

## Zeranol Enhances the Proliferation of Pre-adipocytes in Beef Heifers

WEIPING YE<sup>1</sup>, PINGPING XU<sup>1,2</sup>, WALTER R. THRELFALL<sup>3</sup>, ROBERT JEN<sup>1</sup>, HONG LI<sup>1</sup>,  
SHU-HONG LIN<sup>1</sup>, CHEIK-TI KUO<sup>1</sup> and YOUNG C. LIN<sup>1,4</sup>

<sup>1</sup>Laboratory of Reproductive and Molecular Endocrinology, Department of Veterinary Biosciences, College of Veterinary Medicine, and <sup>3</sup>Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Ohio State University, Columbus, OH 43210, U.S.A.;

<sup>2</sup>Jinhua College of Profession and Technology, Jinhua, Zhejiang, 321017, P.R. China

<sup>4</sup>Ohio State University Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, U.S.A.

**Abstract.** *Background:* The high morbidity and mortality of breast cancer among women is a serious problem. The adverse effects of the consumption of beef with zeranol (Z, a growth promoter widely used in beef industry in North American) residue on human health are still unknown. *Materials and Methods:* The effects of Z implantation on the growth of heifer pre-adipocytes were evaluated. The stimulatory effects of Z and estradiol-17 $\beta$  (E<sub>2</sub>) on the proliferation of pre-adipocytes isolated from control heifers and Z-implanted heifers were measured. Real-time PCR and Western-blotting analysis were performed to evaluate the expression of cyclin D1 and p53 at both mRNA and protein levels. *Results:* The growth of pre-adipocytes from heifers bearing for 2 months of Z-implants was about 12-fold faster than that observed in control heifers. The pre-adipocytes isolated from Z-implanted heifers were more sensitive to treatment with Z and E<sub>2</sub>. Z up-regulated the expression of cyclin D1 and down-regulated p53 in pre-adipocytes isolated from Z-implanted heifers. *Conclusion:* The implantation of Z increases body weight gain by enhancing growth of pre-adipocytes. The stimulation of pre-adipocytes division by Z and E<sub>2</sub> might be partially mediated by up-regulation of cyclin D1 and down-regulation of p53 at mRNA and protein levels.

Breast cancer remains one of the most prevalent neoplasms in the US. According to clinical cancer statistics, it was estimated that 192,370 new cases arise each year and 40,170 women would die from breast cancer in 2009 (1).

*Correspondence to:* Dr. Young C. Lin, 1900 Coffey Rd. Ohio State University, Columbus, OH 43210, U.S.A. Tel: +1 6142929706, Fax: +1 6142926473, e-mail: lin.15@osu.edu

**Key Words:** Breast cancer, Zeranol, pre-adipocytes, body weight gain, doubling time, cyclin D1, p53.

Epidemiological studies have suggested that there are many risk factors associated with breast cancer such as dietary fat and environmental estrogenic endocrine disruptors. The relationship between dietary fat intake and risk of breast cancer has been studied in ecological, etiological, and intervention research and animal experiments, yet the conclusion is still under debate. Rose *et al.* have reported that the quantity of dietary fat consumed does affect human breast cancer cell growth and expression of the metastatic phenotype (2). The association between dietary fat intake and reduced survival has been confirmed in both postmenopausal and premenopausal breast cancer patients (3, 4). However, opposing results from several studies have been reported. Thus, in a 20-year follow up cohort study, Kim and colleagues observed no association between total fat intake and breast cancer risk using the cumulatively averaged and updated percentage of energy derived from total fat in postmenopausal women (5). Another prospective cohort study performed by Swedish researchers showed that total fat, monounsaturated fat, polyunsaturated fat and saturated fat were not associated with overall risk of breast cancer (6). Many studies have been criticized for weak study design, poor dietary assessment tools, measurement errors, improper statistical analyses and the lack of proper quantification of fat intake among the study population. Therefore, it is difficult to establish a robust association between dietary fat and the development of breast cancer. Additional studies with appropriate experimental design are needed to provide a better perspective in examining this critical issue.

A well established risk factor is estrogen agonists, non-steroidal zeranol (Z). Z is a beta-resorcylic acid lactone, a mycotoxin which originates from fungi of the *Fusarium* family. It is one of the six growth promoters approved by the FDA and widely used in feedlot beef production in the US, Canada and other countries. Recent studies have reported that Z is not as safe as previously thought. In the early 1970s,

Peters found that Z and its precursor, zearalenone have strong estrogenic effects (7). It was shown by Sheir *et al.* that the estrogenic activity of Z was higher than that of zearalenone in stimulating the growth of cultured MCF-7 cells (8). Additionally, Takemura *et al.* found that Z was able to occupy the ligand-binding domain of human estrogen receptor alpha and beta (ER $\alpha$  and ER $\beta$ ) in a strikingly similar manner to estradiol-17 $\beta$  (E $_2$ ) (9). Our laboratory has reported that long-term exposure to either Z or E $_2$  can induce transformation of human breast epithelial MCF-10A cells with similar potency, possibly through the oxidation-reduction (redox) pathway and/or ER  $\beta$ -mediated pathway (10). Our previous work also found that both E $_2$  and Z have the ability to suppress by 80% the mRNA expression of the estrogen-regulated candidate tumor suppressor gene, protein tyrosine phosphatase  $\gamma$  (PTP $\gamma$ ) in primary cultured human normal breast tissues (11). Furthermore, we recently reported that a protein disulfide isomerase, which is considered a potential cancer marker, was up-regulated 5-fold in primary cultured normal human breast epithelial cells when exposed to Z (12). The European Union prohibited the import of beef products from the US or Canada where Z serves as a growth promoter. This issue indicates that concerns over the use of Z in the beef industry still remain, and the proper evaluation of the safety of Z is of both public health and economic importance.

