Zeranol Induces Cell Proliferation and Protein Disulfide Isomerase Expression in Mammary Gland of ACI Rat

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Abstract. *Background: Zeranol is a non-steroidal anabolic growth promoter with potent estrogenic activity that is widely used as a growth promoter in the US beef industry. Consumption of beef derived from Zeranol-implanted cattle may be a risk factor for breast cancer. Protein disulfide isomerase (PDI) has been studied extensively as a key enzyme involving in the formation of the correct pattern of disulfide bonds in newly synthesized proteins. The relationship between PDI expression and cancer development has attracted interest of cancer researchers in recent years. Materials and Methods: We implanted ACI rats with 12 mg Zeranol pellet and harvested the mammary tissues and tumor at day 110 after implantation and investigated the effect of Zeranol-implantation on cell proliferation by histological examination and proliferation in vitro. We also evaluated PDI mRNA expression in primary epithelial cells isolated from normal mammary glands and primary tumor cells from tumor specimens using real-time RT-PCR. To further confirm, we also evaluated the effect of Zeranol on PDI mRNA expression in primary epithelial cells isolated from normal mammary gland of ACI rats. Results: We observed a palpable mammary tumor in one of three Zeranol-implanted ACI rats at day-110 post Zeranol-implantation. Zeranol-implantation significantly promoted the cell proliferation of primary mammary epithelial and stromal cells isolated from the mammary gland of normal ACI rats. PDI mRNA is over-expressed in primary tumor cells isolated from the tumor specimen and in Zeranol-treated primary cultured epithelial cells from the mammary gland of normal ACI rats. Conclusion: These findings suggest that up-regulated expression of PDI may play a critical role in mammary tumorigenesis and cell proliferation in response to Zeranol. Our findings implicate PDI as a biomarker for mammary tumorigenesis.*

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Estrogen is a steroidal hormone that is essential for the normal development and growth of the mammary gland and organs important for reproductive functions. It helps control a woman's menstrual cycles and is necessary for reproduction. However, lifelong exposure to estrogen has been associated with an increased risk of breast cancer, and evidence suggests that estrogen exposure has an important role in determining breast cancer risk (1). Increasing evidence suggests that endogenous estrogens are involved in the etiology of certain types of breast cancer (1, 2). Estrogens increase breast epithelial cell proliferation and may facilitate growth of estrogen-responsive neoplastic or pre-neoplastic epithelial cells (3-5). It is known that estrogen promotes proliferation of epithelial cells in the mammary gland mainly by binding to estrogen receptors (ER) and modulating expression of downstream genes (6).

Based on the tumor initiation properties of endogenous estrogen, researchers have hypothesized that some chemical compounds which exert estrogen bioactivity, also have the potential to initiate tumors. Research has shown that numerous environmental pollutants cause mammary gland tumors in animals, are hormonally active, specifically mimicking estrogen, which is a breast cancer risk factor; or affect susceptibility of the mammary gland to carcinogenesis (7). In the past few years, environmental estrogens or synthetic xenoestrogens, which have been found in the environment or food, have been established as carcinogens (8-10). However, there is minimal information on the effects of these chemicals or their metabolites on humans (11-13).

Zeranol (α-Zearalanol) is a non-steroidal mycotoxin produced by Fusarium species on several grains. α-Zearalanol food contamination is caused either by direct contamination of grains, fruits and their products or by 'carry-over' of mycotoxins in animal tissues, milk and eggs after intake of contaminated feedstuff. Moreover, Zeranol (Ralgro®) has been widely used in the USA as a growth promoter to improve feed efficiency, weight gain, meat-to-fat-ratio, and carcass quality in beef cattle. About 95% of the U.S. beef feedlots industry uses growth-promoters; Zeranol's market share is about 60%. Administration of Zeranol is by the subcutaneous implantation in the ear of a pellet containing 72 mg of Zeranol per beef cattle. Thus, people are exposed to Z as a consequence of consumption of beef products derived from Zeranol-implanted beef cattle.

