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MAZ DRIVES TUMOR SPECIFIC EXPRESSION OF PPAR GAMMA 1 IN BREAST CANCER CELLS

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Abstract

The peroxisome proliferator-activated receptor gamma 1 (PPAR γ 1) is a nuclear receptor that plays a pivotal role in breast cancer and is highly over-expressed relative to normal epithelia. We have previously reported that the expression of PPAR γ 1 is mediated by at least 5 distinct promoters and expression in breast cancer is driven by a tumor specific promoter (pA1). Deletional analysis of this promoter fragment revealed that the GC-rich, 263 bp sequence proximal to the start of exon A1, is sufficient to drive expression in breast cancer cells but not in normal, human mammary epithelial cells (HMEC). By combining the disparate technologies of microarray and computer-based transcription factor binding site analyses on this promoter sequence the myc-associated zinc finger protein (MAZ) was identified as a candidate transcription factor mediating tumor-specific expression. Western blot analysis and chromatin immunoprecipitation (ChIP) assays verify that MAZ is overexpressed in MCF-7 cells and is capable of binding to the 263 bp promoter fragment, respectively. Furthermore, the over-expression of MAZ in HMEC is sufficient to drive the expression of PPAR γ 1 and does so by recruiting the tumor-specific promoter. This results in an increase in the amount of PPAR γ capable of binding to its DNA response element. These findings help to define the molecular mechanism driving the high expression of PPAR γ 1 in breast cancer and raise new questions regarding the role of MAZ in cancer progression.

Introduction

Breast cancer is the second leading cause of malignancy related deaths among American women [1]. The majority of these breast tumors arise from the ductile epithelia and infiltrating ductal carcinomas account for over 70% of all cases of breast cancer [2]. Current chemotherapies entail significant toxicity and benefit only a limited number of patients. Due to limitations in current therapeutic options, and the high degree of prevalence, a great deal of research has focused on the search for new and more selective molecular targets in the treatment of breast cancer. A number of nuclear hormone receptors have been identified as potential candidates for use as drug targets including peroxisome proliferator-activated receptor gamma 1 (PPAR γ 1). Our identification of PPAR γ 1 in breast cancer [3] and the subsequent elucidation

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of its role in mediating differentiation [4,5] has lead to the intensive examination of its role in mediating similar programs in breast adenocarcinomas [6–8].

PPAR γ 1 is a member of the nuclear hormone receptor superfamily and plays a critical role in adipogenesis [4,5,9], insulin mediated glucose homeostasis [10], and development [11]. Ligands for PPAR γ 1 include 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), dietary fatty acids and the thiazolidinedione class (TZDs) of hypoglycemic drugs [12–15]. At the protein level, two forms of PPAR γ (γ 1 and γ 2) are expressed from the same gene, γ 2 containing an additional 30 N' terminal amino acids not present on γ 1 [9,16,17]. PPAR γ 1 is expressed in normal, human mammary epithelial cells (HMEC) [7] and established breast cancer cell lines where it is functionally responsive to ligand-mediated transactivation [3,18]; however, expression of PPAR γ 1 is higher in several different tumor types when compared to noncancerous tissue [19–23]. In the case of mammary tissue, normal epithelial cells also express much lower level of PPAR γ 1 compared to breast carcinoma cell lines [6,7,15,18,24,25]. Furthermore, Mueller *et al.* demonstrated that PPAR γ 1 expression was higher in metastatic lesions in the lung compared to the primary breast tumor from the same patient indicating that increasing PPAR γ 1 expression correlates with the progression of breast tumors from formation through metastases [26].

