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RNA interference screen identifies NAA10 as a regulator of *PXR* transcription

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Abstract

The pregnane X receptor (PXR) is a principal xenobiotic receptor crucial in the detection, detoxification, and clearance of toxic substances from the body. PXR plays a vital role in the metabolism and disposition of drugs, and elevated PXR levels contribute to cancer drug resistance. Therefore, to modulate PXR activity and mitigate drug resistance, it is imperative to fully understand its regulation. To this end, we screened a transcription factor siRNA library in pancreatic cancer cells that express high levels of PXR. Through a comprehensive deconvolution process, we identified N-alpha-acetyltransferase 10 (NAA10) as a factor in the transcriptional machinery regulating *PXR* transcription. Because no one single factor has 100% operational control of *PXR* transcriptional regulation, our results together with other previous findings suggest that the transcriptional regulation of *PXR* is complex and that multiple factors contribute to the process including NAA10.

Keywords

PXR; xenobiotic receptor; transcriptional regulation; gene expression

1. INTRODUCTION

The body is under constant assault from ingested, inhaled, and/or absorbed xenobiotics and therefore must protect itself against this constant bombardment. The cytochrome P450 (CYPs) enzymes, which comprise a large family of heme-thiolate proteins, play a central role in the oxidative, peroxidative, and reductive metabolism of an immense array of

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

endogenous (endobiotics) and exogenous (xenobiotics) toxic compounds in which the body is constantly exposed. Thus, CYPs are important constituents of the body's defense mechanism against xenobiotics (Gonzalez and Gelboin, 1992; Goodwin et al., 2002; Kliewer et al., 2002). In addition to CYPs, other phase I and phase II drug-metabolizing enzymes and drug transporters, such as aldehyde dehydrogenase, alcohol dehydrogenase, carboxylesterase, UDP-glucuronosyltransferase 1A1, sulfotransferase, multidrug resistance gene 1, ATP-binding cassette transporter C 2, and organic anion transporting polypeptide 2, are essential for detoxifying and eliminating toxic substances from the body (Chai et al., 2013; Guzelian et al., 2006; Maglich et al., 2002; Rosenfeld et al., 2003; Zhou et al., 2009). The expression of genes encoding several of these drug-metabolizing enzymes and transporters, in addition to at least 30 other genes, are under the control of the ligand-activated transcription factor and master xenobiotic receptor pregnane X receptor (PXR) (Hariparsad et al., 2009). PXR is the primary regulator of CYP3A4 (Wei et al., 2016), which is the most abundant hepatic and intestinal phase I enzyme that metabolizes approximately 50% of marketed drugs (Harris et al., 2003).

Although several studies have investigated how PXR transcriptionally regulates its target genes (Kodama et al., 2004; Smutny et al., 2013; Staudinger et al., 2011), few have focused on the transcriptional regulation of *PXR* itself. Among these, Zhang *et al.* demonstrated that the CYP1A inducers β -naphthoflavone and 3-methylcholanthrene marginally increase rat PXR (*Nr1i2*) mRNA levels, which shares 78% sequence identity with the human PXR. Similarly, the CYP2B inducer phenobarbital also minimally increases *Nr1i2* mRNA levels (Zhang et al., 1999). The CYP3A inducers isoniazid, dexamethasone, pregnenolone-16 α -carbonitrile, and troleandomycin exert differential effects. These inducers moderately increase *Nr1i2* levels, whereas troleandomycin decreases *Nr1i2* (Zhang et al., 1999). Interestingly, ligands of the fatty acid sensor peroxisome proliferator-activated receptor (PPAR α), clofibrate and perfluorodecanoic acid, also strongly increase *Nr1i2* mRNA levels (Zhang et al., 1999). Although clofibrate and perfluorodecanoic acid are not known to directly bind to PXR, PPAR α directly binds the proximal promoter of *PXR* approximately 1.3 kb upstream of its transcriptional start site, suggesting that PPAR α regulates *PXR* transcription (Aouabdi et al., 2006). Furthermore, Pascussi *et al.* reported that *PXR* mRNA levels, as well as those of its obligate cofactor retinoid X receptor- α , increase in response to the glucocorticoid receptor ligand dexamethasone in human hepatocytes (Pascussi et al., 2000). *PXR* promoter activity also increases when hepatocyte nuclear factor 4 α (HNF4 α) binds direct repeat 1 elements located at -88/-76 upstream of *PXR* promoter, suggesting that HNF4 α transactivates *PXR* gene by binding to its promoter (Iwazaki et al., 2008). Moreover, *PXR* mRNA levels are positively correlated with those of HNF4 α in human liver samples, further implicating HNF4 α in the regulation of *PXR* expression (Iwazaki et al., 2008).

PXR was initially discovered as a xenobiotic receptor; however, recent studies have uncovered other physiologic roles of PXR. For example, PXR plays an anti-inflammatory role by negatively regulating toll-like receptor 4 (Venkatesh et al., 2014) and regulates cell proliferation by modulating inhibitors of cell cycle progression (Dubrac et al., 2010; Eijkelenboom and Burgering, 2013; Misawa et al., 2005; Sun et al., 2008) and apoptosis (Robbins et al., 2016; Zhou et al., 2008; Zucchini et al., 2005). PXR is also implicated in

pathophysiologic conditions, such as in vascular disease (Xiao et al., 2014) and cancer, in which *PXR* confers resistance to chemotherapeutic agents and enhances tumorigenicity (Pondugula et al., 2016; Qiao et al., 2013). Therefore, it is imperative to understand the molecular basis of *PXR* expression in such diseases states.

