Pregnane X Receptor PXR Activates the GADD45β Gene, Eliciting the p38 MAPK Signal and Cell Migration*

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Pregnane X receptor (PXR) was originally characterized as a transcription factor that induces hepatic drug metabolism by activating cytochrome P450 genes. Here we have now demonstrated a novel function of PXR, that of eliciting p38 mitogen-activated protein kinase (MAPK) phosphorylation for cell migration. Upon xenobiotic activation of ectopic human PXR, human hepatocellular carcinoma HepG2 cells were found to exhibit increased phosphorylation of p38 MAPK and to subsequently change morphology and migrate. p38 MAPK was responsible for the regulation of these morphological changes and cell migration because the p38 MAPK inhibitor SB239063 repressed both. Prior to this phosphorylation, PXR directly activated the early response GADD45β gene by binding to a distal direct repeat 4 site of the GADD45β promoter. Ectopic expression of GADD45β increased p38 MAPK phosphorylation, whereas siRNA knockdown of GADD45β decreased the PXR-induced p38 MAPK phosphorylation, confirming that GADD45β can regulate PXR-induced p38 MAPK phosphorylation in HepG2 cells. These results indicate that PXR activates the GADD45β gene, increasing p38 MAPK phosphorylation, and leading HepG2 cells to change morphology and migrate. The GADD45β gene is a direct target for PXR, eliciting cell signals to regulate various cellular functions.

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2 The abbreviations used are: PXR, pregnane X receptor; DMSO, dimethyl sulfoxide; DR4, direct repeat 4; MEM, minimum Eagle’s medium; qRT-PCR, quantitative RT-PCR; RIF, rifampicin; RXR, retinoid X receptor; CAR, constitutive active/androstane receptor.
PXR and has provided us with the basis to investigate the molecular mechanism by which PXR regulates cell signals and fates such as morphology and migration. Because humans are constantly exposed to numerous therapeutics and xenobiotics, a PXR-elicted cell signal may become a critical factor in understanding human susceptibility to the toxicity and carcinogenicity caused by chemical exposures.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs (Beverly, MA). Mouse monoclonal antibody to human PXR was from Perseus Proteomics Inc. (Tokyo, Japan). Mouse monoclonal antibody to V5 was from Invitrogen. Antibodies to phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-MKK3/6 (Ser189/Ser207), MKK6, phospho-MK2 (Thr389), MK2, phospho-JNK (Thr83/Tyr185), JNK, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-AKT (Ser473), AKT, phospho-p38 MAPK (Thr172), AMPK, and phospho-c-Jun (Ser63) were from Cell Signaling Technology (Beverly, MA). Normal mouse IgG, anti-GADD45α, c-Jun, and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Vectors**—pC3/hPXR, pcDNA3.1/hPXR, pCMX/hRXR, pcR3/hPGC1α, adeno-hPXR, and adeno-β-galactosidase were described previously (5). A full-length cDNA of human GADD45β, that was cloned from HepG2 with the use of primer pair 5’-GGTACCATGAGCGTCGAGAAGCTCGTG-GG-3’, and 5’-CTCAGCCTTCTGAAGAGAGATGTAG-GG-3’, was inserted into pcDNA3.1/V5-His-TOPO (Invitrogen) to produce pcDNA3.1/hGADD45β. pcR3/hPXR and pcDNA3.1/hPXR were generated from pcR3/hPXR and pcDNA3.1/hPXR, respectively, by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and a proper pair of mutagenic oligonucleotides. Human PXR R98C cDNA, digested from pcR3/hPXR R98C, was inserted into pAdtrackCMV vector (American Type Culture Collection, Manassas, VA) to produce adeno-hPXR R98C. The −1565/+47 region of the human GADD45β promoter was amplified from human genomic DNA (Promega, Madison, WI) using LA Tq DNA polymerase (TaKaRa, Otsu, Japan) and the following primers: 5’-GGAGTCTGTATGTGGATGCTGTAACACATTCAT-3’ and 5’-CAAGCTTGCAGGAGATCCAGGAGACATG-3’. Amplified DNA was digested with BglII and HindIII and was generated DNA was digested with BglII and HindIII and was generated pGL3 Basic (Promega) to yield pGL3/hG45β. To generate pGL3/hG45βcore containing the −66/+47 region of GADD45β promoter, a Xhol site was inserted into pGL3/hG45β by site-directed mutagenesis following digestion with SphI and self-ligation. A double-stranded oligonucleotide 5’-CAGGCAGATCATTTGAGTGTCAGAAGGAGGACATC-AGTGTCAGAAGGGAACATTTGAGTGTCAGAAGGAGGACAT-3’ was inserted into the Xhol site of pGL3/hG45βcore to generate pGL3/3’/DR4hG45βcore.