The safety of using Z as a growth promoter has been debated for many years because of its potential to cause cancer. Zeranol is able to bind to the active site of human estrogen receptor ERα and ERβ in a similar manner as 17β-estradiol (E_2) (14). An epidemiological investigation found that the sperm quality in sons of 'high beef consumers' was lower than that in males whose mothers ate less beef during their pregnancy (15). The results of our previous studies provide evidence that Zeranol exerts estrogenic mitogenic activity in primary pre-adipocytes isolated from heifers and in primary human breast cancer cells (16-19). Moreover, we also found that meat and serum from Zeranol-implanted beef cattle possess heat-stable mitogenicity for cultured human breast epithelial cells, and that both human normal breast epithelial and cancer cells exhibit estrogenic responses to Zeranol (20-22). Our previous data also showed that implantation of Zeranol in beef cattle greatly promotes growth of primary beef pre-adipocytes by up-regulating cyclin D1 and down-regulating p53 expression (23). Furthermore, we found that Zeranol transforms the human normal breast epithelial cell line MCF-10A and increases growth of primary cultured human normal breast epithelial and cancer cells in a dose-dependent manner (24). Zeranol increases estrogen biosynthesis by enhancing aromatase mRNA expression in human pre-adipocytes (25).

However, the mechanism of cell proliferation in response to Zeranol is unknown. Protein disulfide isomerase (PDI), a key enzyme involved in the formation of the correct pattern of disulfide bonds in the maturation of newly synthesized proteins may play a role (26). Previous studies implicated that PDI appears to have anti-apoptotic activity. Tanaka *et al*. (27) reported that PDI is specifically up-regulated in response to hypoxia/brain ischemia in astrocytes, and overexpression of PDI resulted in attenuation of the loss of cell viability in neuroblastoma cells induced by hypoxia and a reduction in the number of DNA fragmented cells in brain ischemic rats. PDI was overexpressed in prostate (28), brain (29), pancreatic (30) and ovarian (31) tumors compared to normal tissues. Moreover, a recent finding showed that PDI was responsible for the accumulation of 17β-estradiol inside mammalian cells and was able to augment estrogen receptor-mediated transcriptional and mitogenic activity of E_2 in cultured cells (32). Lai *et al*. (33) reported that expression of PDI was up-regulated in human breast cancer MCF-7 cells but down-regulated in triple negative human breast cancer cell line, MDA-MB-231 cells compared to that of MCF-10A. We showed that a protein designated as Zeranol-induced protein (ZIP) was up-regulated 5-fold in the human normal breast epithelial cells exposed to Zeranol and that the amino acid sequence derived for the DNA sequence of ZIP protein was identical to that of protein disulfide isomerase (PDI) (18). These findings suggest that PDI may play an important role in tumor initiation in the mammary gland and increased cell proliferation in response to Zeranol. However, the roles of PDI *in vivo* are poorly understood.

We hypothesize expression of PDI may serve as a molecular bio-marker of human breast tumorigenesis caused by Zeranol or other estrogenic endocrine disruptors. In the present study, we implanted female ACI rats with 12 mg Zeranol pellet and harvested mammary tissues at day 110 after implantation and investigated the effect of Zeranol-implantation on cell proliferation and PDI expression of epithelial cells isolated from the mammary gland. Furthermore, we demonstrated that one of the Zeranol-implanted ACI rats developed a mammary tumor at day 110 after implantation.

Materials and Methods

Animal implantation with Z and harvest of mammary tissues. Ralgro Magnum® (RM, commercial Z product) was purchased from Merck Schering-Plough Corp, NJ, USA, in the form of six pellets per cartridge, each containing 12 mg Zeranol Female ACI rats (Harlan Laboratories, Indianapolis, IN, USA) were obtained at 6-8 weeks of age and housed in the Laboratory Animal Facility at the College of Veterinary Medicine, The Ohio State University under controlled temperature, humidity and lighting conditions. After one week of acclimatization, 6 rats were randomly divided into two groups. One group was implanted a Zeranol pellet subcutaneously into the back region of each rat, whereas the control group did not receive implantation. Animals were monitored for tumorigenesis weekly and euthanized by CO_2 asphyxiation after 110 days. The mammary gland (both tumor and normal) was harvested from control and Zeranol-implanted ACI rats for pathological analysis. Epithelial/cancer cells were isolated from mammary gland tissues.

Histological examination of mammary tissue and tumor. Mammary tissue and tumor tissue were fixed in 10% phosphate-buffered formalin for 16 h, and they were then dehydrated and embedded individually in paraffin blocks. Paraffin-embedded mammary tissue and tumor tissue were cut into 4-μm-thick sections and mounted on microscope slides. The sections were deparaffinized in xylene, dehydrated in graded alcohol, and processed routinely and stained with hematoxylin and eosin (H&E) by the Comparative Pathology and Mouse Phenotyping Shared Resource Histology & Immunohistochemistry Shared Resource Core at the College of Veterinary Medicine, The Ohio State University.