The long-term goal of our research is to investigate the effects of Z residues in beef and their effects on human health. The present study was focused on the effect of Z on the growth of pre-adipocytes (PA) isolated from both Z-implanted (RMIHPA) and control heifers (CHPA).

## Materials and Methods

**Animal treatment and tissue sampling.** Ralgro<sup>®</sup> Magnum (RM, commercial Z product) was purchased from Schering-Plough Corp, Kenilworth, NJ, USA, in the form of cartridges, each containing 72 mg Z. Twenty cross-bred Angus beef heifers (about one year old) purchased from the Department of Animal Science were randomly divided into two groups according to their initial body weights. The treatment group consisting of 10 heifers was subcutaneously implanted with 72 mg RM in the ear while the other 10 heifers without implantation were employed as the control group. RM implantation was performed on Sept 14, 2007, through in strip cartridges with the Dur-A-Tract<sup>®</sup> pellet injector (Schering-Plough Corp). Both groups were raised at the same Beef Cattle Barn located at the Ohio State University Livestock Facilities. The body weight of each heifer was measured twice a month. Biopsies were taken by a veterinary surgeon at the beginning of each month from September to December, 2007. Muscle and fat tissue samples were taken from the dorsal side of each heifer and divided into four portions. The first portion of the tissue was fixed in 4% formalin for histopathologic examination. The second portion was transferred to a 50 ml centrifuge tube for proteomic analysis, and the third tissue portion was collected in a cryovial for RNA isolation. The remaining fat tissues were used as the material for PA isolation. A 100 ml blood sample from the jugular vein, urine and feces were also collected at the same time

from the selected heifers. All of the biological samples were then placed into a coolant with ice for transfer to the laboratory. Five heifers in the control group and five heifers in the experiment group were slaughtered at the slaughter house of Meat Research Laboratory, the Department of Animal Science, Ohio State University, after two months of Z-implantation and the remaining 10 heifers were slaughtered at the same place after four months of Z implantation. Blood, adipose tissue, muscle, mammary gland, liver, kidney, pancreas, ovary and pituitary gland were collected during slaughter. Small pieces of each organ were fixed in 4% formalin immediately after transfer to the laboratory, and the remaining parts were stored at -20°C for future analysis.

**Adipose tissue digestion, PA isolation and culture.** Fat tissues harvested from two randomly selected control and RM-implanted heifers (after 2 months of RM implantation) were sterilized in 70% ethanol for 30 seconds and then washed three times with fresh Dulbecco's modified Eagle's medium and Han's F12 Nutrient Mixture (DMEM/F12) (Atlanta Biologicals, Lawrenceville, GA, USA). The tissues were then minced and digested for 18 h at 37°C with 0.1% collagenase solution (Sigma, St. Louis, MO, USA). The digested mixture was centrifuged at 200  $\times$  g (1200 rpm) for 5 minutes at 25°C. The cell pellet was washed three times and then re-suspended in 10 ml DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals). PA was identified under a phase-contrast microscopy (Nikon Inc. Garden city, NY, USA) and pictures were taken using a digital camera connected to a computer (Digital sight DS-Fi1; Nikon Corporation, Japan). The cell suspension was then transferred to a 75 cm<sup>2</sup> culture flask. The media were changed every two days. When the cells reach 85-90% confluence, they were washed once with 10 ml of calcium and magnesium-free phosphate-buffered saline (PBS, pH=7.3) and then treated with 3 ml of 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 2 minutes at 37°C. After most of the cells were dissociated from the flask, 10 ml of DMEM/F12 containing 5% FBS were added to neutralize the effect of trypsin. The suspension was then transferred to a 15 ml tube and centrifuged at 200  $\times$  g for 5 minutes. The supernatant was discarded, and the cell pellet was re-suspended in the DMEM/F12 medium with 5% FBS and sub-cultured into 75 cm<sup>2</sup> flasks.

**Doubling time (DT) measurement.** The first passage and the fifth passage (frozen in liquid nitrogen for 6 months) of PA derived from the control heifer (CHPAs) and PA derived from the RM implanted heifer (RMIHPAs) after 2 months' Z implantation were separately seeded in 25 cm<sup>2</sup> flasks with 5000 cells per flask in a volume of 5 ml DMEM/F12 supplemented with 5% FBS. The cells were grown for several days and counted every 6 h using the trypan blue exclusion method, with a hemacytometer (13). Experiments were performed in triplicate for each time-point and a cell proliferation curve was generated. DT was calculated in the growth log phase by using the formula:  $N_t = N_0 \times 2^{t/DT}$ , where  $N_t$  and  $N_0$  are the cell numbers at different time-points  $T_t$  and  $T_0$  ( $T_t > T_0$ ,  $t = T_t - T_0$ ) (14).

