PPARγ Ligand-induced Annexin A1 Expression Determines Chemotherapy Response via Deubiquitination of Death Domain Kinase RIP in Triple-negative Breast Cancers

Luxi Chen1,2,3, Yi Yuan1, Shreya Kar1,2, Madhu M. Kanchi1, Suruchi Arora4, Ji E. Kim1, Pei F. Koh1,2, Einas Yousef5,6, Ramar P. Samy4, Muthu K. Shanmugam2, Tuan Z. Tan1, Sung W. Shin1, Frank Arfuso6, Han M. Shen4,9, Henry Yang1, Boon C. Goh1,2,10,11, Joo I. Park7, Louis Gaboury5, Peter E. Lobie1,2,11,12, Gautam Sethi2,13, Lina H.K. Lim4,9,14, and Alan P. Kumar1,2,11,15,16

Abstract

Metastatic breast cancer is still incurable so far; new specifically targeted and more effective therapies for triple-negative breast cancer (TNBC) are required in the clinic. In this study, our clinical data have established that basal and claudin-low subtypes of breast cancer (TNBC types) express significantly higher levels of Annexin A1 (ANXA1) with poor survival outcomes. Using human cancer cell lines that model the TNBC subtype, we observed a strong positive correlation between expression of ANXA1 and PPARγ. A similar correlation between these two markers was also established in our clinical breast cancer patients’ specimens. To establish a link between these two markers in TNBC, we show de novo expression of ANXA1 is induced by activation of PPARγ both in vitro and in vivo and it has a predictive value in determining chemosensitivity to PPARγ ligands. Mechanistically, we show for the first time PPARγ-induced ANXA1 protein directly interacts with receptor interacting protein-1 (RIP1), promoting its deubiquitination and thereby activating the caspase-8–dependent death pathway. We further identified this underlying mechanism also involved a PPARγ-induced ANXA1-dependent autoubiquitination of cIAP1, the direct E3 ligase of RIP1, shifting cIAP1 toward proteosomal degradation. Collectively, our study provides first insight for the suitability of using drug-induced expression of ANXA1 as a new player in RIP1-induced death machinery in TNBCs, presenting itself both as an inclusion criterion for patient selection and surrogate marker for drug response in future PPARγ chemotherapy trials.

Introduction

In the past two decades, the risk of breast cancer has been increasing worldwide, as well as in Asia (Japan, Korea and Taiwan; refs. 1–3). The rate of breast cancer incidence in Singapore has also become one of the highest in the world with 5.2% per year increase in premenopausal and 3.9% per year of postmenopausal women (4). Breast cancers can be classified into several classes, with each representing unique molecular or genetic characteristics. Triple-negative breast cancer (TNBC), which is estrogen receptor (ER), progesterone receptor (PR), and C-erb B2 receptor (ERBB2R)-negative (5), is known to be heterogeneous in nature of disease. Anthracycline–taxane regimens remain the current standard for TNBC patients who tend to have a higher risk of relapse and worse overall survival rates with mixed outcomes (6, 7). However, the natural heterogeneity of TNBC has made it difficult to evaluate the true clinical value of the tested drugs, making the cohorts’ selection based on specific biomarkers associated with distinct TNBC subgroups a prerequisite (8, 9). A “Genome-first Approach” concept has been introduced where patients will be prestratified and assigned to clinical trials designed to address the therapeutic hypotheses, based upon analysis of individual tumor profiles (8, 9).
PAPARγ belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. PAPARγ ligands, 15-deoxy-prostaglandin I2 (15d-PGJ2) and the synthetic thiazolidinedione (TZD) family, are currently available in the market as therapeutic agents targeting type II diabetes mellitus (10). The role of PAPARγ ligands as potential antitumor agents has been reported by many, including breast cancer (11–13). However, results from clinical trials have not been consistent among the various breast cancer subgroups, making novel biomarkers for patient selection and/or drug response for PAPARγ ligand trials, a necessity.

Annexin-A1 (ANXA1), a 37-kDa calcium-dependent phospholipid binding protein, has been implicated in various biological processes including tumorigenesis and anti-inflammatory activity of glucocorticoids (14, 15). ANXA1 expression has been suggested to play roles in the progression and development of many human cancers including breast cancer (16, 17).

NFkB complex is sequestered by inhibitor of kappa B (IkB) in the cytoplasm of normal cells. Breast cancer cells with invasive and metastatic characteristics, express constitutively activated NFkB (18, 19). We have reported baseline ANXA1 expressed in the MDA-MB-231 breast cancer cells interacts with NFkB essential modulator (NEMO, also known as IKKγ) and receptor interacting protein 1 (RIP1), driving activation of NFkB in cancer cells (20).

As our previous observations have shown that baseline ANXA1 interacts with NEMO and RIP1 to drive activation of NFkB signaling in TNBC lines (20) and having identified a putative PAPARγ binding consensus within the promoter of ANXA1, we asked whether activation of PAPARγ would have an effect on this multiprotein complex. Herein, we show, using a series of cell-based assays, antitumor activity of PAPARγ ligands is regulated via disruption of the ANXA1–NEMO–RIP1 ubiquitinated complex “switching to” an increased ANXA1–RIP1 deubiquitinated interaction, activating a caspase-8–dependent death pathway.

Materials and Methods

Cell lines and cell culture conditions

MDA-MB-231, BT549, MCF-7, and T47D breast cancer cell lines were purchased from ATCC within one year of the study in 2016. Authentication was based on growth rate, morphology, and absence of mycoplasma. MCF-7 EV and MCF-7 V5 cells were generated in our laboratory via stable transfection, followed by microarray gene expression analysis. These analysis were performed as described previously (22) and details outlined in Supplementary Methods.