Isolation and culture of epithelial and stromal cells from mammary tissues from non-Zeranol-implanted ACI rats. Normal mammary tissues were sterilized in 70% ethanol for 30s and then washed three times with fresh DMEM/F12. They were minced and then digested in phenol red-free high calcium Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) (1.05 mM $CaCl_2$) supplemented with 2% bovine serum albumin (Invitrogen, Carlsbad, CA, USA), 10 ng/ml Cholera toxin (Sigma, St. Louis, MO, USA), 6300U/ml Collagenase (Invitrogen), and 100U/ml Hyalurinidase (Calbiochem, Gibbstown, NJ, USA). After the tissue mixture was incubated in a humidified incubator (5% CO_2, 95% air, 37˚C) for 16 h, the resulting cell suspension was transferred to a 50-ml conical centrifuge tube and centrifuged at 200×g for 5 min. The upper, middle and lower layers were carefully removed and centrifuged again. The upper layer containing pre-adipocytes, the middle layer containing stromal cells, and the lower layer containing epithelial cells were individually centrifuged to pellet the respective cells. All the pellets were washed by DMEM/F12 medium with antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 g/ml streptomycin sulfate and 0.25 g/ml amphotericin B) (Gibco BRL, Bethesda, MD, USA). This wash procedure was repeated three times. The pelleted epithelial cells were resuspended in low calcium DMEM/F12 (0.04 mM $CaCl_2$) supplemented with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA)-treated 10% FBS (GIBCO Cell Culture™ , Grand Island, NY, USA) and the pelleted stromal cells were resuspended in same high-calcium DMEM/F12 supplemented with 5% FBS and 5 μg/ml insulin. These two isolated cell types were plated separately in 75-cm^2 culture flasks in a humidified incubator (5% CO_2: 95% air, 37˚C). The media of all primary cultured mammary cells were changed every 2 days. When cells were grown to 85% confluence, they were trypsinized with 0.5% trypsin-5.3 mM EDTA (Gibco BRL) and split 1:5.

Doubling time assay. A total of 5×10^4 epithelial cells or 1×10^5 stromal cells were seeded in T25 flasks, and were grown for 4 days and counted via trypan blue exclusion every 8 h. Cell doubling time (T) was calculated by using the formula (24):

$$N_j=N_i\times2(t_j-t_i)/T \text{ or } T=(t-t_0)lg2/lg(N/N_0)$$

where N_j or N_i are the cell numbers at time point t_j or t_i of harvested time.

Cell treatment, RNA isolation and cDNA synthesis. Primary cultured epithelial cells (1×10^5) isolated from the mammary gland of normal ACI rats were seeded in 5 ml low calcium (0.04 mM $CaCl_2$) DMEM/F12 in 6-well plates and incubated for 24 h. The medium was replaced with low calcium DMEM/F12 supplemented with 10% dextran-coated charcoal (DCC) stripped Chelex-100-treated FBS for 16 h. The cells were then treated with different concentrations of Zeranol (2, 10 or 50 nM) for another 24 h.

Normal tissues and tumor from ACI rats were rapidly frozen in liquid nitrogen and then homogenized with a mortar and pestle in the presence of TRIZOL Reagent® (Invitrogen). Total RNA was isolated using 1 ml TRIZOL according to the manufacturer's instructions. RNA concentrations were determined by measuring their absorbance at 260 nm using a DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA).

The reverse transcription reaction consisted of total RNA (1 μg), 200 U M-MLV Reverse Transcriptase (Invitrogen), 0.2 mM dNTP (1 μl mixture of 10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH, Invitrogen), 1 μM random hexamers (Amersham, Piscataway, NJ, USA), 10 μl 5X First Strand buffer, 5 μl 0.1M DTT and 40 U RNase Inhibitor (Invitrogen) in a total volume of 50 μl. The reaction was incubated at 37˚C for 50 min followed by inactivation at 70˚C for 15 min in a gradient master¬cycle (Eppendorf®, USA).