**Cell Culture, Drug Treatment, Transfection, and Infection**—Human hepatocellular carcinoma HepG2 and Huh7 cells were maintained in MEM supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO2 at 37 °C. For qRT-PCR and Western blotting, respectively, total RNAs and whole cell lysates were prepared from HepG2 cells that were treated with 10 μM RIF (Sigma-Aldrich) or 3 μM SR12813 (Sigma-Aldrich) in FBS-free MEM for a given time. For luciferase reporter assays, Huh7 cells were co-transfected with human GADD45β promoter-firefly luciferase, pRL-CMV for Renilla luciferase control (Promega), and pCR3/hPXR or pCR3/hPXR R98C using FuGENE 6 (Roche Applied Science). The final amounts of transfected DNAs were adjusted by adding pcDNA3.1-V5-His as empty vector control. Twenty four hours after transfection, these cells were subsequently treated with a given drug in FBS-free MEM for an additional 24 h. Luciferase reporter activities were measured as described previously (2). For ectopic expression of GADD45β, transcribed HepG2 cells were reverse-transfected with increasing amount of pcDNA3.1/hGADD45β, using FuGENE 6. The final amounts of transfected DNAs were adjusted by adding pcDNA3.1-V5-His. After 30 h, whole cell lysates were prepared. For adenoviral infection, HepG2 cells were cultured in MEM containing adeno-β-galactosidase, adeno-hPXR, or adeno-hPXR R98C at 10 of multiple of infection. After 30 h, these cells were treated with 10 μM RIF or 3 μM SR12813 in FBS-free MEM for a given time. Then, total RNAs and whole cell lysates were prepared. For siRNA knockdown, transcribed HepG2 cells were reverse-transfected with 40 μM ON-TARGETplus SMART pool GADD45β (catalogue number L-003894-00) or ON-TARGETplus siCONTROL nontargeting pool (catalogue number D-001810-10) from Dharmacon Research (Lafayette, CO) in MEM for 48 h, using Lipofectamine 2000 (Invitrogen). Then, these cells were treated with dimethyl sulfoxide (DMSO) or RIF in FBS-free MEM for 1 h, from which total RNAs and whole cell lysates were prepared for qRT-PCR and Western blotting, respectively. For knockdown of p38 MAPK, cells were reverse-transfected with ON-TARGETplus SMART pool p38 MAPK (catalogue number L-003512-00) for 36 h and were subsequently treated with RIF for a given time.

**ShP51 Cells That Stably Express Human PXR**—HepG2 cells were transfected with pCR3/hPXR by FuGENE 6 and were selected in MEM containing G418 (Invitrogen) at a concentration of 800 μg/ml. Drug-resistant colonies were further selected and verified by Western blotting of PXR and qRT-PCR of CYP3A4 to establish ShP51 cells.

**Western Blotting**—Cells were lysed and denatured in a fixed volume of NuPAGE LDS sample buffer (Invitrogen), from which a fixed volume was separated on an 8%, 10%, or 12% SDS-polyacrylamide gel and were transferred onto PVDF membrane. This membrane was blocked with 5% milk in 10% SDS-polyacrylamide gel and were transferred onto PVDF membrane. This membrane was blocked with 5% milk in TBS-T for 1 h at room temperature and then incubated with a given primary antibody in TBS-T containing 5% milk for 2 h at room temperature. Immunoreactive bands were visualized using ECL plus Western blotting detection reagents (GE Healthcare).