Estimation of epithelial–mesenchymal transition score

An epithelial–mesenchymal transition (EMT) scoring method was developed using ovarian carcinoma cell lines expression profiling as described previously by our group (21, 24). A higher or lower EMT score indicates that the cell line exhibits a more mesenchymal or epithelial phenotype, respectively.

Statistical analysis

Statistical analyses for all in vitro work were performed using paired Student t test. A P value of less than 0.05 was considered significant. Statistical significance evaluation for microarray analysis were performed using Mann–Whitney test and Spearman correlation test, which were computed using Matlab R2012a (MathWorks). Dot plots were done using GraphPad Prism version 5.0 (GraphPad Software). Statistical analyses for IHC work were performed using the Institutional Review Board, Centre Hospitalier de l’Université de Montréal (CHUM) in 2005 with a renewal in 2010. Archived formalin-fixed paraffin-embedded (FFPE) tumor tissues from 164 breast cancer patients were selected from the archives of the Department of pathology, CHUM, University of Montreal at IRIC. Normal breast tissue samples were collected from healthy women undergoing plastic surgery. IHC was performed as described previously (22) and details outlined in Supplementary Methods.
Figure 1.
High level of expression of ANXA1 and PPARγ positively correlates with the invasive, high-grade phenotype. A, ANXA1 gene expression values in various breast cancer subtypes collected from public database Gene Omnibus (GEO). B, Correlation of ANXA1 expression to breast cancer survival. Kaplan-Meier plot shows ANXA1-high (red color) and ANXA1-low groups (blue color) defined by median of ANXA1 expression in basal and claudin-low breast cancer samples (n = 172). Log-rank test was used to compute the \( P \) value. HR, hazard ratio. C and D, Clinicopathologic examination of patient tissue samples with ANXA1 positive expression in IHC staining. Bar chart (left panels) indicate percentage of patient breast tumor tissues stained positively/negatively for ANXA1, categorized according to molecular classification of breast cancer subtypes. Corresponding anti-ANXA1 IHC staining (right) shows ANXA1 expression detected across different molecular subtypes of breast cancer (BC). (Continued on the following page.)
carried out using different packages of R language (http://www.R-project.org/). The distribution of ANXA1 among different histologic grades and molecular subtypes is depicted using bar charts. Nonparametric tests are used due to the nature of ordinal and categorical data. The overall relationship between ANXA1 scores and other characteristics (i.e., histologic grades, and molecular subtypes) was evaluated by χ² test. Correlation analysis for IHC expression levels was carried out using the Spearman ρ correlation coefficient. Statistical significance was considered, with a P value < 0.05.

Results
ANXA1 gene expression positively correlates to PPARγ gene expression in patients’ specimens and breast cancer models
Twenty-six cohorts containing 3,992 breast cancer tumors, 22 normal breast tissues collected and compiled from the public database Gene Omnibus (GEO) and ArrayExpress were analyzed for mRNA expression levels of ANXA1. ANXA1 expression is highest in Normal-like and claudin-low TNBC subtypes, followed by ERBB2+, luminal-A, and luminal-B subtype, while TNBC has significantly higher basal ANXA1 expression compared with luminal-A, luminal-B, and ERBB2+ breast cancer subtypes (Fig. 1A).

ANXA1-high patients had a markedly reduced percentage of patient survival than ANXA1-low patients (log-rank test, P = 0.015; HR = 0.4815; Fig. 1B). In addition, the epithelial–mesenchymal transition (EMT) scoring system shows ANXA1 was strong and positively correlated with EMT score (Spearman correlation test, ρ = 0.6367, P < 1e−128; Supplementary Fig. S1A). To validate our ANXA1 mRNA analysis from Fig. 1A, we obtained patients’ specimens from Centre hospitalier de l’Université de Montréal (CHUM) for IHC analysis. Normal breast tissue samples were collected from healthy women who underwent plastic surgery. Clinicopathologic features of these patients are shown in Table 1. IHC analyses showed higher levels of ANXA1 expression were associated with the more aggressive tumor subtypes such as TNBC and ERBB2+ compared with the luminal subtypes (Fig. 1C).

Higher ANXA1 expression was also detected in grade III than the lower grade breast tumors (Fig. 1D) with positive correlation observed between ANXA1 expression and cellular proliferation marker, Ki-67. Notably, the TNBC subtype expressed both high levels of ANXA1 and Ki-67 (Fig. 1E). Interestingly, clinical data analyzed using Spearman correlation test results showed ANXA1 gene expression is indeed positively correlated with PPARγ expression in patient samples (ρ = 0.29, P < 1e−128; Fig. 1F). In agreement with our mRNA correlation in Fig. 1F, ANXA1 protein levels also positively correlate to PPARγ protein expression in patient breast tumors by IHC scoring (Fig. 1G) as well as in breast cancer cell lines by relative mRNA quantification (Supplementary Fig. S1B).

In vitro, baseline gene and protein expression levels of ANXA1 and PPARγ were performed in luminal subtype lines (MCF-7and T47D) and claudin-low TNBC subtype lines (MDA-MB-231 and BT549). Baseline ANXA1 mRNA and protein levels were significantly higher by 2.5-times in TNBC than luminal breast cancer cell lines (Fig. 1H). Importantly, we observed a similar corresponding pattern of baseline levels of PPARγ mRNA and protein levels were higher in the two TNBC cell lines as well (Fig. 1I). Concomitant to the differences in baseline PPARγ expression levels (Fig. 1I), lower PPARγ activity is observed in normal MCF10A and luminal type T47D compared with that in TNBC type MDA-MB-231 cells (Fig. 1J).

