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Development: Peroxisome Proliferator Activated Receptors

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13. ABSTRACT <i>(Maximum 200 words)</i>  This IDEA grant tested our hypothesis that peroxisome proliferator-activated receptor (PPAR) are expressed in the mammary gland and regulated during physiologic and pathologic events. The PPARs are nuclear hormone receptors which bind to fatty acids as ligands and control transcription of lipid metabolic genes. Due to the link between dietary fat, obesity, and breast cancer, we postulated that PPARs are potential targets for chemoprevention or chemotherapy. Based on northern blot analyses, we determined that virgin murine mammary glands express mRNA for PPARs alpha, beta, and gamma. During pregnancy and lactation, the levels of PPARs alpha and gamma decreased. Levels of PPAR gamma mRNA were below detection in rodent mammary tumors. Using a rat model of mammary tumorigenesis, we found that synthetic PPAR ligands did not inhibit tumor formation. However, these agents appeared to stimulate regression of established tumors. Additional studies will be required to establish statistical significance to this work.			
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FOREWORD

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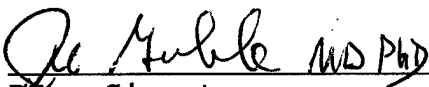
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#### (4) TABLE OF CONTENTS

<u>Page</u>	<u>Content</u>
1	Front Cover
2	SF 298
3	Foreword
4	Table of Contents
5	Introduction
6-12	Body
6-8	Experimental Methods
8-12	Results and Discussion
13	Conclusions
14	References
A-1-5	Appendix I

## (5) INTRODUCTION:

This IDEA proposal was originally designed to test the hypothesis that peroxisome proliferator activated receptors (PPARs) are differentially expressed during normal mammary gland development and carcinogenesis. The PPARs belong to the nuclear hormone receptor family [1]. Upon binding of natural ligands (free fatty acids, arachidonic acid metabolites) or synthetic compounds (thiazolidinediones, troglitazone, Wy14,643), the PPARs take on an active conformation and serve as transcriptional regulators of genes involved in lipid metabolism [1]. There are multiple PPAR genes, known as  $\alpha$ ,  $\beta$ , and  $\gamma$ , which can be expressed from multiple promoter elements leading to PPAR $\gamma$ 1 or PPAR $\gamma$ 2 isoforms [1]. Our previous studies had demonstrated that bone marrow stromal cells express all three PPAR genes [2]. During adipogenesis, the stromal cells specifically increased their level of PPAR $\gamma$ 2. Moreover, synthetic thiazolidinedione PPAR $\gamma$  ligands would induce stromal cell adipogenesis. Based on these findings, we expected that similar changes would occur in mammary stromal adipocytes during physiologic and pathologic events. Mammary stromal adipocytes were expected to express high levels of PPAR $\gamma$ 2 in virgin or non-lactating animals. When fat reserves were depleted during lactation, PPAR $\gamma$ 2 levels were expected to fall. It was unknown if (1) stromal cell PPAR $\alpha$  and PPAR $\beta$  levels would change in a coordinate manner and (2) epithelial cells expressed any of the PPAR genes.

There were compelling reasons to associate PPARs with breast cancer development. In vitro studies had shown that adipocytes promoted the proliferation of mammary epithelial cells as compared to fibroblast feeder layers [3,4]. Epidemiologic data had suggested that the percentage of calories obtained from fats influenced the incidence of breast cancer in a given population [5-7]. In North American and Northern European societies, breast cancer incidence was elevated in comparison to Sub-Saharan African and Asian countries [5,6]. In addition, the incidence of breast cancer was elevated in obese as compared to lean women in the U.S. Animal studies suggested that the type of dietary fats was also important [8-11]. While omega-6 fatty acids increased mammary tumor development, omega-3 fatty acids were associated with tumor protection [6,7]. Because the PPARs directly regulate key metabolic enzymes involved in lipid metabolism, we hypothesized that their expression in the mammary gland was an important component in carcinogenesis. The original specific aims/technical objectives were:

**Aim 1.** Determine the expression pattern of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  during normal mammary development (virgin, pregnant, lactating, involutinal gland) using protein and RNA analytical methods.

**Aim 2.** Determine the expression pattern of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$  in mammary tissues from rodents treated with 7,12-dimethylbenz(a)anthracene (DMBA) using protein and RNA analytical methods.

## (6) BODY:

### Experimental Methods:

**Northern Blots:** Mammary glands were dissected from virgin, pregnant or lactating 6-10 week old Balb/c or C57BL6 mice (obtained from breeding colonies maintained at the Oklahoma Medical Research Foundation Laboratory Animal Care Facility) or from Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis IN). All studies were approved by the institutional animal care and utilization committee. Tissues were frozen on dry ice and stored at -70°C prior to RNA preparation by a modification of the method of Chomzynski and Sacchi [12]. Special care was taken to centrifuge the homogenized tissue at 4°C prior to acid/phenol/chloroform extraction to remove the contaminating fatty layer. Northern blots prepared with 10 µg of total RNA per lane were hybridized with murine cDNA probes for actin and PPARs  $\alpha$ ,  $\beta$ , or  $\gamma$  or with an oligonucleotide probe for the 28s ribosomal RNA at 55° C according to a modification of the method of Church and Gilbert [13]. The cDNA probes were labeled with random hexamers according to the method of Feingold and Vogelstein [14] and the oligonucleotide was labeled with terminal deoxytransferase.