Quantitative real-time PCR. Real-time PCR was used to measure PDI mRNA expression. Conditions were optimized for each primer pair and performed in Stratagene Mx3005p (Agilent Technologies, TX, USA). Briefly, Newly synthesized cDNA (2 μl) was used as a template for the reaction in a total volume of 20 μl reactants, which included 10 μl of 2X real-time master mix (Applied Biosystems, Warrington, UK), 3 μl ultra-pure water and 5 μl of primer mixer. The reactants were first incubated at 95˚C for 10 min, and then 40 cycles of amplification were carried out with each cycle consisting of denaturing at 95˚C for 30 sec, annealing at 60˚C for 1 min and elongation at 72˚C for 1 min. Dissociation curves were created at the completion of each run to ensure that the PCR reactions produced the correct products as anticipated. The primer sequences for PDI (ZIP) were 5'-TTGCACTGCCAACACTAACA-3' (sense) and 5'-AGTGGCTGACAATTCCATCA-3' (antisense). Primer sequences for 18s rRNA were 5'-TCCGATAACGAACGAGAC-3' (sense) and 5'-CTAAGGGCATCACAGACC-3' (antisense). The results of the relative mRNA expression (PDI to 18s rRNA) in cells were analyzed using the ΔΔCt method (34).

Statistical analysis. All tests and analyses were run in three replicates. Analysis was performed using SAS for window (SAS Institute Inc., Cary, NC, USA). Statistical differences were determined using Student's *t*-test for independent groups. *P*-values of less than 0.05 were considered to be statistically significant.

Results

In vitro proliferation of cultured epithelial and stromal cells isolated from mammary tissue of ACI rat. We performed a proliferation study by means of doubling cell assay to determine whether Zeranol has a mitogenic effect on mammary epithelial and stromal cells were isolated from control and Zeranol-implanted female ACI rats (Figure 1). The growth curve of both cell types is shown in Figure 2A and C. The doubling times of epithelial cells isolated from control and Zeranol-implanted ACI rat were 34 and 26 h respectively (Figure 2B). The doubling times of stromal cell isolated from control and Zeranol-implanted ACI rat were 46 and 40 h, respectively (Figure 2D).

Histological examination of mammary tissues and tumor of Zeranol-implantation ACI rat. A palpable mammary tumor (Figure 3C) was observed on day 110 after Zeranol implantation, in one of three ACI rats which were implanted with Zeranol. The normal mammary tissue from control (no Zeranol implanted) ACI rat and tumor tissue and adjacent mammary tissue from the Zeranol-implanted ACI rat were examined by histopathologic analysis. The mammary tissue of control ACI rat showed normal lobular