**Cell proliferation analysis.** Z and E $_2$  (Sigma, ST. Louis, MO, USA) were prepared in dimethyl sulfoxide (DMSO, Sigma) at a stock concentration of 1  $\mu$ M. Both CHPA and RMIHPA cells were separately seeded in a 96-well plate at a density of 5000 cells per well and cultured in 100  $\mu$ l DMEM/F12 medium with 5% FBS supplement overnight. The media were replaced using DMEM/F12 containing

0.2% BSA and cultured for another day. The cells were treated with 0.1% (DMSO, as vehicle control), 0.2, 1, 5 and 25 nM Z or E<sub>2</sub> in DMEM/F12 with 0.2% BSA for 24 h. Cell proliferation was measured using a non-radioactive cell proliferation assay according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). To each well, 20 µl phenazine methosulfate (PMS) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) mixture (20:1) were added. The plates were placed in a humidified incubator (5% CO<sub>2</sub>; 95% air, 37°C) until the brown color was observed in each well. The optical density for each well was measured under UV light at wavelength 490 nm using a kinetic microplate reader and analyzed by Softmax Pro (version 2.1.1) software (Molecular Devices Corporation, Menlo Par, CA, USA).

**Total RNA extraction.** CHPAs and RMIHPAs were separately plated in a 6-well plate at a density of 1×10<sup>5</sup> viable cells/well in 5 ml DMEM/F12 supplemented with 5% FBS and cultured overnight. The media were then changed to DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC, Dextran T-70; Pharmacia; activated charcoal, Sigma) treated FBS to minimize the effects of growth factors in FBS. After 24 h, both cell types were then treated with 2, 10 or 50 nM of Z or 0.1% DMSO as vehicle control in DMEM/F12 supplemented with 5% DCC-treated FBS for 24 h. The total RNA was isolated in 1 ml TRIZOL® Reagent (Invitrogen) according to the manufacturer's instructions.

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** RNA concentrations were determined using a DU®-70 spectrophotometer (Beckman Instruments Inc. Fullerton, CA, USA). RT-PCR was performed in a gradient mastercycler (Eppendorf®, Westbury, NY, USA) in which 1 µg of total RNA was reverse transcribed at 37°C for 50 minutes and 70°C for 15 minutes in the presence of 5 mM dATP, dCTP, dGTP and dTTP (Invitrogen), 10 µl 5× 1st strand buffer (Gibco BRL, Carlsbad, CA), 5 µl 0.1M dithiothreitol (DDT) (Gibco BRL), 1 µl RNase inhibitor (Gibco BRL), 1 µl of 50 mM random hexamers, and 3 µl nuclease-free sterile distilled water and 1 µl (200 units) of Moloney murine leukemia virus reverse transcriptase (M-MLV) (Invitrogen) in a total volume of 50 µl.

**Real-time PCR analysis of cyclin D1 and p53 mRNA expression.** The primers and annealing temperature were optimized for the amplification of the following PCR products: cyclin D1, p53 and 36B4 (internal control). The efficiency of each reaction was also determined and were all 98-110%. Real-time PCR was performed in Stratagene Mx3005p (Agilent Technologies, Cedar Creek, TX, USA) with 2 µl of the newly synthesized cDNAs used as a template for the reaction in a total volume of 20 µl reactants, which included 10 µl of 2× real-time PCR master mix (Applied Biosystems, Woolston, Warrington, UK), 3 µl ultra pure water and 5 µl of primer mixer. The reactants were first incubated at 95°C for 10 minutes followed by 40 cycles of amplification with each cycle consisting of denaturing at 95°C for 30 seconds, annealing at 55°C for 1 minute and elongating at 72°C for 1 minute. A dissociation curve was also generated at the completion of PCR in order to ensure that the reaction produced the correct products as anticipated. The primer sequences for cyclin D1 were 5'-AACGGCTTCCTC TCCTATC 3' (sense) and 5'-GTCCAAGTTCTGCTGCTG-3' (antisense); for p53 were 5'-GGTGAAGTCCTCCTGAAG-3' (sense) and 5'-ATGTCCAGATGCCTCAAG-3' and for 36B4 were 5'-ACATGCTCAACATCTCCC-3' (sense) and 5'-GCGGCACT

TCTCCTGCTCC-3' (antisense). The results of the relative mRNA expression (cyclin D1 or p53 to 36B4) of the PA were analyzed using the  $\Delta\Delta C_t$  method (15)

**Western blotting assay.** CHPAs and RMIHPAs were plated in a 60 mm culture dish at a density of 2×10<sup>5</sup> viable cells/well within 5 ml DMEM/F12 containing 5% FBS and cultured overnight. The media were replaced with DMEM/F12 supplemented with 5% DCC treated FBS and the culture continued for another 24 h. The cells were then treated with 2, 10 or 50 nM of RM or 0.1% DMSO as a vehicle control for 24 h. Proteins were isolated using M-PER® mammalian protein extraction reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, the culture media were discarded from each dish and 200 µl of extraction reagent was added. Each dish was placed on an orbital shaker for cell digestion over 5 minutes. The digested products were collected and transferred to a fresh 1.5 ml eppendorf tube, centrifuged at 10,000 × g for 5 minutes, and the supernatant containing the total protein was then transferred to a new 0.5 ml centrifuge tube. Protein concentrations were measured using a Micro BCATM protein assay reagent kit (Pierce) following the manufacturer's protocol. Fifty µg of proteins from each treatment group were separated by SDS-PAGE and then transferred to a piece of polyvinylidene fluoride (PVDF) membrane. The membrane was first blocked in PBS Tween-20 (PBST) containing 10% fat free milk for 1 h at room temperature and then incubated with primary antibody (cyclin D1, 1: 1000 dilution; p53, 1: 1000 dilution, Cell Signaling Technology®, Danvers, MA, USA;  $\beta$ -actin, 1:2000 dilution, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) for 1 h. The membrane was washed in PBST three times, for 5 minutes each time. Then, the membrane was incubated with the second antibody (donkey anti-goat IgG-horseradish peroxidase (HRP) for  $\beta$  actin, 1: 2000, Santa Cruz Biotechnology, Inc.; ECLTM anti-rabbit IgG-HRP for detecting cyclin D1, and p53, 1:1000, Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) for 1 h at room temperature. After washing the membrane in PBST three times, it was then incubated with ECL-Plus Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 5 minutes and then the image of the membrane was taken by a FujiFilm LAS-300 imagine system (Fujifilm Medical Systems USA Inc., Stamford, CT, USA). The protein ratios of cyclin D1, p53 to the internal standard  $\beta$ -actin were calculated by the density of specific bands using Multi-Gauge (v3.0, Fujifilm Medical Systems USA Inc.).