**Real-time PCR**—Total RNAs were extracted using TRIzol reagent (Invitrogen) to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
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qRT-PCR was performed with an ABI prism 7700 sequence detection system (Applied Biosystems). Assays-on-demand probes (Applied Biosystems) were used for PCR with the TaqMAN PCR Master Mix (Applied Biosystems), HS00430021_m1 for the human CYP3A4 gene. The following PCR primers were used with the SYBR Green Master Mix (Applied Biosystems): hGADD45β-RT-5', 5'-CCGTTGAGGAGCCTTTTGTG-3' and hGADD45β-RT-AS, 5'-GCTGTCTGAGTTCCACATTCA-3' for the human GADD45β gene. The TaqMAN human β-actin control regents kit (Applied Biosystems) was used as the internal control.

Gel Shift—cDNAs for human PXR in pCR3/hPXRx, human PXR<sub>r</sub> in pCR3/h PXRx<sub>r</sub> and human RXR in pCMX/hRXR were used for competition assays. Using these proteins and probes, gel shift assays were performed as described previously (19).

The following primers were used for PCR: for hGADD45β/H9252 and hGADD45β/H11032 probes (Applied Biosystems) were used for hGADD45β/H9252 and hGADD45β/H11032 gene after treatments with two classic activators RIF and SR12813 for 24 h (Fig. 1A). Western blot analysis of whole cell lysates prepared from these same parental HepG2 and ShP51 cells revealed that phosphorylation of p38 MAPK was greatly increased after drug treatments (Fig. 1B). Given this finding, time-dependent increases of phosphorylation of p38 MAPK and its upstream and downstream kinases were examined; MAPK kinase (MKK) 3/6 and MAPK-activated protein kinase 2 (MK2), respectively (Fig. 1C). In ShP51 cells after being treated with RIF, phosphorylation of p38 MAPK was dramatically increased as early as 30 min and reached an apparent peak at 60 min. This high level of this p38 MAPK phosphorylation remained 240 min after RIF treatment. Increase of phosphorylation of MKK3/6 was found to be as fast as that of p38 MAPK after RIF treatment, whereas MK2 exhibited a delayed increase of phosphorylation compared with those of MKK3/6 and p38 MAPK. Phosphorylation of MKK2 was first increased 60 min after RIF treatment and thereafter remained increased in the ShP51 cells. Any of these phosphorylations were not observed in RIF-treated parental HepG2 cells. These results indicate that PXR activated MKK3/6-p38 MAPK-MK2 signal in response to drug treatment. Subsequently, phosphorylation levels of JNK1/2, ERK1/2, AKT, and AMPK were investigated to examine specificity of PXR-activated phosphorylation in ShP51 cells (supplemental Fig. S1). Phosphorylation of JNK1/2 was increased, but this increase was weak until 120 min after RIF treatment. Those of ERK1/2, AKT, and AMPK were not increased in any time of RIF treatment. Thus, the PXR-induced increase of p38 MAPK was a quick and relatively specific response to RIF treatment in ShP51 cells.

PXR Activates the GADD45β Gene—Because PXR is a ligand-dependent transcription factor, its primary targets are genes. We asked whether PXR elicited p38 MAPK phosphorylation via activating a gene. Naturally occurring mutation of arginine 98 to cysteine abolished DNA-binding ability of PXR (21). Utilizing this mutation, we examined whether or not PXR required its DNA-binding ability to elicit phosphorylation...
tion of p38 MAPK. For this purpose, wild-type PXR and its PXRR98C mutant were ectopically expressed in HepG2 cells. Consisting with the lack of DNA binding, adenovirus-based expressing PXRR98C did not activate the expression of CYP3A4 gene in HepG2 cells following RIF treatment (Fig. 2A). As can be seen in Fig. 2B, PXRR98C was not able to increase the phosphorylation levels of both MKK3/6 and p38 MAPK after RIF treatment (Fig. 2B). These results suggested that PXR increased phosphorylation of p38 MAPK by activating a gene in RIF-treated HepG2 cells.