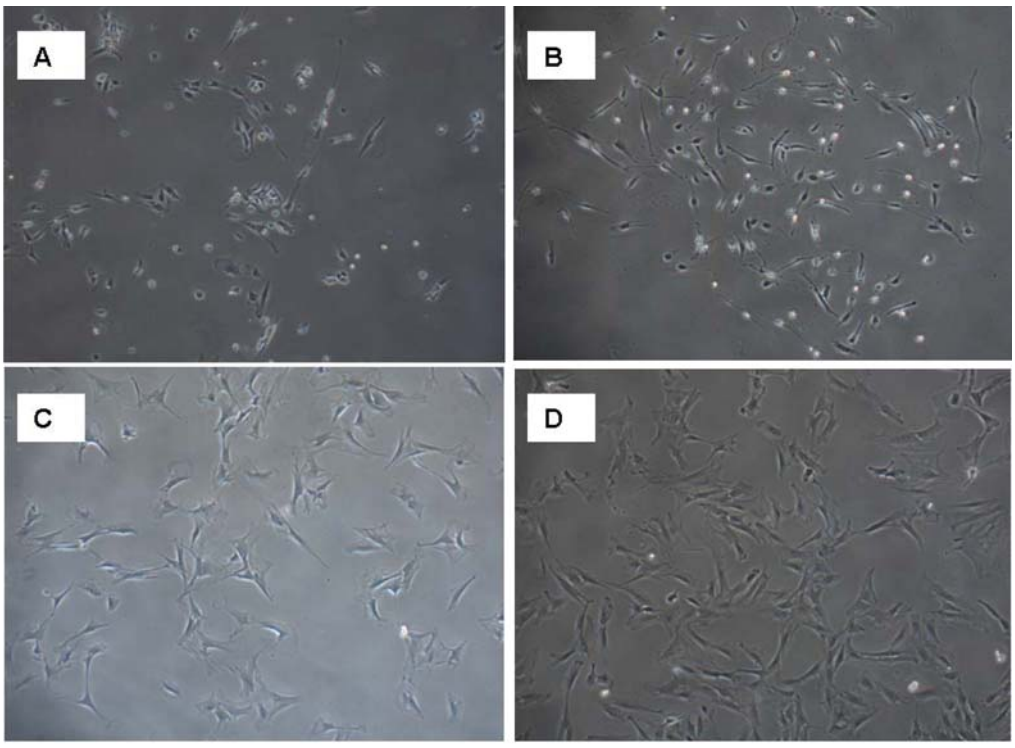

Figure 1. *Morphology of the primary cultured mammary epithelial cells (A, B) and stromal cells (C, D) from control ACI rat (A, C) and Zeranol-implanted ACI rats (B, D).*

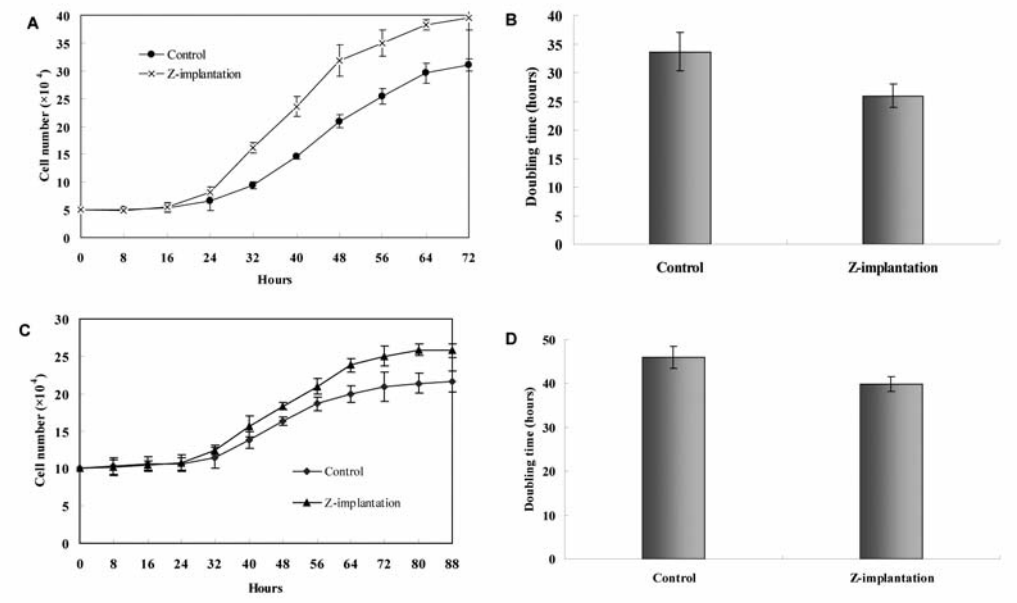

Figure 2. *Cell growth curves (A, C) and doubling time (B, D) of mammary epithelial cells (A, B) and stromal cells (C, D) from control and Zeranol-implanted ACI rats.*

architecture (L) with branched ducts (Du) and normal distribution of adipose tissue (Figure 3A&D), and the mammary tissue from Zeranol-implanted ACI rats displayed increased proliferation with dilated ducts and expansion of terminal lobular units accompanied by compression of and expansion into the surrounding fat tissue (Figure 3B&E), which was similar to the tumor characteristics of hyperchromasia (Figure 3F).