**Quantitative PCR:** Total RNA (5 µg) isolated from virgin murine mammary glands was reverse transcribed according to published methods [2]. Competitive DNA fragments specific for PPAR $\gamma$ 1 and PPAR $\gamma$ 2 were prepared using the following combination of forward (F) and reverse (R) oligonucleotide pairs:

PPAR $\gamma$ 1 Oligo #1F: TGTGTGACAGGAAACAGCTATGACCATG

PPAR $\gamma$ 1 Oligo #2F: TTTGAGCTCTTCTGACAGGACTGTGTGACAG

PPAR $\gamma$ 2 Oligo #1F: TGAAACTCTGGAAACAGCTATGACCAT

PPAR $\gamma$ 2 Oligo #2F: TTTGAGCTCGCTGTTATGGGTGAAACTCTG

PPAR $\gamma$ 1/2 Oligo #1R: ATGCAGGTTTCGTAAAACGACGGCCAGT

PPAR $\gamma$ 1/2 Oligo #2R: TTTGAGCTCATAAGGTGGAGATGCAGGTTTC

The first reaction was conducted with either the unique PPAR $\gamma$ 1 or  $\gamma$ 2 forward primer #1F and the common reverse primer #1R, using pBluescript SKII as template to generate 246 bp DNA fragments. These products then served as template in a second reaction using the appropriate PPAR $\gamma$ 1 or  $\gamma$ 2 forward primer #2F together with the common reverse primer #2R to generate distinct 287 bp PPAR $\gamma$ 1 or PPAR $\gamma$ 2 products. Reactions were performed using a Perkin-Elmer Cetus DNA Thermal Cycler for 35 cycles for 45 seconds at 94°C, 45 seconds at 55° C, and 2 minutes at 72 °C. The final 287 bp products (competitor DNA fragments) were purified on a non-denaturing acrylamide gel and their concentration determined by densitometry performed on an ethidium bromide stained analytical gel with a Stratagene Eagle Eye Video Imager system (San Diego CA). Quantitative PCRs were performed with serial ten-fold dilutions of the competitor DNA fragment at concentrations ranging from 25 pg to 0.025 fg of DNA in a 50 µl volumes containing equal quantities of reverse transcribed virgin mammary gland cDNA (equivalent to 0.7 µg RNA per reaction), 0.04 OD<sub>260</sub> units of oligos #2F and #2R, 1 X reaction buffer (Promega Madison WI), 1.5 mM MgCl<sub>2</sub>, and 200 µM dNTP. Reaction products were analyzed on 6% non-denaturing acrylamide gels.

**Culture of NMuMG Cells:** The NMuMG cell line (ATCC CRL 1636) was obtained from the American Type Culture Collection (Herndon VA) and cultured in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% fetal bovine serum (Hyclone, Logan UT), 100 units penicillin/ml, 100 µg streptomycin/ml, 1 mM glutamate, and 1 mM sodium pyruvate. Cells ( $5 \times 10^4$ ) in a 2 ml volume were inoculated on a 35 mm plate, cultured overnight at 37° C in 5% CO<sub>2</sub>, and induced with the thiazolidinedione, BRL49653 (10 µM) (obtained courtesy Dr. D.C. Morris, Glaxo/Wellcome, Research Triangle Park NC) and/or the MAP kinase inhibitor, PD98059 (5 µM) (New England Biolabs, Beverly MA). Control cultures were treated with the carrier vehicle (0.2% DMSO) alone. Cultures were maintained with fresh media containing the inductive agents changed every three days. Plates were harvested for total RNA on days 3, 6, or 8 following induction. Representative cultures were fixed in paraformaldehyde and stained for neutral lipids with Oil Red O.

**Immunohistochemistry:** Tissues from virgin, pregnant or lactating mice or tumors from DMBA exposed rats were fixed in 10% formalin/phosphate buffered saline and paraffin embedded. Tissues were sectioned (5 µm thick) onto Superfrost Plus slides (Fisher Scientific, Dallas TX), deparaffinized, and the antigen unmasked by microwave treatment in 10 mM citrate buffer pH 6.0. Alternative unmasking procedures were performed using pepsin, proteinase K, or saponin. Immunohistochemistry was performed with (1) an affinity purified goat polyclonal antibody prepared against the common C terminal peptide of the PPAR $\gamma$  proteins (amino acids 482-499 of PPAR $\gamma$ 2); (2) a rabbit antiserum prepared against a unique domain of the PPAR $\gamma$  protein (amino acids 294-298 of murine PPAR $\gamma$ 2) (Cat # SA-206, BioMol, Plymouth Meeting, PA); or (3) a rabbit antiserum prepared against amino acids 2-21 of PPAR $\alpha$  (cross reactive with murine PPAR $\alpha$ ) (Cat # sc-1985, Santa Cruz Biotechnology, Santa Cruz, CA). Tissues were incubated with preimmune goat or rabbit antibodies or the specific anti-PPAR antibodies for 60 minutes (longer incubations of up to 18 hours showed no appreciable difference in staining pattern). In some experiments, the peptide immunogen used to develop the affinity purified antibody against the murine PPAR $\gamma$  C terminus was included in the incubation to act as a specific competitor. The primary antibodies were detected with anti-goat or anti-rabbit avidin-biotin-horseradish peroxidase coupled reagents (Santa Cruz Biotechnology, Santa Cruz CA). Slides were counter stained with hematoxylin and eosin (H&E) or methyl green. To allow for direct comparison between specimens, immunohistochemical studies were performed simultaneously to control for chromogen intensity. Photomicrographs were captured using an AutoCyte Image Analysis system with an Image Manager software package (Elon College, NC) and processed using the Microsoft Powerpoint program.

Additional human normal and malignant mammary gland tissues were obtained from archival materials within the Department of Pathology, University of Oklahoma Health Sciences Center. Patient confidentiality was maintained in accordance with the Institutional Review Board guidelines.