PXR is greatly expressed in exocrine-like pancreatic ductal adenocarcinoma (PDAC) cells at levels comparable to those in liver cells. Exposure of PDAC cell models to erlotinib, dasatinib, and paclitaxel induces *CYP3A5* expression, rendering the cells insensitive to chemotherapeutic agents (Noll et al., 2016). Under physiologic conditions, *PXR* levels in the pancreas are much lower than they are in the liver, suggesting that increased *PXR* expression in PDAC may function in the tumor environment. In fact, *PXR* expression is a proposed novel biomarker for predicting drug resistance in patients with non-small cell lung cancer (Kong et al., 2016).

Although several reports have described specific components of the mechanisms contributing to *PXR* transcription, a comprehensive understanding of the overall transcription machinery regulating *PXR* remains unclear. To address this knowledge gap, we used for the first time an unbiased approach to identify and characterize the transcriptional regulators of endogenous *PXR* in AsPC-1 cells, a PDAC cell model with high *PXR* expression. Based on a desired and validated phenotype in response to siRNAs (i.e., the decreased transcription of *PXR*), and through a comprehensive deconvolution process, including identifying the true target from the false-positive action of a siRNA, we identified N-alpha-acetyltransferase 10 (NAA10) as a transcriptional regulator of *PXR* and further delineated its mechanism of regulating *PXR* expression.

2. MATERIALS AND METHODS

2.1 Materials

Fetal bovine serum and horse serum were purchased from HyClone (Logan, UT). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were authenticated by short tandem repeat DNA profiling. Cells were expanded immediately upon receipt from ATCC within three passages and frozen for future use. G418 and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Anti-mouse IRDye and anti-rabbit IRDye secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Anti-NAA10 antibody; anti-HNF4 α antibody; and *PXR*, KLF14, KLF15, KLF16, NAA10, PPP2R3B, SP8, HNF4 α , 18S, and custom ChIP-qPCR TaqMan probes and the Pierce agarose ChIP kit (26156) were purchased from Thermo Fisher Scientific (Waltham, MA). Anti- β -actin (clone AC-15) was obtained from Sigma-Aldrich (St. Louis, MO). Anti-CYP3A5 was obtained from Abcam (cat # ab108624; Cambridge, MA). SiGenome NR1I2 siRNA (M-003415-02), siGenome NAA10 siRNA (M-009606-00), siGenome KLF15 siRNA (M-006975-01), siGenome SP8 siRNA (M-007163-01), siGenome IKBKB siRNA (M-003503-03), siGenome HNF4 α siRNA (M003406-02), and siGenome NT siRNA pool #1 (D-001206-13) were purchased from GE Dharmacon (Lafayette, CO). Individual IKBKB (D-003503-01, D-003503-02, D-003503-03, D-003503-04), KLF13 (D-006994-01, D-006994-02, D-006994-04, D-006994-17), NAA10 (D-009606-01, D-009606-03), SP8 (D-007163-01, D-007163-18) and NT siRNAs (D-001210-01, D-001210-02, D-001210-

03, D-001210–04) were purchased from GE Dharmacon (Lafayette, CO). Lipofectamine RNAiMax was purchased from Invitrogen (Carlsbad, CA). The 384-well low-volume assay plates were obtained from Corning Inc. (Tewksbury, MA). Compounds are purchased from various suppliers as indicated: docetaxel, gemcitabine, erlotinib, dasatinib, and capecitabine were from LC Laboratories (Woburn, MA); vincristine was from Tocris (Bristol, UK); bortezomib and dactinomycin were from Apexbio (Cambridgeshire, UK); 5-fluorouracil and irinotecan were from Sigma (St. Louis, MO); doxorubicin, oxaliplatin, and paclitaxel were from Carbosynth (San Diego, CA); and doxycycline (dox) was from Research Products International (Mt. Prospect, IL).

2.2 Cell culture

All cell lines were maintained in a humidified incubator at 37°C with 5% CO₂. All cells were maintained in the media suggested by ATCC. Cells were routinely verified to be mycoplasma free by using the MycoProbe Mycoplasma Detection Kit (R&D Systems, Minneapolis, MN). AsPC-1 cells stably expressing dox-inducible shRNA against PXR 5' and 3' untranslated regions (AsPC-1 shPXR) were established with the lentiviral pLKO.1 plasmid (Wiederschain et al., 2009) (Addgene #21915) (Addgene, Cambridge, MA). Knockdown of *PXR* was confirmed by qPCR. AsPC-1 cells stably expressing doxinducible human *PXR* (AsPC-1 PXR OE) was established in the AsPC-1 shPXR background with the lentiviral pLVX-TRE3G plasmid (Liu et al., 2018) (Addgene #113405). Lentiviruses were generated in 293T cells in 6-well plates. We combined 1 µg of human pLKO vector, 0.75 µg of psPAX2 (gift from Didier Trono, Addgene plasmid #12260), and 0.25 µg of pMD2.G (gift from Dider Trono, Addgene plasmid #12259) with 5 µL of Lipofectamine 3000 in Opti-MEM for transfection. Transfection medium was replaced with fresh medium after 6 h, and viruses were collected after 48 h. To remove cells and debris, the medium was filtered with a 0.45 µm PES filter and frozen at –80 °C. Viral transduction was accomplished with 1 mL of virus-containing medium mixed with 3 mL of fresh medium added to a 6-cm dish of AsPC-1 cells at 40% cellular confluence with 8 µg/mL Polybrene (Sigma-Aldrich) for 16 h. The viruses were transduced for 1 day, and transduction medium was replaced with fresh medium containing 0.4 µg/mL puromycin for AsPC-1 shPXR cells or 0.4 µg/mL of G418 for AsPC-1 PXR OE cells. The cells were grown in culture for 4 days to establish pooled antibiotic-resistant stable cells. To maintain the pooled stable cells for future experiments, 0.2 µg/mL of puromycin was used for AsPC-1 shPXR cells, and 0.2 µg/mL each of G418 and puromycin was used for ASsC-1 PXR OE cells. For induction, the cells were grown in medium with tetracycline-free fetal bovine serum before adding dox.