**Statistical analysis.** The body weight, DT and cell proliferation assay results are presented as the mean and 95% confidence interval (CI). Student's *t*-test was performed using Minitab statistical software for Windows Version 15.1.1.0 (Minitab Inc., State College, PA, USA). The statistical differences between the ratio of cyclin D1, p53 and 36B4 mRNA expression in CHPAs and RMIHPAs were determined using one-way ANOVA for each independent treatment group. A *p*-value less than 0.05 was considered statistically significant.

## Results

**Effect of RM on body weight gain.** The initial average body weight of the control group was 792 kg (95% CI=584.2 to 999.8) and the experimental group was 794.6 kg (95% CI=649.2 to 940.0). There was no statistically significant difference between the two groups (*p*=0.958). Table I shows



Table I. Average body weight gained in heifers of the control group and heifers implanted with Ralgro® Magnum.

Month	Average body weight gained (95% CI) (kg)		p-Value
	Control group	Ralgro® Magnum implantated group	
1	68.30 (6.88 to 129.70)	75.00 (0.00 to 157.0)	0.690
2	117.22 (38.98 to 195.46)	150.30 (94.77 to 259.14)	0.140
3	188.20 (134.90 to 241.50)	227.40 (186.24 to 268.56)	0.038
4	206.50 (139.37 to 273.60)	259.33 (190.57 to 328.10)	0.047

CI: Confidence interval.

that the body weight gain in the RM-implanted group was significantly larger than that in the control group after RM had been implanted for 3 months ( $p<0.05$ ). After 1, 2, 3 and 4 months of RM implantation, the average body weight gain in the RM-implanted group was higher (7 kg,  $p=0.69$ ; 33 kg,  $p=0.14$ ; 39 kg,  $p=0.038$  and 53 kg,  $p=0.047$ ) than that in the control group.

Figure 1 illustrates the net body weight gain of two heifers randomly selected from the control group and RM-implanted group during 0- 90 days period. The net body weight gain in the RM-implanted heifer was larger than that of the control heifer at each time-point after RM implantation.

**DT of CHPAs and RMIHPAs.** As Figure 2 shows, the growth of RMIHPAs was noticeably faster than that of CHPAs. The DT of RMIHPAs was about 9.4 h (95% CI=8.42 to 10.38 h), which was about 12-fold less than that of CHPAs (109.1 h, 95% CI=84.8 to 133.4 h;  $p=0.02$ ). This result suggested that RMIHPAs divide rapidly after 2 months of RM implantation. The growth of the fifth passage of CHPAs and RMIHPAs which had been frozen in liquid nitrogen for 6 months was also compared and the RMIHPAs grew about 1.5-fold faster than the CHPAs (data not show).

**Effect of Z and  $E_2$  on proliferation of RMIHPAs and CHPAs.** Both Z and  $E_2$  stimulated RMIHPAs more potently as compared to CHPAs. Figure 3 shows that following 0.2, 1, 5, 25 nM Z treatment, the percentage increase of cell number in CHPAs was 45, 48, 45 and 43%, respectively, while at the same Z concentration, it was 218, 218, 245 and 236% in RMIHPAs. Figure 4 illustrates that treatment with 0.2, 1, 5 and 25 nM of  $E_2$  increased the growth of CHPAs to 35, 31, 36 and 23%, respectively. The percentage increase of RMIHPAs was 200, 218, 263 and 254% with exposure to the same concentrations of  $E_2$ .

**Cyclin D1 and p53 mRNA expression.** In order to explore the mechanisms underlying the different growth rate and response to treatment with Z and  $E_2$  in the CHPAs and RMIHPAs, cyclin D1 and p53 mRNA expression was investigated in both PA groups treated with 2, 10 or 50 nM of Z for 24 h using

real-time PCR. Ten and 50 nM Z treatment significantly increased cyclin D1 mRNA expression in the RMIHPAs by about 1.4- (95% CI=1.2 to 1.59,  $p=0.02$ ) and 2.03- (95% CI=1.25 to 2.81,  $p=0.047$ ) fold compared to CHPAs, while Z treatment did not significantly affect cyclin D1 mRNA expression in the CHPAs (Figure 5).

Figure 6 shows that Z treatment in CHPAs and RMIHPAs down-regulated p53 mRNA expression in a dose-dependent fashion. For CHPAs, 2, 10 and 50 nM Z treatment reduced the expression of p53 mRNA by 5, 36, and 42%, respectively. For the RMIHPAs, the same concentrations reduced the p53 mRNA expression by 4, 26 and 47%. No statistical differences of p53 mRNA expression were found between CHPAs and RMIHPAs at the same concentration of Z treatment.

**Effects of RM on cyclin D1 and p53 protein expression.** The expression of cyclin D1 protein in both CHPAs and RMIHPAs increased after treatment with Z (Figure 7). In the CHPAs (A), 2, 10 and 50 nM of RM increased cyclin D1 protein expression by 3.1, 10.9 and 22.5%, respectively. No significant modification of p53 protein expression was observed in the CHPAs. In the RMIHPAs (B), the same concentrations of RM increased cyclin D1 protein expression by 5, 31 and 96% and reduced p53 protein expression by 36.7, 47.8 and 56.9%, respectively.