Activation of PPARγ ligands leads to an ANXA1-dependent cell death in TNBC lines
Crystal violet staining assay was performed on the breast cancer cells using two PPARγ ligands, 15d-PGJ2, or rosiglitazone. Dose-dependent decrease in cell viability was observed in MDA-MB-231 and BT549 cells after 48-hour treatments (Fig. 2A), while marginal decrease in viability of MCF-7 and T47D cells was observed (Fig. 2B). These findings were further validated by microscopic examinations in TNBC (Supplementary Fig. S1C and S1D) versus luminal breast cancer (Supplementary Fig. S1E and S1F). Colony-forming abilities of MDA-MB-231 and BT549 cells were also greatly affected by PPARγ ligand treatment (Fig. 2C). In contrast, MCF-7 and T47D cells were relatively resistant to PPARγ ligand treatment (Fig. 2D). Interestingly, in both MDA-MB-231 (Fig. 2E) and BT549 (Supplementary Fig. S2A and S2B), the significant decrease in cell viability after exposure to PPARγ ligands was abrogated when ANXA1 depleted in these cells. MDA-MB-231 cells transfected with scrambled siRNA showed 15.3% and 21.8% increase in total apoptosis after exposure of 15d-PGJ2 (10 μmol/L) and rosiglitazone (80 μmol/L), respectively (Fig. 2F and G). However, less than 5% increase in apoptosis was observed in cells with depleted ANXA1 after PPARγ ligand treatments in MDA-MB-231 cells (Fig. 2H). Conversely, in the MCF-7-V5 cells (an MCF-7 cell line stably overexpressing ANXA1 ectopically), treated with the same concentrations of 15d-PGJ2 or rosiglitazone resulted in up to 40% reduction in cell viability compared with MCF-7-EV cells (stably transfected with empty vector; Fig. 2I), with a 17.7% and 33.8% increase in total apoptosis when treated with 15d-PGJ2 or rosiglitazone, respectively. However, only 5% increase in total apoptosis was observed in MCF-7-EV cells after PPARγ ligand treatments (Fig. 2I).

Activation of PPARγ induces ANXA1 gene activity and expression in TNBC cells
As we observed manipulation of ANXA1 expression influences the effect of PPARγ ligands, we next investigated whether PPARγ ligand activity could influence ANXA1 expression. ANXA1 promoter activity increased 2.5-fold and 1.5-fold after treatment

(Continued) E, Clinicopathologic examination of patient tissue samples with ANXA1 and Ki-67–positive expression in IHC staining. Bar chart indicates the percentage (%) of patient’s breast tumor tissue stained for either positively/negatively of ANXA1 or Ki-67 and categorized according to the molecular classification of breast cancer subtypes. Corresponding anti-ANXA1 and anti-Ki-67 IHC staining was detected the expression of ANXA1/Ki-67 across in different molecular subtypes of breast cancer. F, Correlation between PPARγ and ANXA1 gene expression in breast cancer patients. The red dotted line is fitted with the linear regression line. G, Correlation between PPARγ and ANXA1 expression in breast cancer tissues based on IHC scoring. The red dotted line is fitted with the linear regression curve. H, Relative basal ANXA1 levels of the same panel of cell lines. Data are the relative percentage (%) of changes ± SEM of three independent experiments (n = 3). Western blot analysis showed the basal ANXA1 expression levels in the same panel of cells. I, Relative basal PPARγ expression levels of the same panel of cell lines. Data are the relative percentage (%) of changes ± SEM of three independent experiments (n = 3). Western blot analysis showed the basal PPARγ protein levels in the same panel of cell lines. β-Actin was used as a protein loading control. J, Baseline PPARγ activity measured by using a pPREluciferase read-out in normal breast cell line, MCF10A compared with that in a luminal type cell line, T47D, and a TNBC cell line, MDA-MB-231.
with 15d-PGJ2 and rosiglitazone, respectively (Fig. 3A). A time-dependent increase in ANXA1 mRNA (Fig. 3B and C) and corresponding protein expression (Fig. 3D and E) were observed in MDA-MB-231 cells after treatment with 10 μmol/L of 15d-PGJ2 or 80 μmol/L of rosiglitazone. These in vitro observations were further confirmed in vivo using a MDA-MB-231 xenograft mouse model previously reported by our group. Mice injected with 15d-PGJ2 showed no significant increase in tumor volume throughout the experimental period, while mice treated with vehicle control exhibited an increase in tumor volume with time (23). Importantly, in agreement with our cell line data, tissue collected from these 15d-PGJ2–treated mice exhibited more positive staining for ANXA1 than the PBS-treated mice (Fig. 3F). On the other hand, the luminal MCF-7 cells that are insensitive to PPAR γ ligand (Fig. 2A and B), did not show an increase in ANXA1 gene expression (Supplementary Fig. S2C). PPARγ ligand–induced ANXA1 gene activity and expression is receptor dependent

Next, we utilized GW9662, an irreversible and selective PPARγ antagonist (25) to ascertain whether the induction of ANXA1 expression was dependent on PPARγ activity. ANXA1 promoter activity induced by PPARγ activation was decreased in MDA-MB-231 cells preincubated with GW9662 (Fig. 4A and B). Preincubation with GW9662 abrogated the induction of ANXA1 mRNA (Fig. 4C and D) and protein expression (Fig. 4E and F) after treatment with PPARγ ligands. Consistent with these observations, preincubation with GW9662 also exerted its inhibitory effect on PPARγ–induced upregulation of ANXA1 gene expression in BT549 cells (Supplementary Fig. S3A–S3C). In addition, inhibition of PPARγ activity by GW9662 effectively blocked PPARγ–induced apoptosis in both MDA-MB-231 and BT549 cells (Supplementary Fig. S3D and S3E).