2.3 siRNA-mediated knockdown

Cells were plated in culture vessels to approximately 80% confluency at the time of transfection. The transfection reagent RNAi max (Invitrogen) at a final concentration of 0.4% with 25 nM NT siRNA or targeting siRNA was pre-incubated at room temperature for 5 min in Dharmacon ACCELL siRNA delivery medium (Thermo Scientific). The mixture was then added to the AsPC-1 cells in culture medium. The cells were incubated at 37°C for 48 h before analysis. The siGENOME NT siRNA pool #1 (Thermo Scientific Dharmacon) was used as a negative control in the siRNA screening.

2.4 siRNA screening

The general procedure for the siRNA screen and analysis has been previously described (Ong et al., 2014). The human transcription factor siRNA library (Dharmacon) was reverse transfected into AsPC-1 cells by using Lipofectamine RNAiMAX (Life Technologies) at a final concentration of 25 nM. Briefly, reverse transfection is a process by which cells are overlaid onto a lipid:siRNA mix. Reverse transfection was employed because it is a process more amenable to high-throughput assays. Culture medium was changed 16 h after transfection, and the cells were incubated for another day. We performed qPCR to assess *PXR* mRNA levels 48 h post transfection by using the Cellsto-CT assay. Cells-to-CT (Van Peer et al., 2012) is an innovative gene expression analysis technique to measure relative gene expression by real-time qPCR without purifying RNA before amplification, and it is amenable for high-throughput workflows. The ribosomal *18S* gene was used as the housekeeping gene in this screen. FC *PXR* mRNA compared with cells transfected with NT siRNA was used for hit selection. We employed a pragmatic approach in which *PXR* level was compared with the control group within each assay plate, making each assay plate an independent experiment. The sequence of individual siRNAs targeting *KLF13* and *IKBKB* used in hit validation and deconvolution of siRNA pools are, *KLF13* (NM_015995) siRNA#1, #2, #3, and #4: UGGCAGGACUGCAACAAGA, CCGACGAGCUGGCGCGGCA, GAGAAGCGCUUCAUGCGCA, and CAAAGUGGUUCUGUUUAA, respectively; *IKBKB* (NM_001556) siRNA #1, #2, #3, and #4, GGAAGUACCUGAACCAGUU, CCAAUAAUCUUAACAGUGU, GGAUUCAGCUUCUCCUAAA, and GUGGUGAGCUUAAUGAAUG, respectively.

2.5 ChIP-qPCR

AsPC-1 cells were grown in flasks, washed three times with PBS, and subjected to a ChIP kit generally following the manufacturer protocol (Thermo Fisher). The following modifications were made to the manufacturer protocol: the samples were precleared with protein G Sepharose beads before ChIP and treated with micrococcal nuclease for 6 min to yield sheared DNA fragments with an average length of 1000 to 4000 bp following assay optimization. We performed qPCR to determine the change in NAA10 occupancy at various known sites of *PXR* promoter. The negative controls consisted of a nonspecific antibody (IgG) and primers targeting the coding regions of *PXR* that do not interact with NAA10. For qPCR, we used Taqman assays and Fast Advanced Master Mix (Life Technologies) with 11.5-μL reactions in 384-well plates. Thermal cycling for qPCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies) in accordance with the TaqMan Fast protocol. The relative occupancy of an immunoprecipitated factor at a particular locus was estimated by using the following equation $2^{-(Ct \text{ mock} - Ct \text{ specific})}$, where *Ct* mock and *Ct* specific are the mean threshold cycles of PCR performed in triplicate on DNA samples from mock and specific immunoprecipitations. Primer sequences are listed in Table 1.

2.6 Protein extraction and immunoblot analysis

Proteins were extracted from cells by incubating them in Pierce RIPA lysis buffer with added Halt protease inhibitor cocktail (Thermo Fisher Scientific) and phosphatase inhibitor

cocktail (Sigma) for 30 min on ice and then sonicating the lysate for 10 s at 50% amplitude to shear the DNA. Protein concentrations were measured with the Pierce BCA protein assay (Thermo Fisher Scientific) in accordance with manufacturer instructions. Protein lysates were resolved on NuPAGE 4%–12% SDS-PAGE gradient gels (Life Technologies). After electrophoresis was completed, separated proteins were transferred to nitrocellulose membranes with an iBlot Dry Blotting System (Life Technologies). All membranes were incubated in Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NE). All antibodies were diluted in Odyssey blocking buffer. For quantitation of CYP3A5, β -actin was used as the loading control. The intensity of each protein band of CYP3A5 was quantified and normalized to that of β -actin to generate the relative intensity, as described previously (Banerjee et al, 2016). Secondary antibodies were diluted in TBS. All immunoblot imaging was performed with a LI-COR Odyssey infrared imaging system.

2.7 Co-immunoprecipitation

Co-immunoprecipitations were performed by first lysing the cells in Pierce RIPA buffer (Thermo Fisher), and pellets were further dissolved in nuclear extraction buffer (Thermo Fisher). Both buffers were supplemented with phosphatase inhibitors and complete protease inhibitors. The combined lysates were precleared with protein A/G beads for 30 min at 4°C and subjected to immunoprecipitation with either an NAA10 antibody or control IgG. Lysates were incubated with antibody and protein A/G beads overnight (~16 h) with rotation. The beads were washed three times in RIPA buffer containing phosphatase and protease inhibitors. Bound proteins were eluted with SDS loading buffer and boiling. Proteins were separated by SDS-PAGE and detected by immunoblotting.