Although PPAR γ 1 is thought to mediate differentiation in most tissues, its role in tumor progression or suppression is poorly understood. Indeed, in some tissues it has been shown that a reduction in the expression of PPAR γ 1 can increase the risk of carcinogenesis. In these studies, PPAR γ 1 heterozygous (+/-) knockout mice had a much greater rate of colon tumor formation following exposure to azoxymethane, an inducer of colorectal cancer [27]. These animals also develop more mammary tumors following exposure to 7,12-dimethylbenz(a)anthracene [28]. By contrast, constitutive over-expression of PPAR γ 1 in animal studies increases the risk of spontaneous breast cancer in mice already susceptible to the disease [29]. It has been suggested that this paradox might be resolved by careful dose-response studies, where both the level of PPAR γ 1 gene expression and transactivation are carefully controlled [30]. This would suggest that the level of expression is a critical factor in determining the physiological outcome of PPAR γ 1 transactivation in a cell-specific context. Since benign breast ducts express lower levels of PPAR γ 1 protein compared to infiltrating carcinoma cells [6] and the expression of PPAR γ 1 is positively correlated with breast cancer metastasis [26], it is critical that we understand the mechanisms that drive these changes in PPAR γ 1 expression that accompany tumor progression. Therefore, we sought to identify the molecular mechanisms that mediate the increase in PPAR γ 1 as cells progress from normal mammary epithelial to breast carcinoma cells.

We have previously reported that the expression of PPAR γ 1 is under complex regulatory mechanisms [17]. Although we identified distinct promoters associated with untranslated first exons that mediate the changes in expression seen during cellular transformation, we were unable to identify the factor(s) mediating this event. Here we report that through the novel combination of standard promoter analysis, computer-based *cis*-element prediction and microarray analysis we have identified the myc-associated zinc finger protein as the tumor specific regulator of PPAR γ 1 in human breast cancer cell lines.

Methods

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM lacking phenol red (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37 °C in a 5% CO₂ atmosphere. Cells were grown in T-75 flasks prior to transfer to 12-well plates (Corning) used in transfection assays.

Normal, human mammary epithelial cells (HMEC) were obtained from Cambrex/Clonetics. HMEC cells were cultured in the Cambrex/Clonetics MEGM[®] media containing SingleQuot[®], tissue culture media supplements optimized for the growth of these cells. HMEC Cells were grown in phenol red free medium at 37 °C in a 5% CO₂ atmosphere. HMEC Cells were grown in T-75 flasks prior to transfer to 12-well plates used in transfection and assays.

Plasmid construction

The pRL-TK vector (Promega, Madison, WI) was used as an internal control in all transient transfection assays. The PPAR γ 3kb promoter section and the series of 5'-end deletion fragments from the 5'-flanking region of exon A1 were cloned into the multiple cloning site of the pGL3-basic plasmid (Promega). The MAZ cDNA from human B-cells was obtained from ATCC. Using a combination of Xho I and EcoR I restriction enzymes, the MAZ gene was released from the pOTB7 plasmid and cloned into the pCI/IRES-hgfp mammalian expression vector where the CMV promoter drives MAZ gene expression. PCR was used to introduce the mutated MAZ response element, which substituted one of the guanine triplet sequences to a non-functional TCC sequence. As shown, the native MAZ response element found in the 263 bp sequence of the PPAR γ pA1 promoter was -217 GGGAGGGA -209, and was changed to the mutant form -217 GGGATCCA -209 while maintaining the context of the 263 bp pA1 promoter in the pGL3-basic luciferase reporter plasmid.

Transient transfection analysis

Cells were transiently transfected with either 2 μ g of a 3XPPRE-TK-pGL3 reporter vector [17], 2 μ g of MAZ expression vector, or 0.5 μ g pRL-TK (Promega) per plate using ESCORT (Sigma, St. Louis). After 18–24 hours, cells were lysed in 100 μ l passive lysis buffer (Promega) and treated according to manufacturer's instructions for use with the Dual-Luciferase Assay Kit. Luminometry was performed on a Berthold Technologies Lumat (LB9507, Bad Wildbad, Germany) and data calculated as raw luciferase units divided by raw *Renilla* units (RLU's). Data are presented as the mean fold induction. These values were obtained by dividing the RLU data from each treatment well by the mean of the control values $\pm$ the standard error of the mean (sem) as shown.

Sequence Analysis for Putative cis-Elements

Transcription Element Search Software (TESS), a string-based search tool similar to local alignment software [31] was used to search the 263bp promoter sequence of PPAR γ pA1. Using sequence position weight matrices from TRANSFAC, IMD, and the CBIL-GibbsMat databases, possible transcription factor binding sites within the 263 bp region of the PPAR γ promoter A1 were identified.