To identify the PXR-activated gene required for the phosphorylation of p38 MAPK, we initially performed microarray analysis (supplemental Table S1), from which the immediate-early response GADD45/H9252 gene was selected for further investigation. This selection was made based on the fact that GADD45/H9252 is known to regulate phosphorylation of p38 MAPK (15, 17, 18) and the hypothesis that the gene needs to quickly respond to PXR activation to stimulate p38 MAPK phosphorylation within 30 min following RIF treatment. Increase of GADD45/H9252 mRNA was, in fact, detected as early as 15 min and peaked 60 min following RIF treatment in ShP51 cells but not in parent HepG2 cells (Fig. 3A). The GADD45/H9252 mRNA remained at this level for 24 h following RIF treatment (data not shown). This quick response of the GADD45/H9252 gene became more evident when it was compared with a slower increase of the CYP3A4 mRNA, the classic PXR-targeted gene: the most significant increase in CYP3A4 mRNA occurred between 60 and 120 min after RIF treatment. However, the RIF-induced increase of GADD45β mRNA was not observed in HepG2 cells infected with adenovirus expressing PXRR98C mutan, substantiating the notion that PXR binds directly to the GADD45β gene and activates its transcription (Fig. 3B). Hepatocellular carcinoma Huh7 cells were utilized to confirm that the observed PXR-mediated increase of GADD45β mRNA and of phosphorylation of p38 MAPK was...
not specific to HepG2 cells (supplemental Fig. S2). These results suggested that GADD45β was the direct target of PXR to elicit p38 MAPK signal.

Direct activation by PXR of the GADD45β gene was further investigated by performing gel shift, luciferase reporter, and ChIP assays. Motif analysis revealed a direct repeat 4 (DR4) sequence (−4306 AGATCATTTGAGGTCA−4371) within a 10-kb GADD45β promoter as a putative site for PXR binding. Gel shift assays showed a specific binding of PXR to the DR4 sequence which was confirmed by supershift using anti-human PXR antibody (Fig. 4A). A triple repeat of the DR4 sequence was placed in front of the −66/47 bp of the hG45bore-Luc reporter plasmid which contains the GADD45β proximal promoter to produce 3×DR4-hG45bore-Luc. These reporter plasmids were co-transfected with either PXR or PXR}_{R98C mutant expression plasmid into Huh7 cells. PXR activated the 3×DR4-hG45bore-Luc, but not the hG45bore-Luc in RIF-treated Huh7 cells (Fig. 4B). PXR}_{R98C did not activate either 3×DR4-hG45bore-Luc or hG45bore-Luc. Finally, ChIP assays confirmed the binding of PXR to the DR4 sequence of the GADD45β promoter, in which V5-tagged PXR and PXR}_{R98C were ectopically expressed in RIF-treated HepG2 cells. PXR, but not PXR}_{R98C, clearly bound to the DR4 of the GADD45β promoter, similar to those observed in the CYP3A4 promoter (Fig. 4C). In contrast, PXR did not bind to a region lacking the DR4. These results indicate that PXR can directly activate the GADD45β gene.

GADD45β Mediates PXR-elicited p38 MAPK Phosphorylation—To examine whether GADD45β was responsible for RIF-induced increase of p38 MAPK phosphorylation, GADD45β was either overexpressed in HepG2 cells or knocked down in ShP51 cells. Ectopic GADD45β was expressed in HepG2 cells by transfecting the GADD45β expression plasmid in a dose-dependent manner. Western blotting showed a barely detectable expression of GADD45β at 0.1 and 0.3 μg of transfected plasmid and a high expression at 0.9 μg (Fig. 5A). Elevated phosphorylation of MKK3/6 and p38 MAPK was already detected at 0.1 μg, which was further increased at 0.3 μg. These phosphorylations were saturated at 0.3 μg and were no longer increased at 0.9 μg. This phosphorylation of MKK3/6 and p38 MAPK, thus, appeared to be very sensitive to GADD45β. Similar results were also obtained in Huh7 cells (data not shown). Next, we utilized siRNA to knock down endogenous GADD45β in ShP51 cells to ascertain whether GADD45β can determine a PXR-dependent p38 MAPK phosphorylation. Transfection of GADD45β siRNA, but not control siRNA, repressed PXR-elicited phosphorylation of MKK3/6 and p38 MAPK in ShP51 cells following RIF treatment (Fig. 5B). In those cells, GADD45β siRNA specifically decreased both basal and induced levels of GADD45β mRNA, but not those of CYP3A4 mRNA. GADD45β, thus, mediated PXR-dependent phosphorylation of MKK3/6 and p38 MAPK, suggesting that PXR activated the GADD45β gene and the induced GADD45β, in turn, stimulated p38 MAPK signal.