2.8 RNA extraction and real-time qPCR

RNA was extracted by using Maxwell simplyRNA Kits and a Maxwell 16 instrument (Promega). RNA concentrations were measured with a NanoDrop 8000 UV-Vis spectrophotometer (Thermo Fisher Scientific). All cDNAs used in mRNA qPCR analyses were synthesized from extracted RNA by using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) in accordance with the manufacturer protocol. The mRNA expression data were generated by using Applied Biosystems TaqMan assays (20 $\times$) and Fast Advanced master mix (Life Technologies). Thermal cycling for qPCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies) in accordance with the TaqMan Fast protocol. The mRNA was standardized to housekeeping genes *18S* and *GAPDH* before normalization. Housekeeping genes did not vary during experimental conditions. Data are shown as the mRNA FC (2^{-CT}) relative to the mRNA level of the corresponding transcript in the control samples, as indicated. Each experiment was performed at least three times, and all samples were analyzed in triplicate.

2.9 Cell proliferation assays

Real-time cell growth in response to the various treatments was measured as the degree of cell confluence in culture plates and was determined by using an IncuCyte ZOOM live-cell imaging system (Essen BioScience, Ann Arbor, MI). In brief, target genes were knocked down by siRNA in cells plated in 10-cm cell culture dishes, as described previously. After 24 h of transfection, cells were trypsinized and plated at a density of 2000 cells/well in 384-

well plates. The cell density was assessed every 6 h for the duration of the experiment. Cells transfected with NT siRNA were used as a negative control. Cell proliferation curves were plotted by using percent confluence from the starting time point at specified time points for each treatment.

2.10 Cell migration assays

Real-time cell migration in response to the various treatments was measured as the number of cells that migrated into the lower chamber at each time point normalized to the starting number of cells in the upper chamber, which was determined with an IncuCyte ZOOM live-cell imaging system (Essen BioScience, Ann Arbor, MI). In brief, target genes were knocked down by siRNA in cells plated in 10-cm cell culture dishes, as described previously. After 24 h of transfection, the cells were trypsinized and plated at a density of 5000 cells/well in IncuCyte ClearView 96-well chemotaxis plates. The number of migrated cells was assessed every 6 h for the duration of the experiment. Cells transfected with NT siRNA were used as a negative control. Cell migration curves were plotted by using the number of migrated cells normalized to the starting number of cells plated and presented in FC at specified time points for each treatment.

2.11 Drug screening

AsPC-1 cells with *PXR* knockdown or overexpression were induced with 1 µg/mL dox (for the induced groups only) and plated at 2000 cells/well in 40 µL of RPMI media containing 10% fetal bovine serum. The Multidrop Combi Dispenser (Thermo Labsystems) was used to dispense the cells into 384-well white polystyrene plates (Corning Life Sciences, #8804BC). Plates were centrifuged for 1 min at 1000 rpm and placed into a 37°C incubator. After 24 h, the cells were treated with either DMSO or respective compounds by using an Echo 555 Liquid Handler (Labcyte), and the plates were returned to incubate. Appropriate volumes of DMSO were added to achieve a final concentration across all plates of 0.4%. After 72 h, the cells were lysed by adding 25 µL of CellTiter-Glo reagent (Promega). All plates were briefly shaken and then incubated at room temperature for 20 min, while shielded from light. Luminescence was recorded with an Envision 2102 multilabel plate reader (PerkinElmer).

2.12 Caspase 3/7 Glo assay

The Caspase-Glo 3/7 assay reagent (Promega, Madison, WI) was used to detect caspase 3/7 activation in cells. The reagent provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. The addition of the Caspase-Glo 3/7 reagent directly to the assay well in an add-mix measure format results in cell lysis followed by caspase cleavage of the substrate and generation of a luminescence signal. The amount of luminescence measured is proportional to the amount of caspase activity in the cells and a measure of apoptosis.

2.13 Statistical analysis

Data from at least three independent replicated experiments were pooled and quantitatively analyzed by analysis of variance and Tukey honest significant difference tests and by Student t-tests with GraphPad Prism 7.0 software. All *P* values less than 0.05 were

considered significant. Results are expressed as the mean $\pm$ SE. The experiments presented in this study were repeated multiple time (usually $n > 3$) which allows more accurate estimates of the true mean, by the mean of the experimental results. It also led to a narrower error margin, and a more precise estimate of the population values.

3. RESULTS

3.1 PXR transcript is highly elevated in AsPC-1 cell line

To conduct high-throughput siRNA screening of potential modulators of *PXR* transcription, we first conducted a selection study of PDAC cell lines that express high *PXR* levels. We performed quantitative RT-PCR (qPCR) on eight PDAC cell lines in comparison to HPNE cells, which revealed that AsPC-1 cells have the greatest level of *PXR* mRNA expression (Figure 1A); therefore, AsPC-1 cells were used for the screening experiment. Although the cause and function of the abundant *PXR* expression in this cell line is yet unknown, it is one of the intriguing reasons underlining this study.