Formaldehyde cross-linking and Chromatin Immunoprecipitation

Cell growth and chromatin preparation were performed according to the manufacturer's instructions included with the ChIP-IT[™] kit (Active Motif North America, Carlsbad, CA). Chromatin from MCF-7 cells was formaldehyde cross-linked in a 1.6% solution for 10 minutes followed by enzymatic shearing for 11 minutes. Soluble chromatin from MCF-7 cells was immunoprecipitated with a MAZ polyclonal antibody (Santa Cruz Biotechnology, sc-28745, Santa Cruz, CA). Specific primer pairs (Integrated DNA Technologies, Coralville, IA) were designed to amplify the PPAR gamma promoter regions from -210 to -1, forward primer: 5' GCCGCTCCCTCCAGTCGTCGCG 3'; reverse primer: 5' CTCGAGGCCGACCCAAGC 3'. PCR fragments were analyzed by 5% polyacrylamide gel (37.5:1, acrylamide–bisacrylamide) in TBE buffer. The 210 bp fragment was purified and subjected to DNA sequence analysis. Elim Biopharmaceuticals, Inc. (Hayward, CA) performed all DNA sequencing.

Nuclear Protein Extraction

Nuclear proteins were prepared with the TransAM[®] nuclear extract kit (Active Motif North America). In brief, cells were scraped into PBS containing phosphatase and protease inhibitors, centrifuged, resuspended in a 1X hypotonic buffer and then kept on ice for 15 min. After the addition of a detergent, the lysates were centrifuged at $14,000 \times g$ for 30 s at 4°C. The pellets were resuspended in complete lysis buffer and vortexed for 10 s at the highest setting. After a 30 min incubation on ice and centrifugation at $14,000 \times g$ for 10 min at 4°C, the supernatants were collected, and the protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL).

PPAR gamma Transactivation Analysis—PPAR γ 1 activation was determined with the TransAM[®] ELISA kit (Active Motif North America). Nuclear extract was added to each well of a 96-well plate into which an oligonucleotide with a PPAR consensus binding site had been immobilized. After 1 hour of incubation with smooth agitation, wells were washed three times with washing buffer and then incubated with PPAR γ 1 antibody (1:250 dilution in 1X antibody binding buffer) for 1 hour at room temperature. The wells were washed three times and incubated for 1 hour with diluted anti-rabbit HRP-conjugated antibody (1:1000 dilution in 1X antibody binding buffer). After 4 wash cycles, 100 μ l of developing buffer was added to each well and incubated for 5–8 min. The reaction was stopped by the addition of 100 μ l stop buffer. The final A_{450} was read on a Kinetic microplate reader with a reference wavelength of 650 nm.

Western Blot Analysis

Western analysis was performed as described previously [17] using the nuclear fractions from MCF-7 and HMEC. The Anti-MAZ polyclonal antibody was purchased from Santa Cruz (1:200 dilution, sc-28745; Santa Cruz Biotechnology). To assess sample loading, α -Tubulin (1:1000 dilution, sc-8035, Santa Cruz Biotechnology) was used as a loading control. An estimation of the relative quantity of MAZ was determined by densitometry using a Kodak Imaging System EL Logic 2200. The observed MAZ protein level was recorded as a ratio of MAZ to tubulin.

Affymetrix Microarray Analysis

Total RNA was isolated from cells following a 3 hour treatment using the Qiagen RNeasy Mini Kit. The University of Kentucky Microarray Core Facility verified the total yield by both gel electrophoresis and Agilent Bioanalyzer. The core facility processed the total RNA samples to produce biotinylated, fragmented cRNA. The cRNA samples then were hybridized to the Affymetrix (Santa Clara, CA) HG-U133A GeneChip and washed with a streptavidin phycoerythrin solution via an Affymetrix Fluidics Station 400. Finally, the resultant fluorescent intensities were collected and analyzed using an Affymetrix GeneArray Scanner coupled to a computer workstation running Affymetrix Microarray Suite (MAS 5.0). The MAS 5.0 data from each Gene Chip was saved and exported into Microsoft Excel spreadsheets (Redmond, WA). MAS 5.0 signal intensity data collected were aggregated into a single Excel spreadsheet using the probe set IDs, signal intensity values, and signal detection flag for each sample and probe set description. Mean signal intensity values ($n = 3$ per cell type) were reported along with SE as an appropriate estimation of error. One factor analysis of variance (ANOVA, $\alpha=0.05$) followed by t-test was used to determine significant differences in the observed treatment effect within a cell type.