## Discussion

Zearalenone, a precursor of Z has been classified as a chemical with positive evidence of carcinogenicity (16), and in 1983, the International Agency for Research on Cancer categorized Z as a potential carcinogenic compound. The current research found that RMIHPAs grew much faster and were more sensitive in response to treatments with estrogenic agents than were CHPAs. Our preliminary data demonstrated that the serum harvested from heifers implanted with RM for one month significantly stimulated the proliferation of human breast cancer MCF-7 cells and primary cultured human normal breast epithelial cells (unpublished data). It suggests that RM-implanted beef might have promoting effects on the growth of both normal and cancerous breast epithelia.

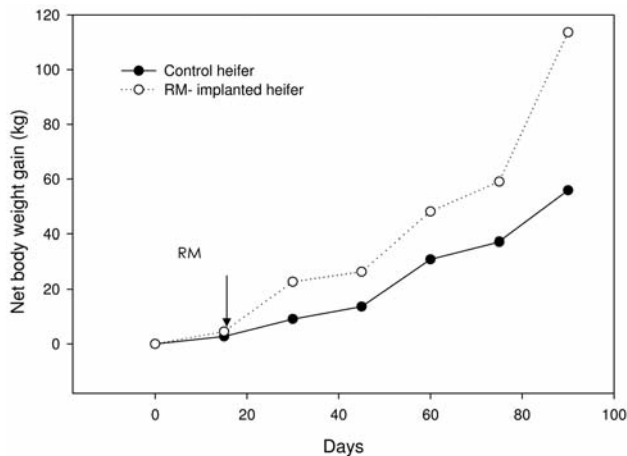


Figure 1. Comparison of net body weight gain between a control heifer and a heifer implanted with RM. During the three-month period, body weights of a randomly selected treated heifer implanted with Ralgro<sup>®</sup> Magnum (72 mg) and a control heifer were monitored at different time-point.

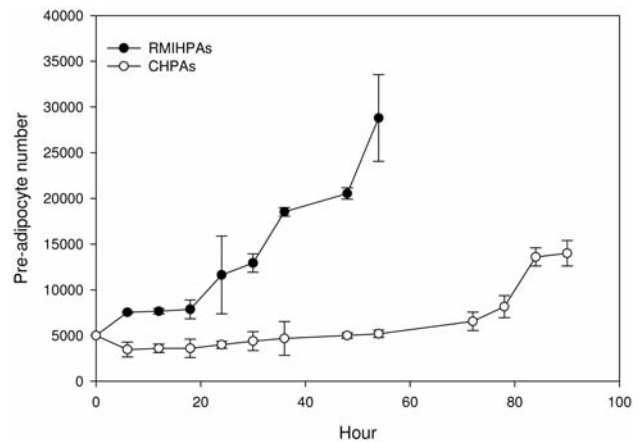


Figure 2. Cell proliferation curve of CHPAs and RMIHPAs. Doubling time was calculated in the growth log phase and was 9.4 h (95% CI=8.42 to 10.38 h) for RMIHPAs which was about 12-fold less than for CHPAs (109.1 h, 95% CI=84.8 to 133.4 h). The difference between the two DTs was statistically significant ( $p=0.02$ ).

The average body weight gain in the RM-implanted heifers was larger than that of the control heifers at different time-points. We surmise that adipose tissue plays an important role in body weight gain. This hypothesis is based not only on the present results, but also on the well-established finding that skeletal myoblasts and adipose cells are derived from a common mesodermal stem cell. Both cells have a closer relationship in the developmental lineage than other somatic cells (17). It is therefore possible PAs differentiate into adipose, muscle and bone tissues following RM implantation leading to weight gain. The function and mechanisms of PAs in myogenesis and osteogenesis heifers remain to be clarified.

In order to elucidate the possible health risks resulting from Z administration, possible mechanisms involved in the growth stimulation by Z on heifer PAs were investigated. The results showed that the expression of cyclin D1 mRNA was exclusively up-regulated in RMIHPAs, and the expression of p53 mRNA was down-regulated in both CHPAs and RMIHPAs. Cyclin D1 expression is induced by estrogen and growth factors and acts as a sensor for growth signal and thus activates downstream cyclin family members. Other research using transgenic or knock-out mice found convincing evidence indicating that cyclin D1 overexpression was involved in mammary carcinoma development (18-19). The oncogenic capability of cyclin D1 has been well established in various studies (18, 20, 21). It has been reported that the mitogenic effects of estradiol-17  $\beta$  ( $E_2$ ) are mediated by cyclin D1. Said and colleagues found that estrogen induced cyclin D1 expression in murine mammary epithelial cells (22). Although Z is a nonsteroidal agent, it has estrogenic activities and, of note, zearalenone was able to stimulate human breast cancer cells to enter the cell cycle (23). Z is 3- to 4-fold more potent