***In situ* Hybridization of PPAR mRNAs:** Fresh frozen tissue sections of murine mammary tissue was hybridized with digoxigenin labeled oligonucleotide sense and antisense probes for

murine PPAR $\alpha$  and PPAR $\gamma$  according to published methods (Boehringer-Mannheim, Indianapolis IN).

Treatment with 7,12-dimethylbenz(a)anthracene (DMBA) and dietary treatment: All studies were approved by the University of Oklahoma Health Science Center Institutional Animal Review and Utilization Committee and conducted under the supervision of the veterinarian staff (Dr. Gary White, Department Chairman). Virgin 55 day old Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis IN) were gavage fed 20 mg DMBA (Sigma Chemical Co, St. Louis MO) suspended in 1 ml of stripped corn oil [15]. Animals were fed a regular chow diet containing approximately 5% of the calories from fat. One week after DMBA exposure, single cohorts of 16 animals were placed on diets supplemented with no additional agents (Control, Cohort #1), 0.1% (wt:wt) troglitazone (Parke Davis, Ann Arbor MI) (Troglitazone, Cohort #3) or 0.1% (wt:wt) Wy14,643 (Eagle Pilcher, Lenexa KS) (Wy14,643, Cohort #4). An additional cohort received a slow-release tamoxifen pellet designed to release 1.5 mg of tamoxifen per kilogram body weight per day (Tamoxifen, Cohort #4) (Innovative Research of America, Sarasota FL); all other cohorts received a placebo pellet instead. Animals were followed weekly for body weight, tumor number, and tumor size. Animals were euthanized 18 weeks after the DMBA exposure. Plasma was harvested for serum lipid analysis (performed by the Lipid Studies Lab, Oklahoma Medical Research Foundation) and liver, ovary, and uterine weights determined. Tumors were preserved in 10% formalin/phosphate buffered saline (Fisher, Dallas TX), paraffin embedded, sectioned, and stained with hematoxylin/eosin. Histologic analysis was performed by Dr. Stan Lightfoot, Department of Pathology, OUHSC, in accordance with the classification of Russo et al [16]. Additional animals (64) were treated with DMBA and kept on a regular chow diet without any additional treatment until the appearance of their first tumor. When the primary tumor exceeded an area of 64 mm<sup>2</sup>, animals were assigned randomly to either a Control (Cohort #5), Tamoxifen (Cohort #6), Troglitazone (Cohort #7), or Wy14,643 (Cohort #8) group. Tamoxifen animals were implanted with a slow release tamoxifen pellet (1.5 mg/kg body weight/day) while all other groups received a placebo pellet. Animals were placed on unsupplemented diets (Control Cohort #5, Tamoxifen Cohort #6) or supplemented diets with 0.1% Troglitazone or 0.1 % Wy14,643. Tumor size and number were followed over the next 6 weeks, at which time the animals were euthanized and harvested as described above. Primary tumors were categorized as “progressing” (greater than 30% increase in area size over 6 week period), “stasis (less than 30% change in area size over 6 week period), or “regressing” (greater than 30% decrease in area size over 6 week period), according to the protocol of Bischoff et al [17].

## **Results and Discussion:**

Detection of PPAR mRNAs in mammary tissues and cell lines: Based on northern blots, the mRNA levels of PPAR $\alpha$  and PPAR $\gamma$  were abundant in mammary tissue isolated from virgin mice but declined during pregnancy and lactation. In contrast, the PPAR $\beta$  mRNA was constitutively expressed independent of the physiologic state (Figure 1 in Appendix #1). Because of the similar size of the PPAR $\gamma$ 1 (1.8 kb) and PPAR $\gamma$ 2 (2.1 kb) mRNAs, we used quantitative

PCR to estimate the relative expression of these two isoforms (Figure 2 in Appendix #2). Based on this approach, the PPAR $\gamma$ 2 mRNA is at least 10-fold more abundant than PPAR $\gamma$ 1 mRNA in the virgin mammary gland. In situ hybridization using digoxigenin labeled oligonucleotide probes for PPAR $\alpha$  and PPAR $\gamma$  was attempted in collaboration with Dr. Jane Jacob, Department of Cell Biology, University of Oklahoma Health Sciences Center. Repeated experiments were unsuccessful due to technical difficulties which related to high background with the control probes and fixation and sectioning of the fatty mammary tissues. While these studies were underway, other groups reported in situ hybridization detecting PPAR mRNAs in the mammary gland [18].

We next determined PPAR mRNA expression in NMuMG cells, an epithelial line derived from normal mammary glands (Figure 4, Appendix 1). Cells were cultured in the presence of a PPAR ligand (BRL49653, a thiazolidinedione) or a MAP kinase inhibitor (PD98059), known to increase PPAR $\gamma$  transcriptional activity. The NMuMG cells accumulated neutral lipids, independent of their treatment regimen. Under all conditions, the cells expressed PPAR $\alpha$ ,  $\beta$ , and  $\gamma$  mRNAs at equivalent levels. Although these exogenous factors had no effect on the expression of PPAR mRNAs, we cannot rule out the possibility that potential ligands contained in the fetal bovine serum had already induced maximum PPAR activation. Nevertheless, these data indicate that murine mammary epithelial cells can express PPAR mRNAs. This is consistent with the multiple reports of mammary epithelial cell expression of PPAR mRNAs by other labs [19-21].