Quantitative Real-Time PCR

A one-step quantitative real-time PCR technique was used to determine relative expression levels of PPAR gamma 1 mRNA using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The RNeasy Mini Kit (Qiagen) was used to isolate the

total RNA. The pre-optimized primers and probe with FAM reporter fluorescent dye for PPAR γ were purchased from Applied Biosystems, assay-on-demand number Hs00234592_m1. For the internal control, *18S*, a pre-optimized primer and probe also was used, assay-on-demand number Hs99999901_s1. A one-step reaction reagent mixture provided in the TaqMan One-step RT-PCR Master Mix kit (Applied Biosystems) was used for all of the amplifications. Cycle parameters for the one-step reverse transcription-PCR included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. The house keeping gene *18S* was used for internal normalization.

Analysis methods as outlined in the ABI Prism 7700 Sequence Detection System User Bulletin 2 (October 2001) were performed using the relative C_t method. Briefly, this method uses the mathematical expression 2^{-C_t} to estimate the relative gene expression based on a calibrated sample, $DC_t = C_{t,x} - C_{t,calibrator}$, and the gene target of interest normalized to the expression of an endogenous housekeeping gene like *18S*, $DDC_t = C_{t,PPAR} - C_{t,18S}$. The mean ($n = 4$ per cell line) values were reported along with the SE of the C_t because an appropriate estimation of error was calculated from the SDs of the C_t values for PPAR and *18S* through the formula

$$SE = s/n \text{ where the SD, } s = \sqrt{(C_{t,PPAR})^2 + (C_{t,18S})^2}.$$

Results

Identifying transcription factors mediating tumor-specific expression of PPAR γ 1

We have previously reported that the regulation of PPAR γ 1 expression is under the control of at least seven different promoters associated with distinct, first exons [17]. We have also demonstrated that the higher levels of PPAR γ 1 expression reported in breast cancer cells results from the recruitment of a distinct tumor specific promoter termed pA1, which is not used in HMECs [17]. To define the factor driving expression from pA1, we initially used 5'-end deletion analysis of the 3000 bp pA1 promoter fragment. These analyses allowed us to more narrowly define the promoter element(s) using luciferase reporters. In addition to the 3 kb pA1 promoter fragment, a 2 kb fragment from -1 to -2000, a 1 kb fragment from -1 to -1000, a 263 bp fragment from -1 to -263 and a 5' end 2 kb fragment from -1000 to -3000 were generated (Fig. 1A). These data indicate that the proximal 263 bp of the pA1 promoter fragment contain the element(s) necessary to drive expression of PPAR γ 1 in breast cancer cells.

The 263 bp pA1 promoter fragment is greater than 90% GC-rich and resisted efforts to more narrowly define the response element by standard deletional analysis (data not shown). To circumvent these limitations and define the element(s) within the 263 bp fragment driving expression, we integrated two distinct technological approaches to identify potential transcription factor binding sites. Using the Transcription Element Search System (TESS, (<http://www.cbil.upenn.edu/tess/>), developed at the University of Pennsylvania Computational Biology and Informatics Laboratory, we identified the potential transcription factor binding sites within this 263 bp tumor specific promoter fragment [32]. This approach identified 29 different transcription factors that had the potential to bind at a total of 209 different DNA binding sites. We then used microarray analysis to determine which of the factors identified by TESS analysis were expressed at significantly higher levels in breast cancer cells relative to HMECs. These analyses revealed that of the transcription factors on the HG-U133A array, myc-associated zinc finger protein (MAZ), was significantly over expressed in estrogen-dependent breast cancer (MCF-7) cells compared to HMECs (Fig. 1B). This message was represented by two different probes sets, and both were significantly higher in the MCF-7 cells relative to HMECs. Densitometric analysis of the western blot confirms that is expressed at significantly higher levels in the nuclear extracts of MCF-7 cells (Fig. 2A, arrow marked bands).