3.2 High-throughput siRNA screen in AsPC-1 cells

To use an arrayed siRNA library to screen for modulators of *PXR* expression in AsPC-1 cells, we developed a reverse transfection protocol by using the lipofectamine RNAiMAX method, which provides high transfection efficiency and minimum cellular toxicity in AsPC-1 cells (see Materials and Methods). To elucidate positive regulators of *PXR* transcription, we conducted siRNA-based high-throughput screening. We transfected AsPC-1 cells with siRNAs and assessed *PXR* mRNA levels after 48 hours. We used an siRNA library targeting 1530 genes (from Dharmacon) involved in transcriptional regulation or chromosome association. To test whether our transfection protocol efficiently knocked down the target genes, an siRNA directed against *PXR* was included as a positive control, and knockdown was confirmed by qPCR. Genes whose silencing decreased *PXR* mRNA levels were classified as positive regulators (below green dots, Figure 1B), whereas silenced genes that increased *PXR* mRNA levels were deemed negative regulators (above green dots, Figure 1B). Relative *PXR* mRNA levels were normalized to multiple positive (*PXR* siRNA) and negative (nontargeting [NT]) siRNA controls on each assay plate. Fold change (FC) and log FC for each gene are determined. Following the screening data analysis, the genes that repressed *PXR* mRNA after knockdown to equal to or greater than *PXR* siRNA controls were selected as hits from each plate ($n = 115$) for a confirmatory screen. Expected hits included *PXR* itself and *HNF4A* (*HNF4A* siRNA was indicated as a blue dot in Figure 1B and 1C), a previously described regulator of *PXR* (Iwazaki et al., 2008; Kamiya et al., 2003), thereby validating our experimental approach.

3.3 Confirmatory screen in AsPC-1 cells

To minimize false positives due to off-target effects, we assembled a new library containing pooled siRNAs, with each pool containing four individual siRNAs targeting each of the genes identified in the primary screen. In total, 115 genes whose silencing decreased *PXR* mRNA in the primary screen were reassessed (Figure 1C). Genes whose silencing had greater than or equal to 65% (65% was arbitrarily chosen in order to focus on the more active siRNAs) efficiency in decreasing *PXR* mRNA when compared with *PXR* siRNA

positive control were selected. According to these criteria, 10 positive regulators, including *HNF4A*- a previously described regulator of *PXR* (blue dot, Figure 1C), *KAT2A*, *ASCL2*, *PDX1*, *IKBKB*, *NR1I2* (*PXR*), *IKZF2*, *KLF13*, *NFYB*, and *TIAL1*, with corresponding FC value of 0.384251, 0.268858, 0.274002, 0.461369, 0.29423, 0.132528, 0.376221, 0.396677, 0.224296, and 0.198105 were confirmed.

3.4 Hit validation and deconvolution of siRNA pools

To validate the hits from the confirmatory screen, each hit was knocked down by a pool of four siRNAs. *PXR* mRNA was significantly repressed after transfection with *HNF4A*, *IKBKB*, or *KLF13* siRNAs (Figure 1D). *KAT2A*, *ASCL2*, *PDX1*, *IKZF2*, *NFYB*, and *TIAL1* were not validated as positive regulators of *PXR* transcription. We next investigated *IKBKB* and *KLF13* further because the transcriptional regulation of *PXR* by HNF4 α has been described previously (Kamiya et al., 2003) and our aim was to identify previously unidentified regulators of *PXR* transcription.

To rule out off-target effects, we individually evaluated the four siRNAs from the pools used in the confirmatory and hit validation screen that specifically targeted the *IKBKB* and *KLF13* genes. We evaluated the eight individual siRNAs in the deconvolution screen (see “siRNA screening” in Materials and Methods) in AsPC-1 cells. To validate the on-target effects on *PXR* transcription, we pre-established that at least two of the four individual siRNAs targeting each gene must significantly decrease ($P < 0.05$) *PXR* mRNA levels over those of the NT siRNA. On the basis of these criteria, we found that neither *IKBKB* nor *KLF13* were on-target validated hits (Figure 2A). None of the individual siRNAs targeting *IKBKB* significantly decreased *PXR* mRNA, although each *IKBKB* siRNA decreased *IKBKB* mRNA by approximately 50% (Figure 2B). Interestingly, only one of the siRNAs targeting *KLF13* (i.e., *KLF13* siRNA 2) significantly decreased *PXR* mRNA (Figure 2A), although each of the individual siRNAs decreased *KLF13* mRNA by at least 50% (Figure 2C). This observation suggests that the effect of the *KLF13* pooled siRNAs on *PXR* mRNA levels was due to an off-target effect on an unexpected gene, likely mediated by *KLF13* siRNA 2. We therefore next elucidated the unexpected targets of *KLF13* siRNA 2 by analyzing its sequence for other potential targets, allowing up to two mismatches, with the online SpliceCenter software (Ryan et al., 2008). We identified *KLF14*, *KLF15*, *KLF16*, *NAA10*, *PPP2R3B*, and *SP8* as possible target genes. We assessed the knockdown of these putative targets after transfecting AsPC-1 cells with *KLF13* siRNA 2 (Figure 3) and observed that the *KLF15*, *NAA10*, and *SP8* were significantly decreased (Figure 3 D,F,H), suggesting that *KLF15*, *NAA10*, or *SP8* may be de facto regulators of *PXR* transcription.

3.5 NAA10 and SP8 positively regulate PXR transcription

To determine whether *KLF15*, *NAA10*, or *SP8* positively regulate *PXR* transcription, we transfected siRNAs targeting each of these genes in AsPC-1 cells and assessed *PXR* mRNA levels. Silencing *NAA10* or *SP8* significantly decreased *PXR* mRNA, whereas *KLF15* knockdown did not (Figure 4A), although each of the genes were efficiently knocked down by their targeting siRNAs (Figure 4 B–D).