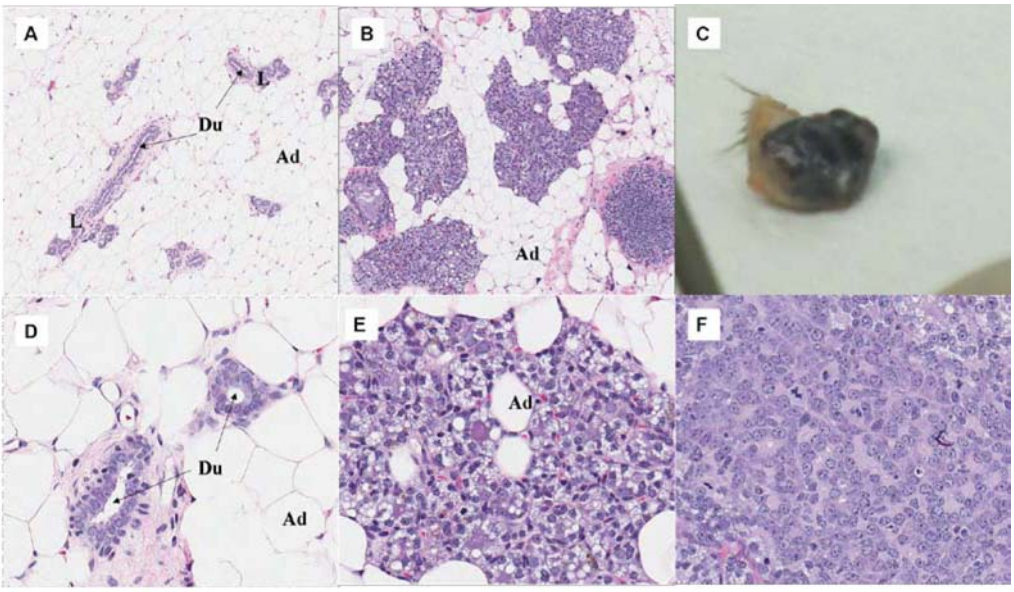

Figure 3. *Histology of mammary tumor in Zeranol-implantation ACI rats. Mammary glands of control (A, D), Zeranol-implanted ACI rats (B, E) and tumor (C, F). The magnification is ×100 for A & B and ×400 for D, D and F.*

Zeranol-implantation increased expression of PDI in ACI mammary tissues, tumor and cultured cells. PDI mRNA in ACI mammary tissues, tumor and cultured epithelial cells isolated from control and Zeranol-implanted ACI rats are shown in Figure 4. PDI was up-regulated 3.1-fold in the tumor compared with normal tissue from control ACI rat (Figure 4A). The PDI mRNA expression of cultured epithelial cells isolated Zeranol-implanted ACI rat was 2.4 times higher than that of epithelial cells from control rats (Figure 4B).

PDI mRNA expression of normal mammary epithelial cells in response to Zeranol. We treated epithelial cells isolated from control normal ACI rats with different doses of Zeranol to determine its effect on PDI mRNA expression. Zeranol from 2-30 nM significantly ($p<0.05$) increased PDI mRNA expression in normal mammary epithelial cells (Figure 5).

Discussion

The synthetic compound Zeranol (Ralgro®) is a potent non-steroidal growth promoter that mimics many of the effects of estradiol. Zeranol is used extensively in the United States and Canada to promote rapid and more efficient growth rates in animals used as sources of meat (35). In the past few years, the use of Zeranol as growth promoter has been controversial because of its potential to cause cancer. Our previous studies have shown that Zeranol exhibits mitogenetic estrogenic activity (16-18, 21-22). Zeranol is able to bind to the active site of human estrogen receptor ERα and ERβ in a similar manner as 17β-estradiol (14). Our previous research also found that Zeranol transforms the human normal breast epithelial cell line MCF-10A and increases proliferation of primary cultured human normal breast epithelial and cancer cells in a dose-dependent manner (24). In addition, we reported that Zeranol suppressed protein tyrosine phosphatase gamma, a breast tumor suppressor gene (36). Furthermore, we showed that Zeranol may increase estrogen biosynthesis by up-regulating aromatase mRNA expression in human pre-adipocytes (25). However, many of the details behind the mechanisms of increased cell proliferation in breast epithelial cells in response to Zeranol remain to be elucidated.

We demonstrated that human normal breast epithelial cells exposed to 30 nM Zeranol for 24 h significantly increased the expression of ZIP (18) and determined that the amino acid sequence of ZIP protein is identical to the PDI, which has been implicated as surface molecular biomarker for many cancer types including those of the prostate, brain, pancreas and ovaries (28-31).

PDI is a key enzyme involved in the formation of the correct pattern of disulfide bonds in newly synthesized proteins (26). In recent years there has been a great deal of interest in the relationship between PDI expression and cancer development. Previous work suggests an intriguing link between PDI and cancer. Protein disulfide isomerase was overexpressed in prostate tumors when compared to normal tissues (28). Goplen *et al*. (29) reported that PDI was overexpressed in invasive low-generation tumors as revealed by immunohistochemistry and Western blots. PDI expression was up-regulated in MCF-7 cells compared to MCF-10A cells (33).