PXR-GADD45β-p38 MAPK Signals Migrate HepG2 Cells—PXR, upon activation by RIF, directly activates the GADD45β gene to elicit p38 MAPK signal in HepG2 cells. Activation of p38 MAPK could have various consequences for cell response, including cell migration and apoptosis. We first observed that ShP51 cells, but not parental HepG2 cells, underwent a striking morphological change wherein cells scattered and flattened upon treatment with RIF or SR12813 (Fig. 6A). The additional 14 clones of HepG2 cell lines stably expressing PXR also changed their morphology in the same manner as ShP51 cells (data not shown). Moreover, adenovirus-based expressing PXR, but not PXR}_{R98C, caused morphological changes after RIF treatment similar to those observed in ShP51 cells (supplemental Fig. S3). Thus, these results clearly indicated that the observed morphological changes occurred as a consequence of PXR activation by drugs. A subsequent immunohistochemical study showed that these morphological changes were accompanied with the reorganization of ac-

FIGURE 3. Drug activation of PXR induces the GADD45β gene. A, increase in GADD45β mRNA levels after RIF treatment. Cells were harvested at each time point after RIF treatment, from which total RNAs were prepared and subjected to qRT-PCR. These GADD45β and CYP3A4 mRNA levels were expressed by taking those in the DMSO-treated cells as 1. Columns represent the mean ± S.D. (error bars). B, PXR}_{R98C mutant. HepG2 cells were infected with adenov-β-galactosidase, adenoh-PXR, or adenoh-PXR}_{R98C for 30 h. After that RIF was added for another 2 h. Subsequently, total RNAs were prepared and subjected to qRT-PCR. These mRNA levels were expressed by taking these in DMSO-treated HepG2 cells infected with adenov-β-galactosidase as 1. Columns represent the mean ± S.D.
tin filaments often observed during cell migration (Fig. 6B): formations of stress fiber, lamellipodia, and filopodia (22, 23). Therefore, transwell migration assays were performed to examine whether or not ShP51 cells would migrate after treatments with RIF and SR12813. Cells that migrated onto the other side of the transwell were visualized, and their numbers were counted in Fig. 6C. ShP51 cells, but not parental HepG2 cells, were found to exhibit a 5-fold increase in migration following treatment with RIF and SR12813. Thus, drug activation of PXR resulted in morphological changes, leading ShP51 cells to migrate. In addition, cell growth was not affected throughout the duration of morphological changes and migration after treatments with RIF and SR12813 (data not shown).

The specific p38 MAPK inhibitor SB239063 was employed to link PXR-mediated morphological changes and cell migration with p38 MAPK in HepG2 cells. Under the assay condition, phosphorylation of an immediate p38 MAPK downstream kinase MK2 was completely inhibited by concentrations as low as 10 μM SB239063 in RIF-treated ShP51 cells (Fig. 7A). Accordingly, ShP51 and parental HepG2 cells were co-treated with RIF and 10 μM SB239063 and were subjected to morphological and transwell migration analyses to examine whether or not p38 MAPK, in fact, regulated PXR-induced morphological changes and migration. No morphological changes were observed in ShP51 cells co-treated with SB239063 (Fig. 7B). A 5-fold increase by RIF treatment of migration of ShP51 cells was completely repressed by co-treatment with SB239063 (Fig. 7C). SB239063 treatment also increased c-Jun phosphorylation in both parental HepG2 and ShP51 cells (Fig. 7A).

To confirm the role of the p38 MAPK in RIF-induced morphological changes, ShP51 cells were transfected with siRNA to knock down endogenous p38 MAPK. This knockdown re-
sulted in significant repression of RIF-induced morphological changes (supplemental Fig. S4). Taken together, p38 MAPK was demonstrated to be responsible for the PXR-induced morphological changes and cell migration.