Detection of PPAR proteins in murine mammary tissues by immunohistochemistry:  
Paraffin embedded, formalin fixed tissues were examined using 2 anti-PPAR $\gamma$  and a single anti-PPAR $\alpha$  antibody. The anti-PPAR $\gamma$  antibodies immunostained the cytoplasm of stromal adipocytes in virgin mammary glands. A minority of adipocyte nuclei stained positive with the antibody. On tissues obtained from day 9 or day 17 of pregnancy, adipocytes remained immunoreactive, while on day 2 of lactation, adipocyte immunostaining was reduced. The anti-PPAR $\gamma$  antibody immunostained the cytoplasm of virgin mammary epithelial cells; nuclear epithelial staining was uncommon. Epithelial cells remained immunoreactive throughout pregnancy and lactation. All immunoreactivity could be competed away by the addition of the immunoreactive peptide. In contrast to the adipocytes and epithelial cells, myoepithelial cells were conspicuously non-reactive with both anti-PPAR $\gamma$  antibodies. A commercial anti-PPAR $\alpha$  polyclonal antibody did not detect immunoreactive protein in any of the mammary tissues examined. Similar results were obtained in human mammary tissue specimens examined under identical conditions.

Since PPAR proteins are "nuclear hormone receptors", the finding of predominantly cytoplasmic immunoreactivity was unexpected. Immunohistochemistry performed using identically processed specimens from healing wounds consistently localized PPAR $\gamma$  protein reactivity to the nucleus of infiltrating tissue macrophages. While this suggested that formalin fixation can be appropriate for maintaining PPAR proteins in a nuclear location, an alternative explanation is that our immunohistochemical methods were introducing artifacts in mammary tissues. Limited studies using paraformaldehyde or acetone fixations did not give superior results. Anecdotal reports from colleagues and companies suggest that the conventional immunohistochemical

methods may not be suitable for PPAR detection. Due to publications from other labs describing the immunohistochemical localization of PPAR proteins in mammary gland [20,21], we elected to pursue another line of investigation described below.

Effect of PPAR ligands on mammary carcinogenesis and tumor progression: With evidence from several laboratories, including our own, that PPAR proteins are expressed in the mammary epithelium, we asked the question, do PPAR ligands influence the mammary carcinogenesis? As a model, we exposed rats to dimethylbenz[a]anthracene (DMBA) to induce mammary tumors. This is a well established system which causes benign and malignant tumors within a 12-16 week period. Animals were fed a regular chow diet, containing approximately 5 % of the calories as fat.

Two groups of animals were examined. (1) In the first group, PPAR ligand treatment began one week following DMBA exposure. Cohorts of 16 rats were placed on diets containing the PPAR $\alpha$  ligand, Wy14,643, or the PPAR $\gamma$  ligand, troglitazone. Two control groups were included. One cohort received no additional factors, thus providing a measure of tumorigenesis with DMBA alone. A second cohort received a slow release tamoxifen pellet. This group provided a measure of "optimal" therapy, since tamoxifen is known to inhibit DMBA induced mammary tumorigenesis. Animals were followed over an 18 week period (once per week) and monitored for tumor formation, number of tumors, body weight, dietary intake, plasma lipid levels, organ size (liver, ovary, uterus) and tumor histology. At the conclusion of the study, neither troglitazone or Wy14,643 protected animals against tumor formation relative to the untreated control group (Table 1). In contrast, tamoxifen reduced the percentage of animals exhibiting tumors and the overall number of tumors per animal. The histology of the tumors was comparable between the untreated, troglitazone, and Wy14,643 groups; however, the tamoxifen tumors were more likely to be malignant, although this difference is not statistically significant (Table 2). Treatment regimens did not significantly alter the plasma lipid profile in the animals, except for an elevation of total cholesterol (Table 3).

**TABLE 1: Tumor Formation in Rats Treated with PPAR Ligands 1 Week Post DMBA**

	Untreated	Tamoxifen	Troglitazone	Wy14,643
% Animals with Tumors	69%	33%	79%	81%
Mean tumor # per rat ( $\pm$ S.E.)	1.81 $\pm$ 0.47	0.58 $\pm$ 0.34	2.07 $\pm$ 0.45	1.81 $\pm$ 0.44
n =	16	12	14	16

**TABLE 2: Percent of Tumors with Malignant Histology (Cribriform, Comedo, Papillary)**

	Untreated	Tamoxifen	Troglitazone	Wy14,643
% Tumors with Malignant Histology	42%	63%	46%	36%
n =	31	8	26	28

**TABLE 3: Plasma Lipid Analyses on DMBA Groups**

	Untreated	Tamoxifen	Troglitazone	Wy14,643
Total Cholesterol	93.3± 4.5	89.3 ± 5.1	101.5 ± 5.1	133.7 ± 5.7*
Triglycerides	70.2 ± 7.2	95.2 ± 15.8	71.1 ± 4.9	100.5 ± 15.4
LDL-Cholesterol	41.6 ± 4.7	39.1 ± 5.7	36.6 ± 3.7	32.0 ± 8.1

(2) A second group of animals were maintained on a control diet following DMBA exposure until the area of their primary tumor exceeded 64 mm<sup>2</sup>. Animals were then assigned at random to one of four groups: an untreated control, tamoxifen pellet treated, troglitazone diet treated, or Wy14,643 diet treated. Tumor size was determined over a six week period, at which time animals were euthanized and tumors harvested for histologic analysis (Table 4). Primary tumors were assessed as “progressing” if their area increased by > 30%; as “stasis” if area remained within ± 30% of the original area; or as “regressing” if area decreased by > 30% over the 6 week period (Table 5). The data suggest that troglitazone or Wy14,643 treatment led to tumor regression, although to a lesser extent than observed with tamoxifen treatment. The sample size is not large enough to achieve statistical significance.