To confirm that NAA10 and SP8 are indeed regulators of *PXR* transcription, we individually evaluated two siRNAs from the siRNA pools targeting *NAA10* and *SP8*. We confirmed that all of the individual siRNAs targeting *NAA10* and *SP8* (Figure 5, B–C) significantly decreased *PXR* mRNA levels (Figure 5A). To determine whether the effect of *NAA10* and *SP8* knockdown on *PXR* transcription is mediated through *PXR* promoter, we performed luciferase reporter assays of *PXR* promoter. Knockdown of *NAA10* decreased *PXR* promoter activity by approximately 50% in agreement with our qPCR data, whereas to our surprise, knockdown of *SP8* increased *PXR* promoter activity (Figure 5D). We also assessed the effect of the *NAA10* and *SP8* siRNAs on other nuclear receptors, including HNF4 α , and we observed that neither of the *NAA10* and *SP8* siRNAs inhibited the transcription of any of the nuclear receptors tested (Figure 6), suggesting that the inhibitory effects of *NAA10* and *SP8* on transcription are not global. Interestingly, the knockdown of *NAA10* and *SP8* significantly increased LXR and RXR mRNA. The significance of this increase is yet to be investigated.

3.6 NAA10 associates with PXR promoter

Because NAA10 interacts with the promoters of the *CDH1* (i.e., e-cadherin) and *MYC* genes to regulate their expression (Lee et al., 2010; Lim et al., 2008), we postulated that NAA10 regulates *PXR* transcription by interacting with its promoter. Therefore, we performed chromatin immunoprecipitation (ChIP)-qPCR in AsPC-1 cells. We found a significantly increased enrichment of NAA10 at the proximal promoter region of *PXR* (region –2200 to –200) (Figure 7, A–C). Because NAA10 is known to interact with the *CDH1* and *MYC* promoters (Lee et al., 2010; Lim et al., 2008), we performed ChIP-qPCR of their promoter regions to serve as positive controls for our experimental approach and to validate our results. NAA10 was enriched on the promoters of both *CDH1* and *MYC* at comparable levels to that of *PXR* (Figure 7 D,E). In contrast, NAA10 was not enriched in the exonic regions of *PXR*, *GAPDH*, or *18S* (Figure 7, F–H). These observations suggest that although NAA10 is not a transcription factor, it associates with *PXR* promoter, most likely in a complex with other factors, to regulate the transcription of *PXR*. We confirmed the validity of the NAA10 antibody used for our ChIP experiments by immunoblot analysis of NAA10 after *NAA10* knockdown in AsPC-1 cells (Figure 7I).

3.7 NAA10 interacts with HNF4 α

HNF4 α is known to regulate *PXR* transcription by binding to *PXR* proximal promoter regions (Iwazaki et al., 2008). To test whether NAA10 and HNF α exert an additive effect on *PXR* transcription, we performed a double knockdown of *HNF4A* and *NAA10* in AsPC-1 and HPAF-II cells (Figure 8). We did not observe an additive effect of the double knockdown in AsPC-1 cells (Figure 9A), suggesting that NAA10 and HNF4 α function in the same pathway. We repeated the same experiment in another PDAC cell line, HPAF-II cells, that also highly expresses *PXR* (Figure 1A), and we observed similar results to those in AsPC-1 cells (Figure 9B). Because our data show that NAA10 is present on the promoter of *PXR* (Figure 7, A–C), and contributes no additive effect to HNF4 α on *PXR* transcription, we posited that NAA10 and HNF4 α function in a complex to regulate *PXR* transcription. To test this, we performed a co-immunoprecipitation experiment and found that NAA10 and

HNFA indeed are present together in a complex in both AsPC-1 and HPAF-II cells (Figure 9C).

3.8 PXR suppresses migration and proliferation of AsPC-1 cells

Because *PXR* is highly expressed in AsPC-1 cells, we next investigated its function in PDAC. To this end, we assessed previously identified functions of *PXR* in other systems, which are also critical in cancer progression (Oladimeji and Chen, 2018). We performed siRNA-mediated *PXR* knockdown (Figure 10B) and examined the activation of caspases –3 and –7 as a measure of apoptotic activity. *PXR* knockdown did not yield any changes in apoptosis, as measured by caspase 3/7 activity (Figure 10A). Because the canonical role of *PXR* is detoxification and drug metabolism, we also assessed the effect of *PXR* knockdown on cell viability in response to commonly used chemotherapeutic agents. We did not observe any differences in drug responses between control cells and those with *PXR* knockdown (Figure 10C) or overexpression (Figure 10D). To determine whether there is a correlation between the levels of *PXR* and those of CYP3A5, we tested a few less toxic concentrations of bortezomib (Bor) and capecitabine (Cap) with and without inducible knockdown (in AsPC-1 sh*PXR* cells) or overexpression (in AsPC-1 *PXR* OE cells) of *PXR*. Figure 11 indicated that there is no correlation between the levels of *PXR* and those of CYP3A5.