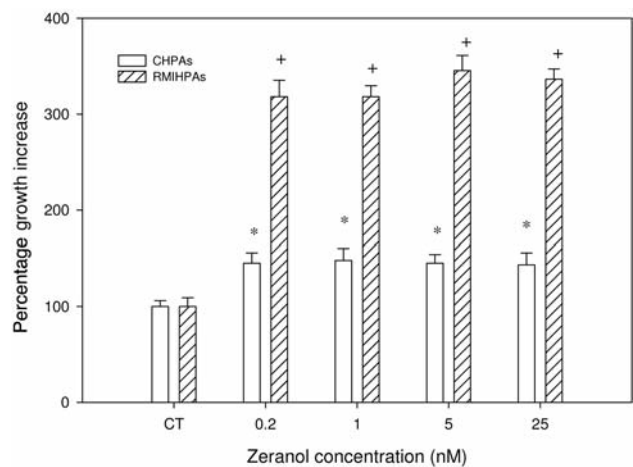


Figure 3. Effect of zeranol on RMIHPAs and CHPAs. Non-radioactive cell proliferation assay was performed after 24 h treatment with zeranol or DMSO as the vehicle control. Compared to each control, \* and +: statistically significantly different compared to their respective control ( $p<0.05$ ,  $N=8$ ).

as an estrogen agonist than zearalenone (24). Our preliminary data illustrated that Z can stimulate human breast cancer, MCF-7, MCF-10A and MDA-MB-231 cell growth. It can also stimulate primary cultured human normal and neoplastic breast epithelial cell proliferation. These cumulative results suggest the potential risk of Z treatment for the human breast. As for the cyclin D1 gene, the p53 gene is a well-studied tumor suppressor gene which is directly involved in cell cycle regulation and one of the most commonly mutated genes described in human neoplasia. It was reported that the

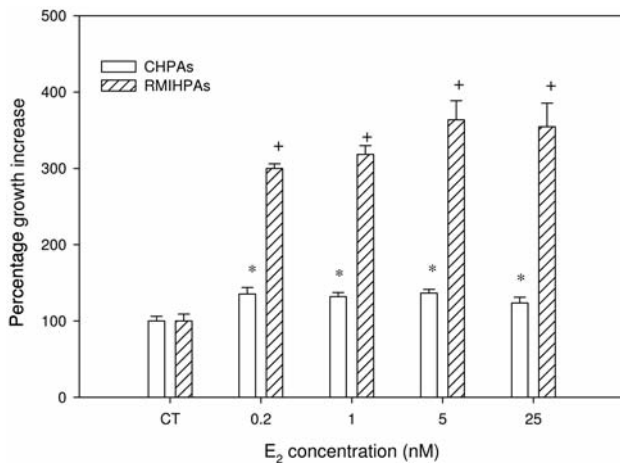


Figure 4. Effect of E<sub>2</sub> treatment on CHPA and RMIHPA proliferation. Each bar represents the percentage growth increase as compared to each control group. \* and +: statistically significantly different compared to their respective control ( $p < 0.05$ ,  $N = 8$ ).

occurrence of p53 mutations in sporadic breast carcinomas was about 20% (25) and p53 expression status was a significant molecular marker of the response to first-line endocrine therapy and for predicting time to endocrine therapy failure in recurrent breast cancer with hormone-sensitive disease (26). In the current study, Z down-regulated both mRNA and protein expression of p53 in RMIHPAs. Whether Z has a similar effect on human normal breast cells or primary cultured human normal breast epithelia needs to be further investigated.

Interestingly, the RMIHPAs divided much faster and responded dramatically to the treatment with Z and E<sub>2</sub>, even after long-term storage and several passages, suggesting that a certain form of “bio-imprinting” might be present. However, much work still needs to be conducted on this topic.

These results raise the concern that dietary fat containing bioactive Z residue might have the ability to perturb the expression of cyclin D1, a key regulatory protein of the cell cycle and alter tumor suppressor gene p53. Whether these aberrant changes occur in humans following the intake of Z-containing beef is an important issue that warrants in-depth investigation.

Recently, we found that Z treatment (2 or 10 nM) up-regulated aromatase mRNA expression 16- to 18-fold in PAs isolated from human normal breast tissue (our unpublished data). In humans, aromatase is expressed in a variety of cells including the ovarian granulosa cell, the placental syncytiotrophoblast and the testicular Leydig cell, as well as various extraglandular sites including the brain and skin fibroblasts (27). Importantly, aromatase is also expressed in human adipose tissues and this is associated with the high risk of breast cancer in obese women. Aromatase possesses the capacity of converting androgen to estrogen and thus

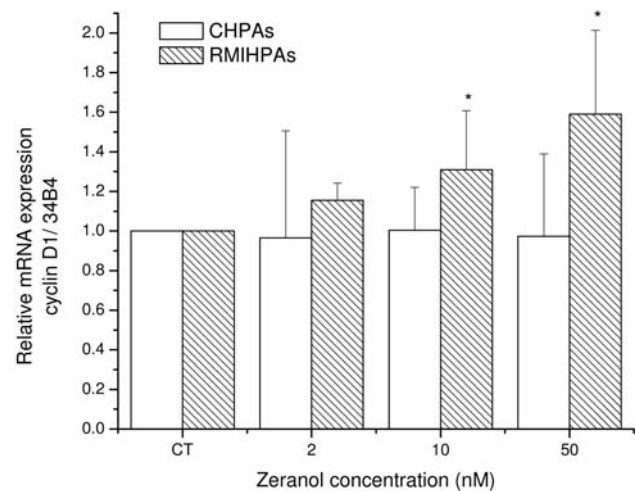


Figure 5. Comparison of the effect of Zeranone (Z) on cyclin D1 mRNA expression in CHPAs and RMIHPAs. Cells were treated with Z or vehicle (0.1% DMSO) control for 24 h. 36B4 was used as internal standard. The mRNA ratios of cyclin D1 to 36B4 were calculated using the  $\Delta\Delta C_t$  method after real-time PCR was performed. Each bar represents the average plus standard error of three independent experiments. \*: Statistically significantly different as compared to the control ( $p < 0.05$ ).