Alternatively, the dependence of ANXA1 gene induction on PPARγ activity was examined by using a dominant-negative (DN) mutant of PPARγ (Supplementary Fig. S3F). Overexpression of DN-PPARγ significantly inhibited the induction of peroxisome proliferator response element (PPRE) luciferase activity by PPARγ ligands (Supplementary Fig. S3G). Importantly, DN-PPARγ effectively prevented the drug-induced increase in ANXA1 promoter activity (Fig. 4G and H). In addition, DN-PPARγ also significantly suppressed the PPARγ–induced ANXA1 mRNA (Fig. 4I and J) and protein expression (Fig. 4K and L).

Exposure to PPARγ ligands promotes a ‘switch’ from ANXA1–NEMO–RIP1 ubiquitinated interaction (non-death) to ANXA1–RIP1 deubiquitinated interaction (death)

We next assessed whether activation of PPARγ influences protein–protein interactions within ANXA1–NEMO–RIP1 complex; as we previously reported (20). Treatment of MDA-MB-231 cells with 10 μmol/L of 15d-PGJ2 leads to induction of both ANXA1 and RIP1 protein expression, but reduction in NEMO protein expression (Supplementary Fig. S4A). A 4-fold increase in RIP1 mRNA and a 30% decrease in NEMO mRNA was observed after treatment with 15d-PGJ2 (Supplementary Fig. S4B and S4C). Moreover, these changes in RIP1 and NEMO mRNA were found to be dependent on PPARγ activity, as preincubation with GW9662 attenuated these changes (Supplementary Fig. S4B and S4C). Activation of PPARγ also resulted in a receptor-dependent reduction of NFκB activation (Supplementary Fig. S4D and S4E).

To gain deeper insight into the mechanism for our observed results in Supplementary Fig. S4A–S4E, immunoprecipitation (IP) was performed for each of the proteins in the tricomplex upon PPARγ activation. MDA-MB-231 cells treated with 10 μmol/L of 15d-PGJ2 exhibited an increase in ANXA1–RIP1 interaction (Fig. 5A), together with a decrease in NEMO–RIP1 interaction (Fig. 5A) and ANXA1–NEMO interaction (Fig. 5A). The role of RIP1 on the cell viability after treatment with PPARγ ligands was also investigated. A 30% to 40% decrease in cell viability was observed in control MDA-MB-231 cells transfected with scrambled siRNA after exposure to 15d-PGJ2 or rosiglitazone (Fig. 5C). Importantly, this reduction in cell viability was attenuated upon RIP1 depletion. Moreover, silencing RIP1 expression also blocked the increase in apoptotic cells upon treatments with PPARγ ligands (Supplementary Fig. S5A). These results suggest that the loss of RIP1 expression prevented cell death stimulated by PPARγ activation. Interestingly, the loss of RIP1 not only reduced the baseline expression of ANXA1, but also prevented the induction of ANXA1 protein expression to the same level of expression by PPARγ activation. However, NEMO protein expression was not downregulated upon PPARγ activation in RIP1-depleted cells (Fig. 5D).

The ubiquitination status of RIP1 determines its role as either a prosurvival or a prodeath agent (26). Interestingly, reduction in global polyubiquitination was observed in both MDA-MB-231 and BT549 cells treated with 10 μmol/L of 15d-PGJ2 in a time-dependent manner (Fig. 5E; Supplementary Fig. S5E). More importantly, treatment with the PPARγ ligands also led to a time-dependent reduction in RIP1–specific ubiquitination in TNBC cell lines (Fig. 5F). To investigate the role of ANXA1 in RIP1 ubiquitination, endogenous ANXA1 was silenced in MDA-MB-231, followed by treatment with 10 μmol/L of 15d-PGJ2. Depletion of ANXA1 prevented a decrease in global protein polyubiquitination levels in MDA-MB-231 cells (Fig. 5G).

### Table 1.

<table>
<thead>
<tr>
<th>Clinicopathologic data of the 315 patients’ specimen from Centre hospitalier de l’Université de Montréal (CHUM)</th>
<th>Number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (35–92 years)</td>
<td>315</td>
</tr>
<tr>
<td>Grade</td>
<td>26</td>
</tr>
<tr>
<td>I</td>
<td>47</td>
</tr>
<tr>
<td>II</td>
<td>242</td>
</tr>
<tr>
<td>Histologic subtypes</td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>239</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Typical medullary carcinoma</td>
<td>46</td>
</tr>
<tr>
<td>Atypical medullary carcinoma</td>
<td>17</td>
</tr>
<tr>
<td>Colloid carcinoma</td>
<td>8</td>
</tr>
<tr>
<td>Estrogen receptors</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>121</td>
</tr>
<tr>
<td>Negative</td>
<td>194</td>
</tr>
<tr>
<td>Progesterone receptors</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>107</td>
</tr>
<tr>
<td>Negative</td>
<td>208</td>
</tr>
<tr>
<td>HER-2 receptors</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>51</td>
</tr>
<tr>
<td>Negative</td>
<td>264</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>120</td>
</tr>
<tr>
<td>No</td>
<td>195</td>
</tr>
</tbody>
</table>