DISCUSSION

Upon drug activation, PXR has now been shown to directly activate the GADD45β gene by binding to a DR4 sequence within its promoter, eliciting a MKK3/6-p38 MAPK signal and migrating HepG2 cells. The PXR regulation of cell migration via the GADD45β gene emphasizes the possibility that PXR may play diverse roles within the realms of cell regulation, because GADD45β is known to regulate various types of cellular functions from apoptosis to cell cycle and DNA repair through its interactions with various signal factors such as the cdc2/cyclin B1 complex and PCNA (13).

GADD45β is rapidly induced by genotoxic/oxidative stresses and cytokines and acts as a stress sensor to coordinate cellular response signals such as cell survival and apoptosis. TGFβ is one such stress-mediated factor that activates the GADD45β gene to promote apoptosis via p38 MAPK signal: the TGFβ-induced apoptosis and p38 MAPK phosphorylation were diminished in the primary hepatocytes prepared from Gadd45β KO mice (24). In our present study with HepG2 cells, induction of GADD45β by PXR had no affect on cell growth under the experimental conditions used; the known TGFβ-induced apoptosis-resistant nature of HepG2 cells could be a factor in this phenomenon (25). Instead, an up-regulated GADD45β-MKK3/6-p38 MAPK signal resulted in epithelial-mesenchymal transition-like morphological changes and migration of HepG2 cells. This finding, however, is consistent with the previous observations that TGFβ causes
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HepG2 cells to invade (26) and that p38 MAPK mediates TGFβ-induced migration of mouse mammary epithelial NMuMG cells (27). Furthermore, it is recently reported that activation of p38 MAPK signal pathway leads hepatocellular carcinoma to migrate (28). MK2, a well known downstream kinase of p38 MAPK, is known to regulate cell migration in various cells by activating signals such as heat shock protein 27 and LIM-kinase 1 (29, 30). Thus, phosphorylation of MK2 may be a critical factor in regulating RIF-induced morphological changes and the migration of HepG2 cells, although it remains to be proven in future investigations.

CAR (NR1I3) belongs to the same NR1I subfamily as PXR does: CAR and PXR can be activated by an overlapping group of therapeutics and activate the overlapping target genes (31). Similar to PXR, CAR activated the GADD45β gene in HepG2 cells (32). Furthermore, CAR was also found to activate the GADD45β gene in mouse livers, and this activation occurred independently from the NFκβ-mediated pathway (32, 33). In mouse primary hepatocytes, CAR up-regulated Gadd45β, repressing TNFα-induced phosphorylation of JNK and JNK-mediated cell death (34). In supporting the hypothesis that CAR may regulate cell death via Gadd45β gene, a recent study with Gadd45β KO mice nicely demonstrated that Gadd45β attenuates TNFα-induced JNK phosphorylation and liver regeneration after a partial hepatectomy (35). Given various overlapping functions of PXR, it is anticipated that further investigations will reveal that PXR may play similar roles as well as yet identified new roles. Using Pxr+/− and Pxr−/− mice, we confirmed that PXR induced Gadd45β and increased phosphorylation level of p38 MAPK in mouse liver in vivo (supplemental Fig. S5). Any physiological/pathophysiological role of induction of GADD45β and activation of p38 MAPK signal pathway by PXR in the liver remains virtually unexplored at the present time. Our present findings may have provided new insights into understanding the molecular mechanisms by which therapeutics causes various cell signal responses such as hepatomegaly, cell proliferation, apoptosis, and migration, thereby affecting human susceptibility to therapeutic exposures (7, 36).

In conclusion, GADD45β as the gene directly activated by PXR presents a novel target for future investigation concerned with the molecular mechanism by which the therapeutic activation of PXR can cause beneficial as well as adverse effects to liver physiology. Because various nuclear hormone receptors are known to cross-talk with the members of the GADD45 family (37), PXR activation by therapeutics and the resulted induction of GADD45β gene may also modulate the nuclear receptor-mediated hormonal responses and homeostasis.

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