**TABLE 4: Percent malignant tumors in 6 week treatment groups**

	Untreated	Tamoxifen	Troglitazone	Wy14,643
Primary Tumor	70%	64 %	73%	83%
n =	10	11	11	6
Total Tumors	61 %	57%	53%	68%
n =	38	23	30	22

**TABLE 5: Primary tumor regression and stasis with 6 week treatment regimen**

	Untreated	Tamoxifen	Troglitazone	Wy14,643
% Regression	8.3 %	66.6 %	41.7 %	36.4 %
% Stasis	16.6 %	0 %	16.6 %	18.1 %
n =	12	12	12	11

## (7) CONCLUSIONS:

Our work supports the following conclusions:

(1) The mRNA levels of PPAR $\alpha$  and PPAR $\gamma$  decrease during pregnancy and lactation in the murine mammary gland. The most abundant PPAR $\gamma$  isoform is PPAR $\gamma$ 2 in the virgin animal.

(2) Murine epithelial cells express mRNA for PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$ .

(3) Levels of PPAR $\gamma$  mRNA are reduced in rat mammary tumors, based on northern blot analysis.

(4) Treatment with a PPAR $\alpha$  (Wy14,643) or PPAR $\gamma$  (troglitazone) ligand does not significantly inhibit mammary tumorigenesis in rats exposed to DMBA and fed a low fat (5% calories) diet.

(5) Treatment with a PPAR $\alpha$  (Wy14,643) or PPAR $\gamma$  (troglitazone) ligand appears to inhibit the progression and increase the regression of an established DMBA tumor in rats exposed to DMBA and fed a low fat (5% calories) diet. Our study does not achieve statistical significance in its present form.

The implications of this study are:

(1) Because of their expression in the mammary epithelium and stroma, the PPAR proteins represent a potential target for chemoprevention and chemotherapy in breast cancer.

(2) Although single agent treatment with synthetic PPAR ligands did not reduce DMBA-induced mammary tumorigenesis in a low fat diet, these agents may be beneficial in animals fed a high fat diet. We have begun further studies to pursue these findings with grant support from the American Institute for Cancer Research. We will examine DMBA-treated rats fed a 20% (wt:wt)  $\omega$ -3 or  $\omega$ -6 fat diet (approximately 40% calories from fat). The  $\omega$ -6 fatty acid diets is expected to accelerate mammary tumorigenesis while the  $\omega$ -3 fatty acid diets may be protective [6,7]. We will determine if troglitazone (0.1 %) or Wy14,643 (0.1%) influence tumorigenesis in these animals. We will use tamoxifen (0.005%) as a control treatment, administering it in the diet rather than as a slow release pellet. Unlike the diet in our previous study, these 20% fat diets approximate the percentage of fat found in the average North American diet. These studies will test the effect of synthetic PPAR ligands on mammary tumorigenesis in animals receiving high levels of natural PPAR ligands.

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**MEETING ABSTRACTS**

Mammary Gland Biology Gordon Research Conference, Plymouth NH, June 18, 1997.

Department of Defense Breast Cancer Research Program Era of Hope, Washington DC, November 2, 1997.

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## Expression of Peroxisome Proliferator Activated Receptor mRNA in Normal and Tumorigenic Rodent Mammary Glands<sup>1</sup>

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The peroxisome proliferator activated receptors (PPARs)  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  are novel nuclear hormone receptors activated by long chain fatty acids and synthetic ligands and which regulate lipid metabolism. Recent studies have detected PPAR $\gamma$  mRNA in human mammary tumor cell lines. The current study examined the expression profile of PPAR mRNAs in normal and malignant rodent mammary tissues. Virgin murine mammary glands contained PPAR  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  mRNAs based on northern blot analysis. The PPAR $\gamma$  isoform was predominantly  $\gamma 2$  based on quantitative PCR analysis. During pregnancy and lactation, the PPAR $\alpha$  and  $\gamma$  mRNAs decreased while the PPAR  $\beta/\delta$  mRNA remained relatively unchanged. NMuMG cells, an epithelial line derived from normal murine mammary gland, expressed PPAR  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  mRNAs, independent of the presence or absence of compounds modifying PPAR activity. In rats, the physiologic expression pattern of PPAR $\gamma$  mRNA paralleled the murine model; levels were detected in virgin but not lactating mammary glands. In addition, the PPAR $\gamma$  mRNA was not detected in several histologically distinct 7,12-dimethylbenz(a)anthracene induced mammary tumors. These findings suggest that PPARs may regulate mammary epithelial and stromal cell function in response to physiologic or pathologic stimuli that profoundly alter lipid metabolism. © 1998

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The peroxisome proliferator activated receptors (PPARs) belong to the nuclear hormone receptor family and play a "master" regulatory role in lipid metabolism and adipogenesis [1-3]. The PPARs are lipid activated transcription factors which control the promoters of multiple genes encoding enzymes in the lipid metabolic pathways [1-3]. Three PPAR genes have been identified in mammalian species: PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  [1-3]. While the physiologic ligand for each of the PPARs has not been identified absolutely, both natural and synthetic ligands exist; these include prostaglandin J, long chain fatty acids such as  $\gamma$ -linoleic acid (primrose oil), the oral antidiabetic thiazolidinedione drugs, and the prostaglandin synthase inhibitors, indomethacin and eicosatetraenoic acid (ETYA) [1-7]. Two PPAR $\gamma$  mRNA isoforms have been described, PPAR $\gamma 1$  and PPAR $\gamma 2$ , reflecting alternative promoter utilization [8]. The PPAR $\gamma 1$  isoform was initially described in hepatocytes and macrophages [9,10]. In contrast, the PPAR $\gamma 2$  isoform is unique to adipocytes and over-expression of the PPAR $\gamma 2$  cDNA and exposure to PPAR ligands is sufficient to convert fibroblast-like cells into fat [1-7,11].