Published OnlineFirst August 15, 2017; DOI: 10.1158/1535-7163.MCT-16-0739
Figure 2.
High ANXA1-expressing TNBC cell lines display greater sensitivity to PPARγ ligands compared with low ANXA1-expressing luminal-like cell lines. A–D, MDA-MB231 cells (basal) were treated with indicated concentrations of 15d-PGJ2 and rosiglitazone for indicated time points. Cell viability and colony-forming assay was measured using crystal violet assay after 48-hour treatment with (A and C) 15d-PGJ2 or (B and D) rosiglitazone, respectively. E, Western blot assay after knockdown with siANXA1 F and G, MDA-MB231 cells (Control or ANXA1 siRNA) were treated with indicated concentrations of 15d-PGJ2 and rosiglitazone for indicated time points. Cell viability was measured using crystal violet assay after 48-hour treatment with (F) 15d-PGJ2 or (G) rosiglitazone, respectively. H, ANXA-V PI staining measured after 48-hour treatment with 15d-PGJ2 or rosiglitazone. Percentage (%) indicates cells that underwent late and early apoptosis (total apoptosis). I and J, Viability of MCF-7 EV and MCF-7 V5 (ANXA1 overexpressing) stable cells were measured using crystal violet assay (I) or ANXV-PI staining after 48 hours of indicated PPARγ drug treatments (J). Data are all average ± SEM; n = 3 experiments; *, P < 0.05.
RIP1 needs to be in a deubiquitinated state to enable its interaction with caspase-8 to promote apoptosis (27). A moderate decrease in procaspase-8, together with a marked increase in caspase-8 cleavage after treatment with 15d-PGJ2 was observed in MDA-MB-231 cells. However, these observations were not seen in ANXA1-depleted cells (Fig. 5H). Moreover, knocking down expression of ANXA1 in MDA-MB-231 cells also blocked the ligand-induced increase in caspase-3 cleavage as well as inhibiting the downregulation of prosurvival proteins such as Bcl-2 and survivin (Fig. 5I).

**Figure 3.**
Activation of PPARγ induces expression of ANXA1 gene expression in vitro and in vivo. MDA-MB-231 cells (basal or ANXA1 siRNA silenced) were treated with indicated concentrations of 15d-PGJ2 and rosiglitazone for indicated time points. A, ANXA1 promoter activity was measured in MDA-MB-231 cells after treatment with the PPARγ drugs. Data are average relative light units/Renilla/µg protein ± SEM of three independent experiments. B and C, ANXA1 mRNA was quantified using qPCR in MDA-MB-231 cells after increasing time points of treatment with 10 µmol/L of 15d-PGJ2 (B) or 80 µmol/L rosiglitazone (C). D and E, ANXA1 protein expression was determined by Western blot analysis in MDA-MB-231 cells treated with different doses of 15d-PGJ2 (D) or rosiglitazone (E) for 48 hours. β-Actin was used as a protein-loading control. F, Tumor tissues from mice were subjected to IHC using antibodies against ANXA1. In all experiments, data are mean ± SEM of at least three experiments. *P < 0.05 was considered as significant. For Western blots, a representative blot was chosen from at least three biological replicates.
Induction of ANXA1 gene expression by PPARγ ligands is receptor-dependent. MDA-MB-231 cells were preincubated with 10 μmol/L of PPARγ-inhibitor GW9662 for 4 hours, prior to treatment with 15d-PGJ2 or rosiglitazone for 18 hours. A and B, ANXA1 promoter activity was measured, using untreated set as a control (dashed line). Data are average relative light units/Renilla/mg protein. C and D, ANXA1 expression of mRNA levels was analyzed in MDA-MB-231 cells after 24-hour treatment with 15d-PGJ2 (C) or rosiglitazone (D). E and F, Alterations of ANXA1 protein levels were detected by Western blot analysis in MDA-MB-231 cells after treatment with 15d-PGJ2 (E) or rosiglitazone (F) for 48 hours. β-Actin was used as a protein-loading control. G and H, After transfection of dominant-negative PPARγ (PPARγ-DN) or empty vector control, cells were treated with 15d-PGJ2 (G) or rosiglitazone (H) for 18 hours and ANXA1 promoter activity was measured. I–L, Cells were treated as above and expression of ANXA1 mRNA and protein were measured. In all experiments, data are mean/C6 SEM of at least three experiments. P < 0.05 was considered as significant. For Western blots, a representative blot was chosen from at least three biological replicates.
Figure 5.
Increased expression of ANXA1 interacts with RIP1 blocking RIP1-NEMO interaction and mediates deubiquitination of RIP1. A, Immunoprecipitation (IP) analysis was performed to confirm the expression levels of ANXA1 and NEMO interaction with RIP1, ANXA1 and RIP1 interaction with NEMO, and RIP1 and NEMO interaction with ANXA1 after 24-hour treatment with 10 μmol/L 15d-PGJ2. B, Expression of all three proteins after treatment with 10 μmol/L 15d-PGJ2. C, Cell viability of MDA-MB-231 cells measured after 48 hours of PPARy drug treatment posttransfection with siRIP1 or siCtrl. Data are mean ± SEM of three biological replicates; *P < 0.05. D, ANXA1, NEMO, and RIP1 protein levels determined after depletion of RIP1, followed by treatment with 15d-PGJ2 or rosiglitazone for 48 hours. E, Global polyubiquitin levels were measured after treatment with 15d-PGJ2 at different time points. F, IP analysis of ubiquitination level of RIP1 protein upon treatment with 15d-PGJ2 for various time points. G, Global protein polyubiquitin expression levels upon depletion of ANXA1, followed by treatment of 15d-PGJ2 for 24 hours. H, Intact and cleaved caspase-8 protein levels upon depletion of ANXA1, followed by treatment with 15d-PGJ2 for 48 hours. I, Caspase-3, Bcl-2, and survivin protein levels upon depletion of ANXA1, followed by treatment with 15d-PGJ2 for 48 hours.
expression is detected only in TNBC tumors and histologic grade III tumors. However, most of the earlier studies on ANXA1 expression in breast cancer did not include tumors of all the different molecular or histologic subtypes or histologic grade of breast cancer (16, 17, 38). This may be the reason for the inconsistency in reporting ANXA1 expression in breast cancers conducted in the earlier studies.