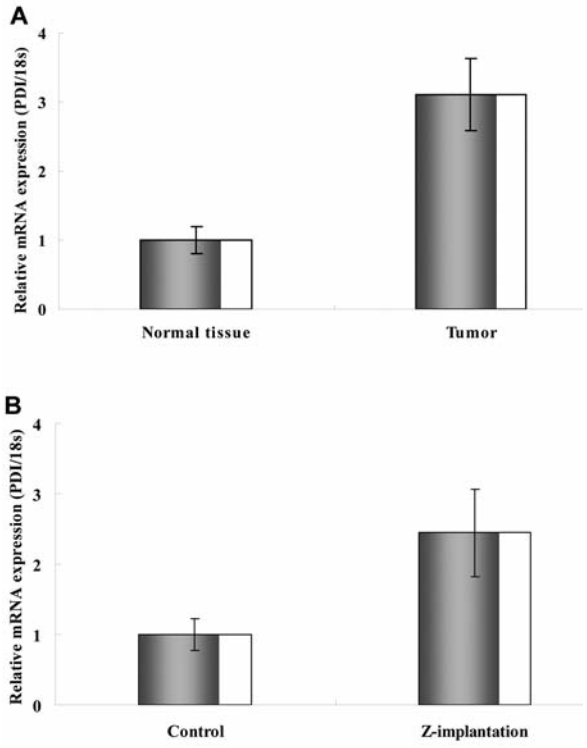

Figure 4. *PDI mRNA expression in (A) normal tissue and tumor and (B) primary cultured epithelial cells from control and Zeranol-implanted ACI rats.*

Although the role of PDI in tumorigenesis is poorly understood, we hypothesize that PDI may be useful as a molecular biomarker for the involvement of Zeranol in implanted cattle in human breast tumorigenesis. In the present study, we implanted female ACI rats with 12 mg Zeranol pellet and harvested mammary tissues at day 110 post implantation and investigated the effect of Zeranol-implantation on cell proliferation and PDI expression in epithelial cells isolated from mammary tissue. We observed a palpable mammary tumor in one of three Zeranol-implanted ACI rats at day-110 post Zeranol-implantation. Our results also showed that Zeranol-implantation significantly promoted the cell proliferation of mammary epithelial and stromal cells. In addition, PDI was up-regulated 3.1-fold in the tumor compared with normal mammary tissue from control ACI rats. Similarly, the PDI mRNA expression in primary mammary epithelial cells isolated from Zeranol-implanted ACI rats was 2.4 times greater than that of mammary epithelial cells from control rats. Furthermore, we demonstrated that primary normal mammary epithelial cells isolated from non-Zeranol implanted ACI rats that were exposed to Zeranol (2-30 nM) in the culture media had significantly ($p<0.05$) increased PDI mRNA expression compared to non-treated cells.

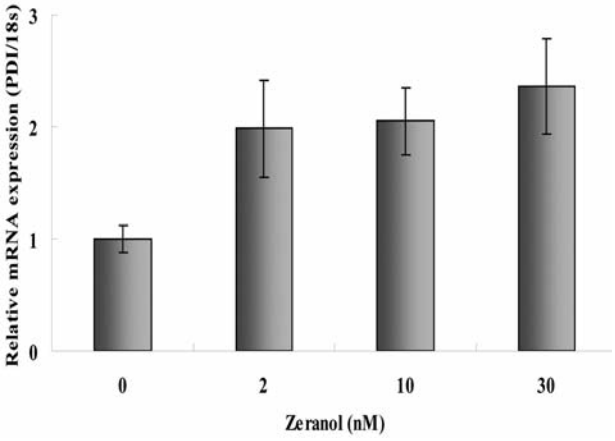

Figure 5. *Effects of Zeranol on PDI mRNA expression in primary cultured epithelial cells from non-Zeranol-implanted ACI rats. Cells were treated with 2 nM, 10 nM or 50 nM Zeranol; 0.1% DMSO was used as control.*

These findings illustrate increased PDI expression in ACI mammary gland and cell proliferation in response to Zeranol. Hence Zeranol may be associated with very early events in mammary cancer initiation. Although the mechanisms responsible for this increased expression of PDI and formation of the mammary adenocarcinoma in one of the ACI rats implanted with Zeranol are unclear at the present time, our studies suggest that PDI may be considered as an additional diagnostic biomarker for breast cancer.

Acknowledgements

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