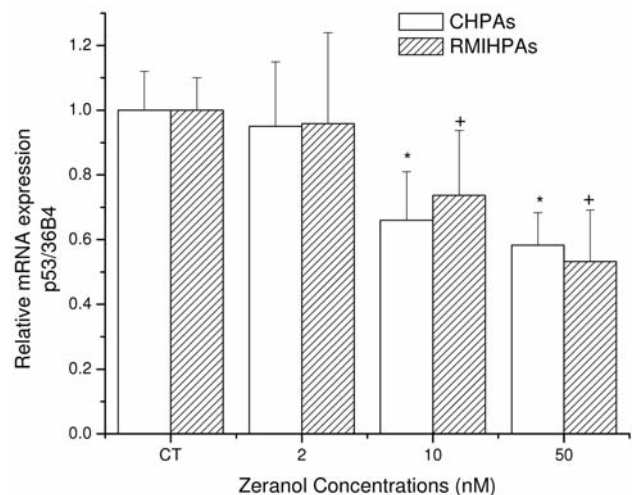


Figure 6. P53 mRNA expression in CHPAs and RMIHPAs. Cells were treated with Z or vehicle (0.1% DMSO) control for 24 h. 36B4 was used as internal standard. The mRNA ratios of p53 to 36B4 were calculated using the  $\Delta\Delta C_t$  method after real-time PCR was performed. Each bar represents the average plus standard error of three independent experiments. \* and +: statistically significantly different compared to their respective control ( $p < 0.05$ ).

resulting in increased serum E<sub>2</sub>, finally leading to breast cancer initiation, promotion and progression (28). This implies that the potential adverse health effect of Z might be more serious in obese women than in normal weight women.

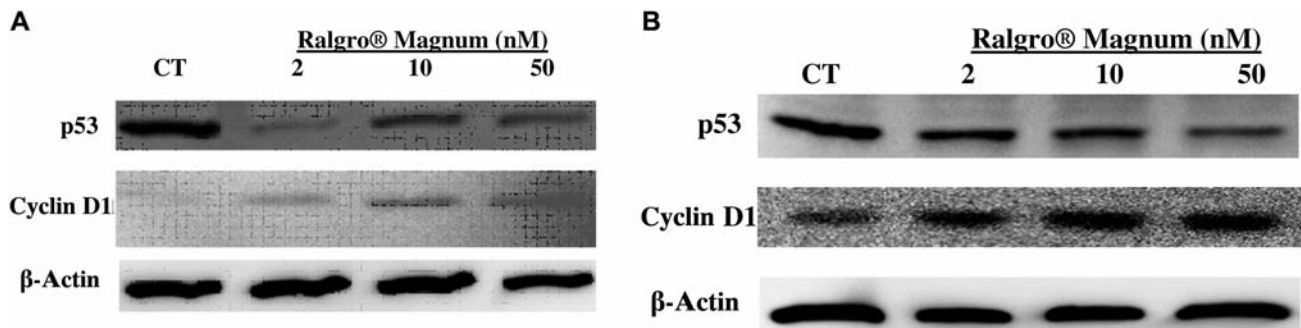


Figure 7. Effect of RM on p53 and cyclin D1 protein expressions in (A) CHPAs and (B) RMIHPAs. Cyclin D1 and p53 protein expressions were evaluated after treatment with RM or 0.1% DMSO as vehicle control for 24 h by Western blotting analysis.  $\beta$ -actin was used to confirm equal loading of the total proteins.

Furthermore, leptin improved the sensitivity of human breast cancer cell line, MCF-7 Adr, to Z treatment (29). Leptin is mainly secreted by adipocytes, and plays an important role in breast cancer development (30). Caldefie-Chezet *et al.* reported that leptin is not expressed in normal breast tissue, but exists in tissues adjacent to malignant breast lesions (31). This, again, suggests a serious adverse health effect in obese women.

In conclusion, implantation of RM in beef heifers greatly induces PA growth, and at the same time bestows these cells with higher sensitivity to the growth-promoting activities of Z and E<sub>2</sub>. This might be related to up-regulation of cyclin D1 and down-regulation of p53 expression at both mRNA and protein levels.

### Acknowledgements

We thank Dr. Walter Threlfall and his team members for collecting the biological samples from the experimental heifers and injecting RM during the first stages of our project. We thank manager Martin Mussard and his team members for taking care of the heifers in the Beef Cattle Barn located at the Ohio State University Livestock Facilities. We also thank the anatomist Christopher Frasure for collecting all the tissue samples during the slaughtering. Thanks also go to the staff in the Histology/ Immunohistochemistry Laboratory of the Department of Veterinary Biosciences for preparing all the histological slides using the tissue samples we collected and fixed. Lastly, we thank the workers in the Meat Research Laboratory of the Department of Animal Science. This study was supported by NIH grants RO1 ES015212-01.