Lipids and their metabolism play an important role in mammary gland physiology. Adipocytes are the most abundant stromal component of the developing mammary gland and they directly regulate epithelial cell growth. *In vitro*, adipocytes are superior to fibroblasts as a feeder layer for mammary epithelial cell differentiation. *In vivo*, the boundary of the mammary fat pad delimits epithelial ducts and alveolar branching [12,13]. During lactation, the lipid metabolic pathways of the mammary adipocytes and epithelial cells undergo profound changes. At this time, adipocytes in close proximity to epithelial ducts decrease their lipid

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vacuole size, consistent with a mobilization of their stored fats by lipolysis [14]. Simultaneously, mammary epithelial cells produce and release milk fat globules [15].

Dietary lipids may influence the initiation and progression of mammary tumors [16]. Epidemiologic studies in man suggest that the incidence of breast cancer is increased in populations consuming a diet rich in unsaturated fatty acids [16]. Rodent breast cancer models provide evidence supporting a link between dietary fat intake and breast cancer risk [16]. The underlying mechanism for this phenomenon is unknown. Recently, PPAR $\gamma$  mRNA was found in human mammary epithelial cell lines where its expression was regulated by PPAR ligands and MAP kinase inhibitors [17–19]. The current study examines the PPAR mRNA expression profile in murine and rat mammary tissues reflecting different physiologic and pathologic states.

## MATERIALS AND METHODS

**Northern blots.** Mammary glands were dissected from virgin, pregnant, or lactating 6–10 week old female Balb/c or C57BL6 mice (obtained from the breeding colony maintained at the Oklahoma Medical Research Foundation Laboratory Animal Care Resource) or from Sprague–Dawley rats (Harlan Sprague Dawley Labs, Indianapolis, IN). All studies were evaluated and approved by the institutional animal care and utilization committee. Tissues were frozen on dry ice and stored at  $-70^{\circ}\text{C}$  prior to RNA preparation by a modification of the method of Chomzynski and Sacchi [11, 20]. Special care was taken to centrifuge the homogenized tissue at  $4^{\circ}\text{C}$  prior to acid/phenol/chloroform extraction to remove the contaminating fatty layer. Mammary epithelial cells (MEC) were isolated from the surrounding stroma of virgin Sprague Dawley rat mammary glands by overnight digestion in 0.2% class 3 collagenase (Worthington Biochemical, Lakewood NJ) and 0.2% dispase (Gibco/BRL, Gaithersburg, MD), followed by filtration and entrapment on  $60\ \mu\text{m}^2$  Nitex filters (Tetko, Lancaster NY) [21]. Northern blots were prepared with  $10\ \mu\text{g}$  of total RNA per lane were hybridized with murine cDNA probes for actin and PPARs  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  or with an oligonucleotide probe for the 28s ribosomal RNA (5'AACGATCAGAGTAGTGG-TATTTACC 3') at  $55^{\circ}\text{C}$  according to a modification of the method of Church and Gilbert [11,22]. The cDNA probes were labeled with random hexamers according to the method of Feinberg and Vogelstein [23] and the oligonucleotide probe was labeled with terminal deoxytransferase.

**Quantitative PCR.** Total RNA ( $5\ \mu\text{g}$ ) isolated from virgin murine mammary glands was reverse transcribed according to published methods [11]. Competitive DNA fragments specific for PPAR $\gamma$ 1 and PPAR $\gamma$ 2 were prepared using the following combination of forward (F) and reverse (R) oligonucleotide pairs:

PPAR $\gamma$ 1 Oligo #1F: TGTGTGACAGGAAACAGCTATGACCATG  
 PPAR $\gamma$ 1 Oligo #2F: TTTGAGCTCTTCTGACAGGACTGTGTGACAG  
 PPAR $\gamma$ 2 Oligo #1F: TGAACTCTGGAACAGCTATGACCAT  
 PPAR $\gamma$ 2 Oligo #2F: TTTGAGCTCGCTGTATGGGTGAACTCTG  
 PPAR $\gamma$ 1/2 Oligo #1R: ATGCAGGTTCGTAACAGCAGGCCAGT  
 PPAR $\gamma$ 1/2 Oligo #2R: TTTGAGCTCATAAGGTGGAGATGCAGGTTTC

The first reaction was conducted with either the unique PPAR $\gamma$ 1 or  $\gamma$ 2 forward primers #1F and the common reverse primer #1R, using pBluescript SKII as template to generate a 246 bp DNA fragment. These products then served as template in a second reaction using the appropriate PPAR $\gamma$ 1 or  $\gamma$ 2 forward primer #2F

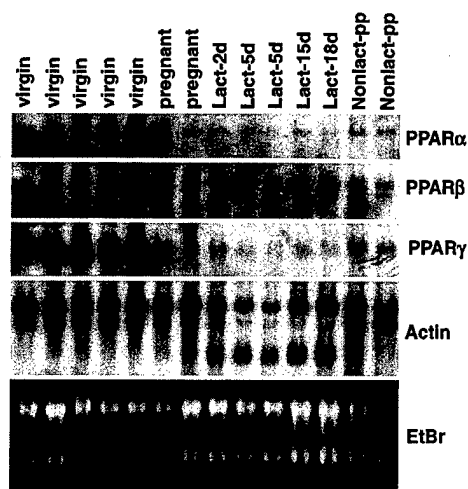
together with the common reverse primer #2R to generate distinct 287 bp PPAR $\gamma$ 1 or PPAR $\gamma$ 2 products. Reactions were performed using a Perkin–Elmer Cetus DNA Thermal Cycler for 35 cycles under the following conditions:  $94^{\circ}\text{C}$ , 45 seconds;  $55^{\circ}\text{C}$ , 45 seconds;  $72^{\circ}\text{C}$ , 2 minutes. The final 287 bp products (competitor DNA fragments) were purified on a non-denaturing acrylamide gel and their concentration determined by densitometry performed on an ethidium bromide stained analytical gel with a Stratagene Eagle Eye Video Imager system (San Diego CA). Quantitative PCRs were performed with serial ten-fold dilutions of the competitor DNA fragment at concentrations ranging from 25 pg to 0.025 fg of DNA in  $50\ \mu\text{l}$  volumes containing equal quantities of reverse transcribed virgin mammary gland cDNA (equivalent to  $0.7\ \mu\text{g}$  RNA per reaction), 0.04 OD<sub>260</sub> units of oligos #2F and #2R, 1 X reaction buffer (Promega, Madison, WI), 1.5 mM MgCl<sub>2</sub> and 200  $\mu\text{M}$  dNTP. Reaction products were analyzed on 6% nondenaturing acrylamide gels as described [11].