IHC staining results demonstrated that TNBC tumors, which express a high level of ANXA1, also demonstrate a higher level of Ki-67 expression. Additional supporting evidence on high ANXA1 expression as an indicator of cancer metastasis and invasion is found in more recent literature (37, 39, 40). Particularly, de Graauw and colleagues reported that ANXA1 expression is functionally related to the progression of breast cancer, and is associated with the highly invasive BLBC subtype (41). Linking back to our in vitro result on the basal gene expression of ANXA1 among breast cancer cell lines, these observations suggest that the ANXA1 expression level is potentially associated with the degree of malignancy of breast cancer tumors.

Importantly, depletion of endogenous ANXA1 (or rather not allowing for PPARγ-induced increased expression) conferred resistance in two TNBC cell lines with exposure to PPARγ agonists. On the other hand, stable clones of MCF-7 cells with ectopic overexpression of ANXA1 showed enhanced responsiveness to PPARγ drugs as compared with the unresponsive parental empty vector control clones. These results proved our hypothesis that high ANXA1 expression in breast cancer cells acts as an indicator of PPARγ agonist response. Interestingly, activation of PPARγ failed to influence baseline ANXA1 expression in these PPARγ-unresponsive cells. It has been reported that ERα could bind to PPRE elements and represses PPARγ transactivation in MCF-7 cells (42). Our group has previously reported ANXA1 as a putative ERα target (43). It is therefore probable that an active ERα may have negated PPARγ activity at the promoter of ANXA1 in MCF-7 cells.

PPARγ activation has been reported to be involved in suppression of tumor proliferation and growth in various types of cancer tissues (13, 23, 32, 44). The functional role of PPARγ was firstly elucidated by us when MDA-MB-231 cells were injected subcutaneously into mice mammary fat pad, followed by treatment with 15d-PGJ2. Mice exposed to 15d-PGJ2 showed inhibited tumor growth in tumor volume as compared with mice treated with control vehicle (23). More interestingly, ANXA1 expression in tumor tissues obtained from 15d-PGJ2–treated mice was significantly higher than that obtained from the untreated animals. Using our published PPRE search engine (45), we located a putative PPRE in the promoter of ANXA1. To verify our hypothesis that perhaps ANXA1 is a novel target of PPARγ activity, we performed in vitro evaluation on changes in ANXA1 gene expression levels upon PPARγ ligand exposure in two TNBC models. We observed induction of ANXA1 expression by PPARγ ligands, from promoter activity to protein expression, suggesting that ANXA1 expression is indeed regulated by PPARγ.

The irreversibility of PPARγ inhibitor, GW9662, and DN-PPARγ, which has no transcriptional activity, was able to block the induction of ANXA1 expression and attenuated the apoptotic effects of PPARγ ligands. These findings further confirm that the upregulation of ANXA1 is mediated by the activity of ligand-activated PPARγ, and the apoptotic effects observed upon incubation with PPARγ ligands are also due to the intrinsic activity of the receptor.

(5). As activation of PPARγ is known to induce proteasomal degradation of various proteins (30, 31), we proceeded to demonstrate that preincubation of MDA-MB-231 cells with MG132 a proteasomal inhibitor, followed by exposure to 10 μmol/L of 15d-PGJ2, for 24 hours, prevented the degradation of cIAP1 protein (Fig. 6B). On the other hand, we observed 15d-PGJ2 reduced the degradation of cIAP1 with a much delayed and weaker when the endogenous ANXA1 was silenced (Fig. 6C). Importantly, we also observed a marked increase in ubiquitinated cIAP in MDA-MB-231 cells after exposure to 15d-PGJ2, which was not observed when ANXA1 was silenced (Fig. 6D). Exposure to 15d-PGJ2 also reduced NEMO-specific ubiquitin levels, which was also not observed when ANXA1 was silenced (Supplementary Fig. S5F). Indeed, we observed an interaction between RIP1 and cIAP1, which was followed by a gradual time-dependent decrease in cIAP1–RIP1 interaction upon treatment with 10 μmol/L of 15d-PGJ2. However, when ANXA1 was depleted, the time-dependent dissociation of cIAP1 from RIP1 after exposure to 15d-PGJ2 was not observed (Fig. 6E). Importantly, the RIP1-specific ubiquitin level remained unchanged with 15d-PGJ2 treatment in cells silenced with ANXA1 (Fig. 6F). Finally, PPARγ ligand treatment induced an increase in RIP1-procaspase-8 association in a time-dependent manner (Fig. 6G).

**Discussion**

To date, several in vitro studies have shown that PPARγ activation causes cell death and induces apoptosis in various cancer cell lines including breast cancer. Nevertheless, applications of PPARγ activators as anticancer options in clinical trials have not yet shown promising results. Particularly, in the context of breast cancer, inconsistent outcomes of PPARγ activators have been reported across different subgroups of breast cancer tissues or cells (32–34). In parallel, the antiproliferative role of ANXA1 has been reported (35, 36); however, there has not been a suggested a role for ANXA1 as a biomarker in any cancer types (17, 37). Our study aimed to address the question of whether the presence of ANXA1 could influence the effectiveness of PPARγ activators in breast cancer.