### References

- Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics, 2009. *CA Cancer J Clin* 59(4): 225-249, 2009.
- Rose DP, Connolly JM and Meschter CL: Effect of dietary fat on human breast cancer growth and lung metastasis in nude mice. *J Natl Cancer Inst* 83(20): 1491-1495, 1991.
- McEligot AJ, Largent J, Ziogas A, Peel D and Anton-Culver H: Dietary fat, fiber, vegetable, and micronutrients are associated with overall survival in postmenopausal women diagnosed with breast cancer. *Nutr Cancer* 55(2): 132-140, 2006.
- Saxe GA, Rock CL, Wicha MS and Schottenfeld D: Diet and risk for breast cancer recurrence and survival. *Breast Cancer Res Treat* 53(3): 241-253, 1999.
- Kim EH, Willett WC, Colditz GA, Hankinson SE, Stampfer MJ, Hunter DJ, Rosner B and Holmes MD: Dietary fat and risk of postmenopausal breast cancer in a 20-year follow-up. *Am J Epidemiol* 164(10): 990-997, 2006.
- Lof M, Sandin S, Laggiou P, Hilakivi-Clarke L, Trichopoulos D, Adami HO and Weiderpass E: Dietary fat and breast cancer risk in the Swedish women's lifestyle and health cohort. *Br J Cancer* 97(11): 1570-1576, 2007.
- Peters CA: Photochemistry of zearalenone and its derivatives. *J Med Chem* 15(8): 867-868, 1972.
- Shier WT, Shier AC, Xie W and Mirocha CJ: Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicon* 39(9): 1435-1438, 2001.
- Takemura H, Shim JY, Sayama K, Tsubura A, Zhu BT and Shimoi K: Characterization of the estrogenic activities of zearalenone and zeranol *in vivo* and *in vitro*. *J Steroid Biochem Mol Biol* 103(2): 170-177, 2007.
- Liu S and Lin YC: Transformation of MCF-10A human breast epithelial cells by zeranol and estradiol-17 $\beta$ . *Breast J* 10(6): 514-521, 2004.
- Liu S, Sugimoto Y, Sorio C, Tecchio C and Lin YC: Function analysis of estrogenically regulated protein tyrosine phosphatase gamma (PTPgamma) in human breast cancer cell line MCF-7. *Oncogene* 23(6): 1256-1262, 2004.
- Updike MS, Sawdy JC, Wang LS, Liu S, Huang YW, Ye W, Farrar WB, Lin YC and Wick M: Primary cultured human breast epithelial cells up-regulate protein disulfide isomerase in response to zeranol. *Anticancer Res* 27(1A): 407-410, 2007.
- Tennant JR: Evaluation of the trypan blue technique for determination of cell viability. *Transplantation* 2: 685-694, 1964.
- Poliseno L, Mariani L, Collecchi P, Piras A, Zaccaro L and Rainaldi G: Bcl2-negative MCF7 cells overexpress p53: implications for the cell cycle and sensitivity to cytotoxic drugs. *Cancer Chemother Pharmacol* 50(2): 127-130, 2002.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4): 402-408, 2001.
- Haseman JK, Crawford DD, Huff JE, Boorman GA and McConnell EE: Results from 86 two-year carcinogenicity studies



- conducted by the National Toxicology Program. *J Toxicol Environ Health* 14(5-6): 621-639, 1984.
- 17 Cornelius P, MacDougald OA and Lane MD: Regulation of adipocyte development. *Annu Rev Nutr* 14: 99-129, 1994.
- 18 Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A and Schmidt EV: Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 369(6482): 669-671, 1994.
- 19 Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ and Weinberg RA: Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82(4): 621-630, 1995.
- 20 Jiang W, Kahn SM, Zhou P, Zhang YJ, Cacace AM, Infante AS, Doi S, Santella RM and Weinstein IB: Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene* 8(12): 3447-3457, 1993.
- 21 Zhou P, Jiang W, Zhang YJ, Kahn SM, Schieren I, Santella RM and Weinstein IB: Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. *Oncogene* 11(3): 571-580, 1995.
- 22 Said TK, Conneely OM, Medina D, O'Malley BW and Lydon JP: Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, *in vivo*. *Endocrinology* 138(9): 3933-3939, 1997.
- 23 Dees C, Foster JS, Ahamed S and Wimalasena J: Dietary estrogens stimulate human breast cells to enter the cell cycle. *Environ Health Perspect* 105(Suppl 3): 633-636, 1997.
- 24 Mirocha CJ, Schauerhamer B, Christensen CM, Niku-Paavola ML and Nummi M: Incidence of zearalenol (Fusarium mycotoxin) in animal feed. *Appl Environ Microbiol* 38(4): 749-750, 1979.
- 25 Pharoah PD, Day NE and Caldas C: Somatic mutations in the *p53* gene and prognosis in breast cancer: a meta-analysis. *Br J Cancer* 80(12): 1968-1973, 1999.
- 26 Kai K, Nishimura R, Arima N, Miyayama H and Iwase H: p53 expression status is a significant molecular marker in predicting the time to endocrine therapy failure in recurrent breast cancer: a cohort study. *Int J Clin Oncol* 11(6): 426-433, 2006.
- 27 Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarnah B, Ito Y, Fisher CR, Michael MD *et al*: Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* 15(3): 342-355, 1994.
- 28 Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, Barbieri RL and Speizer FE: Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. *J Natl Cancer Inst* 90(17): 1292-1299, 1998.
- 29 Xu PP, Ye WP, Jen R, Lin SH, Kuo CT and Lin YC: Mitogenic activity of zeranol in human breast cancer cells is enhanced by leptin and suppressed by gossypol. *Anticancer Res* 29: 2009 (in press)
- 30 Castracane VD and Henson MC: *Leptin*. Springer Verlag. pp. 201-223, 2007
- 31 Caldefie-Chezet F, Damez M, de Latour M, Konska G, Mishellani F, Fusillier C, Guerry M, Penault-Llorca F, Guillot J and Vasson MP: Leptin: a proliferative factor for breast cancer? Study on human ductal carcinoma. *Biochem Biophys Res Commun* 334(3): 737-741, 2005.

Received June 15, 2009

Revised November 5, 2009

Accepted November 11, 2009