To resolve whether the binding site identified by TESS analysis is recognized and bound by MAZ, Chromatin Immunoprecipitation (ChIP) assay was preformed. Using the polyclonal antibody to MAZ and the gene specific primers, the expected band size of 210 bp was amplified (Fig. 2B). This fragment was purified and sequenced confirming this to be the pA1 promoter. Electrophoretic mobility shift assays (data not shown) confirmed the ChIP assay that MAZ binds to the MAZ response element (MAZ-RE) identified by TESS. Together these demonstrate that MAZ is overexpressed in tumors and physically binds to the endogeneous response element within the -263 bp pA1 promoter in MCF-7.

The expression of MAZ drives PPAR γ 1 expression in MCF-7 cells

Initially, we examined the ability of MAZ to mediate reporter activity of the 263 bp promoter shown to control tumor-specific expression of PPAR γ 1. In MCF-7 cells, this promoter is sufficient to confer transcriptional regulation compared to the basic (pGL3-basic) luciferase reporter (Fig. 3A). Furthermore, when a MAZ expression plasmid is introduced into these cells reporter activity is significantly higher than MCF-7 cells not overexpressing MAZ (Fig. 3A). To confirm the need of the MAZ response element, this element was scrambled by site directed mutagenesis where the second G triplet sequence was replaced with TCC. When this mutated MAZ-RE was placed within the context of the 263 bp promoter, reporter activity was significantly reduced (Fig. 3B).

Expression of MAZ in HMEC cells drive PPAR γ expression from the tumor specific promoter

Transient transfection of HMEC with the MAZ expression vector dramatically increased mRNA levels of PPAR γ 1 as seen by real-time PCR (Fig. 4A). This also resulted in a small but significant increase in protein levels (Fig. 2A, band marked by arrow). Since breast cancer cells drive expression of PPAR γ 1 from a tumor-specific promoter not used by HMEC, probes were designed to determine whether MAZ drives PPAR γ 1 expression from the tumor-specific promoter in HMECs as well. Exon-specific probes to the first exon (A1) present on PPAR γ mRNA from MCF-7 cells and the first exon present on PPAR γ 1 from HMEC (A3)[17] were used to amplify the mRNA from HMEC expressing MAZ. These data demonstrate that MAZ drives expression of PPAR γ 1 in HMEC (Fig. 4A, insert) from pA1, the tumor-specific promoter [17]. Finally, to determine whether the MAZ-driven increase in PPAR γ 1 increases the amount of protein capable of binding DNA, an ELISA-based assay to quantitate the binding of PPAR γ to its response element was used. These data demonstrate that MAZ expression increases not only the level of PPAR γ 1 protein in HMEC but also the amount of PPAR γ 1 capable of binding to DNA.

DISCUSSION

A rapidly growing body of work has demonstrated that the transactivation of PPAR γ 1 by various exogenous ligands mediates a wide range of responses including growth arrest, differentiation and apoptosis making this nuclear receptor a possible target for cancer therapy [7,8]. We have shown that PPAR γ 1 is highly over expressed in many tumors including breast, colon and lung [18]. In order to examine the transcriptional regulation of PPAR γ 1, we have determined its genomic structure and shown that the rise in expression from normal, human mammary epithelia to breast cancer is due to the recruitment of a distal, tumor-specific promoter element, termed pA1 [17]. The studies outlined in this report were designed to identify the mechanism driving this increase in PPAR γ 1 expression. Through standard 5'-end deletion analysis, we sought to determine the transcription factor that mediates the recruitment of the pA1 promoter. Although we were able to narrow the response element to a 263 bp fragment immediately flanking the start site of transcription, the GC-rich nature of this region made further promoter analysis by standard methods intractable. Therefore, we chose to take a novel approach that combined two disparate technologies to identify the factor mediating the

tumor specific expression of PPAR γ 1 in breast cancer. In addition to having identified a novel transcription factor whose expression could have an important role in cancer biology, this approach could find broader use in locating response elements and identifying the transcription factors that drive the expression of genes of interest.