Preliminary analysis across 3,992 breast cancer tumors showed ANXA1 mRNA expression was higher in basal and claudin-low breast cancer (grouped as TNBC) subtypes correlating to poor patient survival. In addition, higher level of ANXA1 expression was positively correlated with mesenchymal phenotype. In agreement with our mRNA analysis, IHC staining of various breast cancer tissues showed that TNBC subtype exhibited high level of ANXA1 expression that was correlated with very high histologic grading scores. These findings strongly support the observation of in vitro screening of breast cancer cells, that the highly invasive TNBC cells express more baseline ANXA1 than the poorly invasive ER+ breast cancer cells. Baseline PPARγ expression and therefore activity also showed a similar trend across the panel. To our understanding, this is the first report on the positive correlation between ANXA1 and PPARγ in breast cancer.

Despite the continuous reporting of ANXA1 expression in many human cancers, the results have been conflicting across different studies and in different types of cancers; thus, the exact role of ANXA1 in cancer development and progression remains undefined. Our IHC staining for ANXA1 expression in tumors of various breast cancer subtypes shows that ANXA1 expression is not evenly distributed in all breast tumor tissues. High ANXA1
Figure 6.
Activation of PPARγ leads to proteosomal degradation of cIAP1 dissociating itself from RIP1 complex to initiating a caspase-8-dependent cell death pathway. A and B, MDA-MB-231 cells were preincubated with/without 10 μmol/L of GW9662 (A) for 18 hours, or 1 μmol/L of MG132 (B) for 2 hours, followed by treatment with 10 μmol/L of 15d-PGJ2 for various time points up to 48 hours. Protein levels of RIP1, ANXA1, and cIAP1 were measured. C, Protein level of cIAP1 was measured after depletion of ANXA1 followed by treatment with 15d-PGJ2 for various time points up to 24 hours. D, Ubiquitinated cIAP1 protein levels were assessed by IP analysis before and after depletion of ANXA1, followed by treatment with 15d-PGJ2 for 24 hours. E, ANXA1 and cIAP1 proteins interaction with RIP1 was assessed by IP analysis after depletion of ANXA1, followed by treatment with 15d-PGJ2 at different time points up to 24 hours. F, Expression level of RIP1 protein was analyzed after depletion of ANXA1, followed by treatment with 15d-PGJ2 at different time points up to 24 hours. G, Caspase-8 interacting with RIP1 protein expression levels was measured after treatment with 15d-PGJ2 of different time points up to 30 hours.
RIP1 exists as a double-edged sword in controlling the cellular fate. Together with NEMO, they cooperate to promote cell survival in response to excessive DNA damage (46). Our earlier study reported that basal ANXA1 in MDA-MB-231 cells interacts with NEMO and RIP1, and contributes to the constitutively active NFκB in these cells (20). To take these findings further, we propose a new mechanism by which activated PPARγ promotes apoptosis in breast cancer cells in ANXA1-dependent manner. MDA-MB-231 cells treated with 15d-PGJ2 exhibit increased ANXA1 and RIP1 expression after treatment, while NEMO expression was downregulated. We also found increases in levels of cleaved caspase-8 and caspase-3 after treatment, while NEMO expression was downregulated. We also found increases in levels of cleaved caspase-8 and caspase-3 after treatment, suggesting the drug treatment leads to apoptosis via activation of caspase-8. These observations indicate that 15d-PGJ2 treatments have shifted the balance of RIP1 activity toward proapoptosis. Interestingly, 15d-PGJ2 exposure for 24 hours led to stronger interaction between RIP1 and ANXA1 but important dissociation of NEMO from both RIP1 and ANXA1.

A previous study suggested the proapoptotic activity of RIP1 requires its dissociation from NEMO (47). This is in line with our expectation of decreased RIP1–NEMO interactions upon drug treatment, due to the fact that PPARγ drugs are able to induce apoptosis. One possible explanation to the inhibitory effect of NEMO on RIP1-dependent cell death is that ubiquitination of RIP1 at lysine 377 recruits ubiquitin-binding proteins, such as NEMO, and this binding sterically hinders RIP1 from associating with caspase-8 and other downstream death mediators (29). On the other hand, ubiquitination of RIP1 may also prevent RIP1 from oligomerization. The oligomerization of the death domain of RIP1 has been implicated to contribute to its cell death–triggering ability.

The increased RIP1–ANXA1 association attracted our attention and led us to investigate the role of ANXA1 on RIP1-mediated cell death. Depletion of ANXA1 reduced apoptosis upon PPARγ ligand treatment and leads to a reduction in caspase-8 and caspase-3 activation and inhibition in the downregulation of prosurvival molecules such as Bcl-2 and survivin.
importantly, the loss of ANXA1 caused a reduction in RIP1 protein and mRNA expression in MDA-MB-231 cells. This indicates the importance of ANXA1 in maintaining both mRNA and protein expression of RIP1 and its role in the activation of caspase-8 and the initiation of apoptosis. Furthermore, activation of PPARγ failed in suppressing NEMO transcription when ANXA1 was absent in MDA-MB-231 cells. This provides possible alternative mechanism for the higher resistance to PPARγ ligands found in TNBC cells with low ANXA1 expression.