**Culture of NMuMG cell line.** The NMuMG cell line (ATCC CRL 1636) was obtained from The American Type Culture Collection (Rockville MD) and cultured in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT), 100 units penicillin/ml, 100  $\mu\text{g}$  streptomycin/ml, 1 mM glutamate, and 1 mM sodium pyruvate [24,25].  $5 \times 10^4$  cells in a 2 ml volume were inoculated on a 35 mm plate, cultured overnight at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub>, and induced with the thiazolidinedione, BRL49653 (10  $\mu\text{M}$ ), generously provided by David C. Morris, Ph.D., Glaxo/Wellcome, Research Triangle Park, NC, and/or the MAP kinase inhibitor, PD98059 (5  $\mu\text{M}$ ), (Cat. # 9900S, New England Biolabs, Beverly, MA). Control cultures were treated with the carrier vehicle (0.2% dimethylsulfoxide) alone. Cultures were maintained with fresh media containing the inductive agents every three days. Plates were harvested for total RNA on days 3, 6, or 8 following induction. Representative cultures on day 8 were fixed in paraformaldehyde and stained for neutral lipids with Oil Red O.

**Treatment with 7,12-dimethylbenz(a)anthracene (DMBA).** Virgin 55 day old Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were gavage fed 20 mg DMBA (Sigma Chemical Co., St. Louis, MO) suspended in 1 ml of stripped corn oil [26]. Mammary glands were harvested after a 16 week period for RNA analysis or fixed in phosphate buffered formalin and paraffin embedded prior to histochemical evaluation of hematoxylin/eosin stained sections. The histologic grade of the tumors was assigned based on the criteria of Russo et al [27].

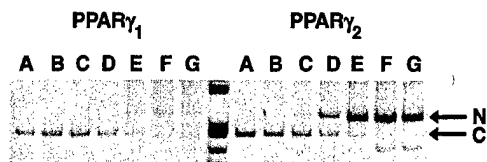
## RESULTS AND DISCUSSION

**Detection of PPAR mRNAs in murine mammary tissues.** Our initial studies set out to determine the expression profile of PPAR mRNAs under different physiologic conditions (Figure 1). Based on northern blots, the mRNA levels of PPAR $\alpha$  and PPAR $\gamma$  were abundant in mammary tissue isolated from virgin mice but declined during pregnancy and lactation. In contrast, PPAR $\beta/\delta$  mRNA was constitutively expressed independent of the physiologic state, although at lower levels. Equal loading between lanes was confirmed by ethidium bromide staining of the gel. The northern blots were hybridized with the housekeeping gene actin as an additional control. Actin levels were reduced during lactation, consistent with reports of decreased housekeeping gene expression during pregnancy and lactation [28,29].

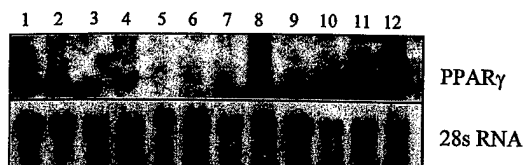


**FIG. 1.** Detection of PPAR mRNAs in murine mammary gland tissues by Northern blot. Total RNA was isolated from mammary glands dissected from virgin, pregnant (day 18), and lactating (day 2, 5, 15, and 18 post-partum) Balb/c mice. Mammary glands were also isolated from 18 day post-partum mice whose pups had been removed at birth and which had never suckled (Nonlact-pp). Northern blots were prepared with 10  $\mu$ g total RNA and hybridized with radiolabeled cDNA probes for murine PPAR $\alpha$ , PPAR $\beta/\delta$ , PPAR $\gamma$ , and actin. Ethidium bromide staining (EtBr) of the Northern blot is included as a control.

PPAR $\gamma$ 2 is the most abundant isoform. Because PPAR $\gamma$ 1 and PPAR $\gamma$ 2 mRNAs are 1.8 kb and 2.1 kb in size, respectively, it is difficult to distinguish them by conventional northern blot analysis. We used quantitative PCR analysis to estimate the relative expression level of these two PPAR isoforms in virgin mammary tissue (Figure 2). Analyses were conducted in the presence of serial 10-fold dilutions of specific PPAR $\gamma$ 1 or PPAR $\gamma$ 2 competitors with equal quantities of reverse transcribed total RNA. When the concentration of the native (N) and competitor (C) templates in each reaction are equal, their PCR products display equal signal intensity. These analyses demonstrated that PPAR $\gamma$ 2 mRNA was 10-fold more abundant than PPAR $\gamma$ 1 mRNA in the virgin mammary gland.