We ascertained whether *PXR* knockdown (Figure 12B) exerts morphologic changes in AsPC-1 cells. We observed that *PXR* knockdown induces a rounded, less spread-out phenotype with reduced cell–cell contacts (Figure 13A), which is associated with increased migratory potential (Roycroft and Mayor, 2016). This phenotype was recapitulated by *NAA10* knockdown (Figure 12C; Figure 13A). To specifically determine whether *PXR* knockdown affects AsPC-1 cell migration, we assessed cell migration with Boyden chamber assays. We observed significantly increased migration of cells with *PXR* or *NAA10* knockdown, when compared with the migration of control cells (Figure 13B). Additionally, *PXR* or *NAA10* knockdown significantly increased AsPC-1 cell proliferation compared to cells transfected with NT siRNA, but the effect was not as striking as that observed for migration (Figure 12A). To further demonstrate that *PXR* is involved in AsPC-1 cell migration, we performed a rescue experiment with AsPC-1 cells with inducible *PXR* knockdown (i.e., AsPC-1 sh*PXR*) and with inducible *PXR* overexpression (i.e., AsPC-1 *PXR* OE) (see Materials and Methods). AsPC-1 sh*PXR* cells treated with doxycycline (dox) to induce *PXR* knockdown (Figure 12D) exhibited increased migration (Figure 13C). In contrast, AsPC-1 *PXR* OE cells treated with dox to induce *PXR* re-expression above basal levels in wildtype cells (Figure 12E) rescued the migratory potential observed with *PXR* knockdown (Figure 13D). The SEM values are much smaller at earlier time points because the starting number of cells were meticulously counted, thus are very similar. As cells migrate over time, the rate of migration differs between different cell populations, hence the counted number of cells at the subsequent time points are more varied, explaining the constant decrease of SEM values from longer time points to 0 h. Together, these data indicate that *PXR* suppresses migration and proliferation of AsPC-1 cells.

4. DISCUSSION

RNAi screening is a high-throughput method of identifying regulators and modulators of specific targets or phenotypes. Often, a number of factors are considered simultaneously in RNAi screens, which can produce flawed results from false positives and false negatives generated by the inherent potential of RNAi-based technology to produce off-target effects (Sharma and Rao, 2009). Off-target effects can be potentially minimized by screening for targets with individual siRNAs, rather than pooled siRNAs targeting the same gene, but this approach is not often used as it is resource-intensive. Most primary screens are performed with pooled siRNAs, leaving the question of how to manage false positives and/or false negatives unanswered. Developing robust and reproducible assays for identifying and further characterizing interesting candidate genes is required to adequately address this challenge. Testing of two or more nonoverlapping siRNAs per gene should also be a general standard for initially verifying primary screen results. The quality of an RNAi screen also greatly depends on the methods used for detecting desired phenotypes in a robust, reliable, and highthroughput format.

As a proof of principle for elucidating the transcriptional regulators of *PXR*, we specifically chose a transcription factor siRNA library for our primary screen. Although reporter assays are typically used for such screens, they do not always provide a complete picture of gene regulation in a genomic context. Distal enhancer elements often play a crucial role in the transcriptional regulation of a gene. Enhancers in reporter constructs are typically placed in proximity to the tested promoter, which does not recapitulate the physiologic environment (Atkinson and Halfon, 2014). Gene regulation depends on a local genomic context that takes into account the multiple local features necessary to maintain gene regulation. For this reason, we evaluated endogenous *PXR* mRNA as our readout and identified HNF4 α , IKBKB, and KLF13 as *PXR* transcriptional regulators.

To eliminate false positives, we deconvoluted the pooled siRNAs targeting *IKBKB* and *KLF13*. This process revealed that IKBKB was not a true hit and that an off-target effect of one of the four individual siRNAs targeting *KLF13* modulated *PXR* transcription. By reverse engineering the target of this specific *KLF13* siRNA, we determined that NAA10 was the true regulator of *PXR*, thereby discovering a true hit from a false-positive result. Therefore, if a desired phenotype is observed, even if it is produced by a false positive, the underlining mechanism can be determined by working backwards from the end results.

NAA10 is the catalytic subunit of the N-terminal acetyltransferase complex and regulates protein function, stability, and/or complex formation of a variety of substrates. Consequently, it modulates many cellular processes, including cell cycle, cancer progression, DNA damage, stress responses, development, and diseases (Dorfel and Lyon, 2015). Although no DNA binding domain has been identified for NAA10, it does interact with the promoters of *CDH1* and *MYC* (Lee et al., 2010; Lim et al., 2008). We observed the presence of NAA10 on *PXR* promoter, most likely in a complex containing HNF4 α , which is a known regulator of *PXR*. Removal of NAA10 from this complex did not completely abolish *PXR* mRNA expression, suggesting that NAA10 contributes to but is not the only factor that modulates *PXR* transcription. Knocking down *HNF4A* decreased *PXR* mRNA by

approximately 50%, and HNF4 α is the most potent regulator of *PXR* transcription identified to date. Altogether, these data suggest that a single principal transcription factor does not control *PXR* transcription. Instead, multiple factors are involved, but no one single factor is sufficient for driving full *PXR* transcription. We identified NAA10 as one such factor.

The canonical function of *PXR* is detoxification and elimination of toxic substances from the body. Because *PXR* is highly expressed in AsPC-1 cells, we assessed the response of these cells to various commonly used chemotherapeutic agents. To our surprise, cells with either *PXR* knockdown or overexpression responded similarly to that of control cells. This suggests that *PXR* serves other functions in AsPC-1 cells. By assessing various cellular functions, we determined that *PXR* plays a tumor suppressive role in AsPC-1 cells by inhibiting cell migration and proliferation. *PXR* knockdown increased both migration and proliferation of AsPC-1 cells, which was further validated by re-expressing *PXR* in *PXR*-knockdown cells. This function of *PXR* in AsPC-1 cells was unexpected, especially because CYP3A5, a transcriptional target of *PXR*, contributes to acquired drug resistance in PDAC (Noll et al., 2016). Perhaps the function of *PXR* is tissue-context dependent. Therefore, caution must be exercised when selecting cell models to study *PXR* functions.