A time-kinetic study with treatment of 15d-PGJ2 showed deubiquitination of RIP1 in MDA-MB-231 cells. Without hindrance of the polyubiquitin chain, RIP1 is free to interact with the pro-caspase-8 and causes the subsequent initiation of apoptotic events. This provides a possible mechanism where activation of PPARγ induces apoptosis in MDA-MB-231 cells via the action of deubiquitinated RIP1. In agreement with our findings, activation of caspase-8 is known to be dependent on deubiquitination of RIP1 (48). The essential role of RIP1 in PPARγ-induced apoptosis was granted with the observation that depleting RIP1 was able to block the apoptosis rate in treated MDA-MB-231 cells. Furthermore, the absence of RIP1 restrains the drug-induced dissociation of NEMO from ANXA1. Interestingly, the expression of ANXA1 exerts influence on the ubiquitination status of RIP1. We observed that depletion of ANXA1 has an inhibitory effect on the reduction of total protein ubiquitin expression after the treatment, while the RIP1-specific deubiquitination is also hindered in MDA-MB-231 cells with depleted ANXA1. Linking back to the earlier observations that knocking down ANXA1 promotes resistance to PPARγ drugs, in TNBC cells, this confirms that ANXA1 is an important intermediate molecule that ensures the prodeath mechanism of ligand-activated PPARγ.

We have demonstrated a highly possible mechanism by which PPARγ activation lead to caspase-8-initiated apoptosis in TNBC cells, through a process of ANXA1-dependent deubiquitination of RIP1. Several earlier studies reported that cIAP1 functioning as an E3 ligase is involved in catalyzing the polyubiquitination of RIP1, while loss of cIAP1 blocks ubiquitination of RIP1 in response to TNF (49, 50). More recently, cIAP1 has been identified as the direct E3 ligase that conjugates ubiquitin chains to RIP1 (28). Treatment with 15d-PGJ2 showed a time-dependent decrease in cIAP1 protein expression level and preincubation with GW9662 was able to block the drug-induced reduction in cIAP1 gene expression. It suggests that cIAP1 expression could be PPARγ regulated. However, the downregulation of cIAP1 in MDA-MB-231 cells may not be due to transcriptional regulation by PPARγ. The activity of PPARγ to cause degradation of cIAP1 was uncovered; we also demonstrated that inhibiting proteasome activity by MG132 was able to attenuate PPARγ-induced degradation of cIAP1 protein expression. Interestingly, silencing of ANXA1 in MDA-MB-231 cells slows down this degradation process on cIAP1 protein upon exposure to the PPARγ ligands. Moreover, the PPARγ ligands caused increased autoubiquitination of cIAP1 and turn its degradation in ANXA1-dependent manner. These findings suggest that drug-induced ANXA1 expression is crucial for the promotion of proteasome-dependent degradation of cIAP1 by PPARγ ligands.

In summary, our study proposes a novel schematic model to describe the mechanism of how PPARγ activation lead to apoptosis in TNBC through the actions of ANXA1 and RIP1 (Fig. 7). Under basal conditions, NEMO binds to polyubiquitinated RIP1 and ANXA1, inducing constitutive activation of NFXB to promote cancer cell survival (20). Agonists that activate PPARγ induce expression of RIP1 and ANXA1. Meanwhile, deubiquitinated RIP1 dissociates from NEMO, while it remains associated with ANXA1. RIP1 then binds procaspase-8 and leads to initiation of caspase-8-dependent apoptosis. ANXA1 expression required for the stabilization of RIP1 protein to ensure function as a mediator of the proapoptotic action of PPARγ ligands. Taken together, we show that drug-induced ANXA1 expression mediates actions of PPARγ ligands in promoting cell apoptosis and that ANXA1 could serve as a clinically useful surrogate biomarker for such therapies in the future trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L. Chen, P.F. Koh, L. Lim, A.P. Kumar.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Chen, Y. Yuan, P.F. Koh, E. Yousef, M.K. Shanmugam, S.-W. Shin, J.-I. Park, L. Gaboury, A.P. Kumar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Chen, S. Kar, P.F. Koh, E. Yousef, T.Z. Tan, H. Yang, L. Gaboury, P.E. Lobie, G. Sethi, L. Lim, A.P. Kumar
Writing, review, and/or revision of the manuscript: L. Chen, Y. Yuan, S. Kar, J. Kim, P.F. Koh, F. Arfuso, J.-I. Park, L. Gaboury, A.P. Kumar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Chen, M. Kanchi, E. Yousef, B.C. Goh, P.E. Lobie, A.P. Kumar
Study supervision: H.-M. Shen, P.E. Lobie, L. Lim, A.P. Kumar
Other (fine tuning and revision of the manuscript): R. Samy

Grant Support
This work was supported by grants from the National Medical Research Council of Singapore and by the NCSI Yong Siew Yoon Research Grant through donations from the Yong Loo Lin Trust (to A.P. Kumar). A.P. Kumar and P.E. Lobie were supported by grants from the Singapore NMRC Clinician Scientist IRC and the National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centers of Excellence Initiative to Cancer Science Institute of Singapore, National University of Singapore. This work was also supported by the NIH U01-CA198866-01A1 (to A.P. Park) and the National Institutes of Health (to J.-I. Park). This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) and the National Research Foundation (grant funded by the Korea government (MEST). This work was supported by a grant from Singapore Ministry of Education Tier 2. This work is supported in part by National Medical Research Council Singapore (NMRC) grants (to H.-M. Shen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 2, 2016; revised May 17, 2017; accepted July 20, 2017; published OnlineFirst August 15, 2017.

References


Molecular Cancer Therapeutics

PPARγ Ligand–induced Annexin A1 Expression Determines Chemotherapy Response via Deubiquitination of Death Domain Kinase RIP in Triple-negative Breast Cancers

Luxi Chen, Yi Yuan, Shreya Kar, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-16-0739

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2017/08/12/1535-7163.MCT-16-0739.DC1

Cited articles
This article cites 50 articles, 14 of which you can access for free at:
http://mct.aacrjournals.org/content/16/11/2528.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/16/11/2528.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/16/11/2528.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.