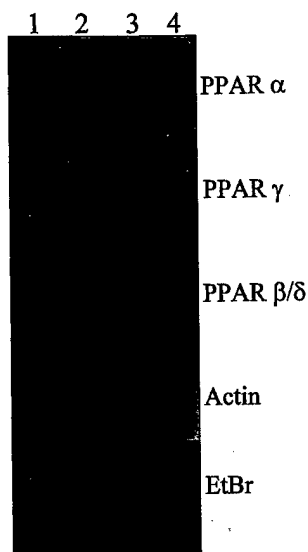
**FIG. 2.** Detection of PPAR $\gamma$ 1 and PPAR $\gamma$ 2 mRNAs by quantitative PCR. Quantitative PCR was performed using cDNA reverse transcribed from virgin mammary gland total RNA and primers specific for PPAR $\gamma$ 1 and PPAR $\gamma$ 2. The size of the competitive PCR fragment is 287 bp (C) while that of the native PPAR $\gamma$ 1 and PPAR $\gamma$ 2 cDNAs is 384 bp (N). The concentration of competitor DNA in each reaction was reduced by ten-fold in each lane, ranging from 25  $\mu$ g (lane A) to 0.025  $\mu$ g (lane G).



**FIG. 3.** Detection of PPAR mRNAs in a murine epithelial cell line derived from normal mammary tissue. The NmuMG cell line was cultured for 6 days under control conditions (lane 1), in the presence of thiazolidinedione BRL49653 at 10  $\mu$ M (lane 2), in the presence of the MAP kinase inhibitor PD98059 at 5  $\mu$ M, and in the presence of both BRL49653 and PD98059 (lane 4). Total RNA isolated from cells was analyzed by Northern blot (10  $\mu$ g/lane) and hybridized with radiolabeled probes for murine PPAR $\alpha$ , PPAR $\beta/\delta$ , PPAR $\gamma$ , and actin. Ethidium bromide staining (EtBr) of the Northern blot is included as an additional control.

**Analysis of PPAR mRNAs in a murine epithelial cell line.** We next determined PPAR mRNA levels in the NMuMG cells, an epithelial line derived from normal murine mammary glands [25,26]. Cells were cultured in control medium alone or in the presence of the thiazolidinedione BRL49653, a PPAR $\gamma$  ligand, and/or the MAP kinase inhibitor, PD98059. Independent of the presence or absence of these exogenous factors, the NMuMG cells accumulated neutral lipid, based on staining with Oil Red O [data not shown]. The cells expressed PPAR  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  mRNAs throughout the incubation period. The PPAR mRNA signals and that of the actin housekeeping gene on day 6 following induction are shown in Figure 3. The cells expressed strong signals for each PPAR isoform throughout the incubation period (data not shown). The additional presence of compounds which modulate PPAR transcriptional function did not alter the PPAR mRNA expression pattern [18]. The thiazolidinedione, BRL49653, induces PPAR $\gamma$  mRNA levels in stromal cells while simultaneously stimulating adipocyte differentiation [11]. The MAP kinase inhibitor, PD98059, inhibits the phosphorylation of PPAR $\gamma$ , an event which down regulates its transcriptional activity [1,18]. The absence of any effect by these agents may result from the culture conditions employed. For example, we cannot rule out the possibility that constituents of the fetal bovine serum may have maximally induced PPAR expression levels, masking the effects of BRL49653.

**Analysis of PPAR $\gamma$  mRNA in physiologic and pathologic rat mammary tissues.** Our final studies compared the PPAR $\gamma$  mRNA level in rat mammary tissues (Figure 4). The mRNA was present only in virgin mammary tissue (ln 1,2). The PPAR $\gamma$ 2 mRNA signal was below the limits of northern blot detection in lactating mammary tissue (ln 3,4) and in histologically distinct tumors induced by the mammary carcinogen, 7,12-dimethylbenz(a)anthracene; these included papilloma (ln 5,6), comedo (ln 7,8), cribriform (ln 9,10), and tubular



**FIG. 4.** Detection of PPAR $\gamma$  mRNA in rat mammary tissues. A Northern blot was prepared with Sprague-Dawley rat mammary gland total RNA isolated from virgin (lanes 1, 2) or lactating day 11 normal tissues (lane 3, 4) or from the following histologic grades of 7,12-dimethylbenz[a]anthracene induced tumors: papilloma (lanes 5, 6); comedo (lanes 7, 8); cribriform (lanes 9, 10); tubular (lanes 11, 12). The blot was successively hybridized with a murine cDNA probe for PPAR $\gamma$  and an oligonucleotide probe for the 28 s ribosomal RNA.

(In 11,12). As a control for equal loading between lanes, blots were probed with an oligonucleotide probe hybridizing to the 28s ribosomal RNA band. In parallel studies, northern blot analysis of RNA isolated from virgin rat mammary epithelial cells (MEC) detected a faint PPAR $\gamma$  signal (data not shown), consistent with the NMuMG studies shown above.

## CONCLUSIONS

The current data demonstrate the presence of PPAR mRNAs in both the stromal adipocytes and epithelial cells of rodent mammary tissue. The levels of PPAR $\alpha$  and PPAR $\gamma$  mRNA decreased during pregnancy and lactation while the levels of PPAR $\gamma$  were reduced in tumors compared to virgin mammary glands. It remains to be determined if the PPAR $\alpha$  and PPAR $\gamma$  protein levels parallel those of their mRNAs. Additional histologic studies will be required to investigate this issue.

Epidemiologic studies associate obesity and high fat diets with an increased incidence of breast cancer in women, although this does not prove a causal relationship [16]. In rodent mammary tumor models, potential PPAR ligands have been reported to reduce tumor incidence and progression [7]. In rats were fed a high fat diet, indomethacin, eicosatetraenoic acid (ETYA), or  $\gamma$ -linoleic acid (primrose oil) all decreased the incidence of mammary tumors following DMBA exposure [30-34]. Similar results with indomethacin were obtained

with a transplantable mammary tumor model [35]. The presence of PPAR proteins in the mammary epithelial cells or stroma may provide a mechanism for the protective action of these drugs. Their actions may be nutritional, depending on the specific lipid composition of the diet. Consequently, these nuclear hormone receptors represent a potential target for chemoprevention and/or chemotherapy in breast cancer.

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