By using the transcription element search system developed at the University of Pennsylvania, we were able to locate potential transcription factor binding sites within the proximal 263 bp PPAR γ 1 promoter fragment. Since PPAR γ 1 is highly expressed in MCF-7 cells relative to HMEC, we sought to determine which of the transcription factors identified by TESS were also over expressed in MCF-7 cells. For this, we employed data from a microarray analysis currently underway in the lab. We examined the expression levels of all the transcription factors identified by TESS that were present on the HG-U133A chip for factors overexpressed in MCF-7 cells compared to HMEC. Microarray analysis indicated that MAZ is expressed at significantly higher levels in MCF-7 cells. Indeed, MAZ is represented by two different probe sets on the HG-U133A chip and both were significantly higher in MCF-7 cells. Western blot analysis confirms that this is true at the protein level as well. Furthermore, ChIP assay proved that *in vivo* MAZ binds to the MAZ response elements in the 263 bp pA1 promoter fragment. The forced overexpression of MAZ in HMEC also drives an increase in the expression of PPAR γ 1. Furthermore, not only does MAZ drive expression of PPAR γ 1 in HMEC, it does so from the tumor-specific promoter. Finally, mutating the MAZ-RE in the context of the tumor-specific promoter inhibits reporter activity and provides further support of its role in mediating the expression of PPAR γ 1 in breast cancer. These data have led us to hypothesize that MAZ plays a critical role in tumorigenesis and this is currently being tested in the lab. The prevalence of MAZ expression in breast cancer is unknown and is also currently under investigation in the lab.

Through development, differentiation and tumorigenesis, genes can be silenced and activated by several mechanisms. Clearly acetylation and deactivation of histones in the region of targeted genes has been shown to regulate expression, as has the methylation of CpG islands. Changes in the expression of transcription factors during these events can also play a critical role. These data demonstrate that the upregulation of MAZ is responsible, at least in part, for driving the increase in PPAR γ 1 expression and may also play a role in tumor progression. Although the tumor specific promoter is very GC-rich, and therefore a potential target for methylation, this does not appear to be preventing use of this element. This is evident by the fact that when normal epithelial cells are forced to express MAZ, not only does this increase the expression of PPAR γ 1, but it does so using the tumor specific, GC-rich, promoter to drive expression. Although MAZ clearly drives an increase in the expression of PPAR γ 1 during tumor formation, it is not known what other genes are regulated by MAZ and what is the underlying mechanism that regulates MAZ expression. Therefore, it will be critical to understand the range of genes under the direct and indirect control of MAZ and define the consequences of MAZ expression to the mammary epithelia function. These questions are critical to our understanding of the consequences of regulation of both PPAR γ 1 and MAZ and are currently under investigation in the laboratory. These studies also highlight the usefulness of integrating the disparate technologies of computer based genomic analysis with expression patterns gleaned from microarray analysis to identify transcription factors involved in gene regulation. This is especially true of complex promoters such as that described here. This approach might also be useful in identifying the combination of factors that coordinate the expression of target genes including those that directly bind DNA as well as co-activators and co-repressors that coordinate the actions of the transcriptosome.

Experiments performed *in vitro* clearly demonstrate the potential of targeting PPAR γ 1 in the treatment of breast cancer [26,28]. In addition, animal studies support the role for using PPAR γ 1 ligands for both the treatment and prevention of breast cancer [28], and clinical data

is now emerging supporting the *in vitro* and animal studies that demonstrate a protective role of PPAR γ 1 ligands in the treatment and prevention of breast cancer in women (<http://www.proactive-results.com>). In these studies, a significant reduction in the occurrence of breast cancer was seen in type 2 diabetic patients taking the thiazolidinedione Pioglitazone. It is critical, however, that we understand what role the level of PPAR γ 1 expression plays in mediating the responses of these ligands on growth suppression and apoptosis. The work outlined in these studies provide a much needed basis for assessing these changes and determining whether the expression of other factors will alter a patients response to drugs targeting PPAR γ 1.

