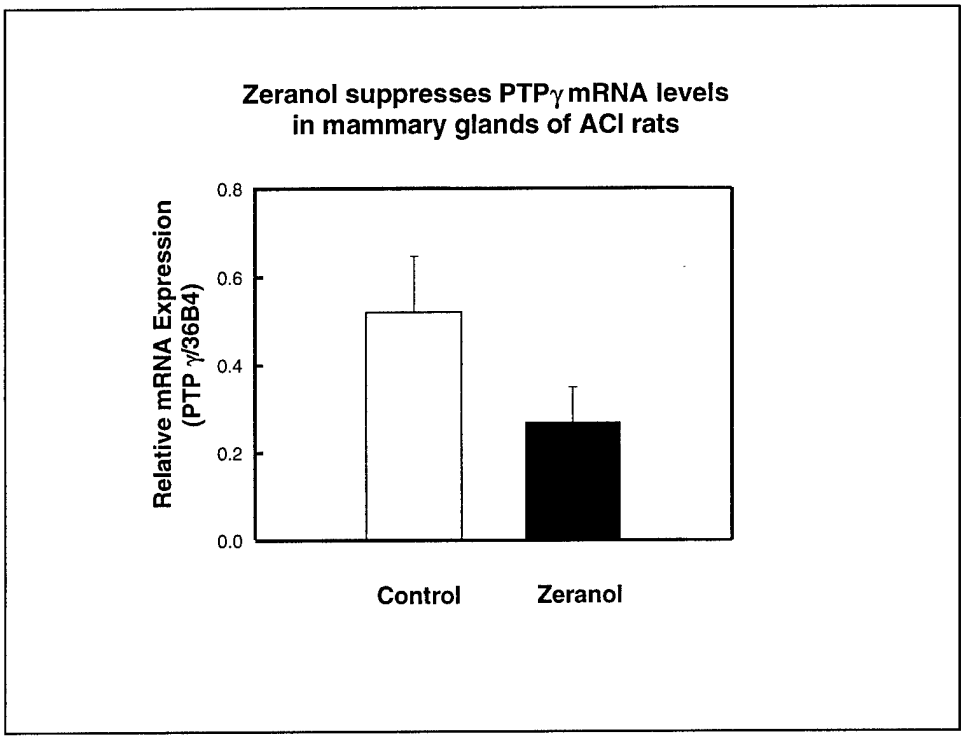


**Figure 9.** Ovary-intact, 8 – 9 week-old ACI rats were treated with Z via sustained-release pellets (5 μg/day) for 112 days. Total RNA was isolated from whole mammary tissues and RT-PCR was performed to determine PTPγ mRNA expression levels.
**Results:** Long-term treatment of female ACI rats with Z resulted in an approximately 2-fold reduction in PTPγ mRNA levels within the mammary gland (Kulp et al., 1999).
**Significance:** This finding supports our in vitro results in human breast cells presented above by demonstrating that exposure to the nonsteroidal estrogenic agent, Z, can suppress PTPγ mRNA levels in the mammary gland in vivo. Given the proposed role of PTPγ in cancers, including breast cancer, the ability of Z to induce changes in mammary PTPγ expression in an in vivo model strengthens the need for further investigation of the risk of exposure to dietary Z for human breast cancer. In addition, in this ACI rat model, PTPγ was immunolocalized to the glandular epithelium (not shown; Kulp et al., 2000), which is also the site of PTPγ immunostaining in the human mammary gland (Figure 5). Also, the changes in PTPγ mRNA expression levels were observed in mammary tissues that were histologically indistinguishable from the mammary glands of control ACI rats (Kulp et al., 2000). Taken together, the findings suggest the potential value of the ACI rat model for the sensitive in vivo detection of estrogenic effects of putative xenoestrogens/endocrine disruptors, and for the continued study of the role of PTPγ in estrogen-induced mammary tumorigenesis.

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