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Fig. 1.

Promoter analysis of the proximal 3 kb of exon A1 (pA1) from PPAR γ 1 in MCF-7 cells. **(A)** Promoter fragments were generated containing -2000 to +1 (2 kb), -1000 to +1 (1 kb), -263 to +1 (0.26 kb) and -3000 to -1000 (3 kb) controlling Luciferase expression. The proximal 263 bp flanking the start site of transcription are sufficient to mediate expression. Constructs were transfected into MCF-7 cells and data were normalized to *Renilla* as a control for transfection efficiency. Error bars represent half of the critical value calculated from the Tukey's pair-wise comparison test and those that do not share a letter designation were determined to be significantly different ($p < 0.001$). The proximal 263 bp flanking the start site of transcription are sufficient to mediate expression. The data shown represent three independent experiments composed of three biological replicates for each treatment for a total of nine observations per treatment. **(B)** The expression of transcription factors identified by TESS present on the HG-U133A chips was compared between HMEC and MCF-7 cells. Data

was analyzed using a two-tailed heteroscedastic t-test. MAZ expression is significantly higher in MCF-7 cells compared to HMECs ($p < 0.01$). The data shown represent a single experiment composed of three Affymetrix GeneChips per cell line.

Fig. 2.

The expression of MAZ in HMEC and MCF-7 cells. **(A)** Western blot analysis confirms that MAZ expression is much higher in nuclear extraction from MCF-7 compared to control. A small but significant increase in MAZ expression (as indicated by the bands marked with an arrow) is seen in HMEC following transfection with the MAZ expression plasmid. Alpha-tubulin was used as nuclear protein loading control. The data shown is representative of a single experiment with only one observation. **(B)** Formaldehyde cross-linking and Chromatin Immunoprecipitation (ChIP) assays were performed to confirm MAZ binding to the tumor-specific binding in MCF-7. The 210 bp amplified by tumor-specific promoter primers is clearly seen on 5% polyacrylamide gel from anti-MAZ sample and this 210 bp fragment was confirmed by DNA sequencing. The data shown is representative of a single experiment with only one observation.

Fig. 3.

Overexpression of MAZ in MCF-7 increases the expression of PPAR γ 1 from the MAZ response element. **(A)** MCF-7 cells were co-transfected with either pGL3 basic or pA1-263 alone or with the MAZ expression plasmid. Overexpression of MAZ increased the expression of the reporter from the -263 bp promoter. **(B)** Mutations (MUT) in the MAZ response element introduced into the -263 bp promoter significantly suppressed reporter activity. The reporter activity was measured by Luciferase assay with *Renilla* used as a transfection efficiency control. Error bars represent half of the critical value calculated from the Tukey's pairwise comparison test and those that do not share a letter designation were determined to be significantly different. Data shown in both panel A and B each represent three independent

experiments composed of three biological replicates for each treatment for a total of nine observations per treatment.

Fig. 4.

MAZ recruits the tumor-specific promoter to drive the expression of PPAR γ 1 in HMEC. **(A)** HMEC were transfected with a control or a MAZ expression plasmid and PPAR γ expression measured by real-time PCR. Both real-time PCR (data not shown) and Western blot analysis (fig. 2, A) confirmed the expression of MAZ. PPAR γ 1 mRNA is dramatically increased in the presence of MAZ expression. PCR, using pA1 specific primers, verify that only in the presence of MAZ is the exon associated with the tumor-specific promoter used (insert). For Real-time PCR, the data shown represent 2 independent experiments with 3 technical replicates for each treatment. **(B)** The ability of MAZ expression to increase the amount of PPAR γ able to bind to a PPRE was measured by ELISA using DNA as the immobilized target. Following the

expression of MAZ the amount of PPAR γ bound to its response element is significantly increased. For transactivation analysis, the data shown represent three independent experiments each with three biological replicates for a total of nine observations per treatment.