RNAi remains a powerful tool for the systematic interrogation of gene functions. Nevertheless, a major limitation of any siRNA library is its efficacy for gene silencing, which we did not take into consideration here. Our screening experience provides a template for identifying true targets from false-positive findings, which are based on the desired and validated phenotypes measured. Given the complex network of factors that regulate *PXR* at the transcriptional level, we elucidated NAA10 as a novel part of this network. *PXR* mRNA has been shown previously to have a significant correlation with *PXR* protein level in human liver (Wei et al., 2013), therefore, fully understanding the transcriptional regulation of *PXR* is an important endeavor because it will create future strategies to better modulate it. Knowing the factors that regulate *PXR* transcription may reveal a novel target for therapeutic intervention in *PXR*-mediated pathophysiology.

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FIGURE 1:

An siRNA screen identifies HNF4 α , IKBKB, and KLF13 as regulators of *PXR* transcription. (A) *PXR* transcript levels were assessed in eight pancreatic ductal adenocarcinoma cell lines and compared with those in the noncancerous pancreatic cell line HPNE. (B) A transcription factor siRNA library was transfected in AsPC-1 cells and *PXR* mRNA levels were assessed 48 h post transfection. A nontargeting (NT) siRNA was used as negative control (green dots, each corresponding to NT siRNA from each assay plate), and *PXR* siRNA was used as positive control (red dots, each corresponding to each *PXR* siRNA from each assay plate). *HNF4A* siRNA was indicated as a blue dot. (C) The 115 transcription factor siRNA hits were selected according to preestablished criteria. The 115

siRNAs were transfected in AsPC-1 cells, and *PXR* mRNA levels were assessed 48 h post transfection. A NT siRNA was used as negative control (green dots), and *PXR* siRNA was used as positive control (red dots). *HNF4A* siRNA was indicated as a blue dot. (D) Nine siRNAs were further validated in AsPC-1 cells, and *PXR* mRNA levels were assessed 48 h post transfection. A NT siRNA was used as negative control, and *PXR* siRNA was used as positive control. The values in A and D represent the mean of three independent experiments, and the bars denote the standard error of the mean (SE). *P* values were determined by ANOVA with Tukey honest significant difference test. **P* < 0.05.

FIGURE 2:

Deconvolution of siRNA hits. (A) The individual siRNAs constituting the pooled siRNAs used to identify hit genes in the screen were transfected in AsPC-1 cells. *PXR* mRNA levels were assessed 48 h post transfection. (B) The knockdown efficiency of the *IKKBK* individual siRNA was assessed in AsPC-1 cells. *IKKBK* mRNA was assessed 48 h post transfection. (C) The knockdown efficiency of the *KLF13* individual siRNA was assessed in AsPC-1 cells. *KLF13* mRNA was assessed 48 h post transfection. The values represent the means of three independent experiments, and the bars denote the standard error of the mean (SE). *P* values were determined by ANOVA with Tukey honest significant difference test. **P* < 0.05.

FIGURE 3: *KLF13* siRNA 2 targets *KLF15*, *NAA10*, and *SP8*. *KLF13* siRNA 2 was transfected in AsPC-1 cells, and the mRNA levels of the indicated genes were assessed 48 h post transfection. The values represent the means of at least three independent experiments, and the bars denote the standard error of the mean (SE). *P* values were determined by ANOVA with Tukey honest significant difference test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

FIGURE 4:

NAA10 and SP8 positively regulate *PXR* transcription. The siRNAs targeting *NAA10*, *SP8*, and *KLF15* were individually transfected in AsPC-1 cells. (A) *PXR* mRNA was assessed 48 h post transfection. (B–D) *NAA10*, *SP8*, and *KLF15* siRNA knockdown efficiency was assessed. The values represent the means of three independent experiments, and the bars denote the standard error of the mean (SE). *P* values were determined by ANOVA with Tukey honest significant difference test. *****P* < 0.0001.

FIGURE 5:

Deconvolution of *NAA10* and *SP8* siRNAs. (A) Two individual siRNAs constituting the pooled siRNAs each targeting *NAA10* or *SP8* were transfected in AsPC1 cells. *PXR* mRNA levels were assessed 48 h post transfection. (B–C) *NAA10* and *SP8* individual siRNA knockdown efficiency was assessed in AsPC-1 cells. (D) AsPC-1 cells were transfected with constructs encoding *PXR*-luciferase and renilla-luciferase. The cells were subsequently transfected with nontargeting (NT), *SP8* (#2), or *NAA10* (#1) siRNA. Luciferase activity was measured 72 h after the initial transfection. Luciferase assays were performed using the Dual-Glo luciferase reagent, and the luciferase activities are represented as the fold change over NT siRNA control cells. The values represent the means of three independent

experiments, and the bars denote the standard error of the mean (SE). *P* values were determined by ANOVA with Tukey honest significant difference test. *****P* < 0.0001.

FIGURE 6:

The regulation of *PXR* by NAA10 and SP8 is not global. *NAA10* pooled siRNA, *SP8* pooled siRNA, and nontargeting siRNA were transfected in AsPC-1 cell, and the levels of the indicated genes were assessed relative to nontargeting siRNA transfected cells. Graph represents the mean of three independent experiments, and the bars denote the standard error of the mean (SE). * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$.

FIGURE 7:

NAA10 interacts with the promoter region of *PXR*. (A–C) Chromatin immunoprecipitation (ChIP) was performed in AsPC-1 cells with –200, –1200, and –2200 *PXR* promoter regions against NAA10 followed by qPCR with probes specific for *PXR* proximal promoter region. Graphs show qPCR results with the relative occupancy method. ChIP-qPCR was applied to confirm NAA10 binding to the *CDH1* (E-cad) (D) and *MYC* (c-Myc) promoters (E) as positive controls, as well as to *PXR* (F), *GAPDH* (G) and *18S* coding sequences (H) as negative controls. (I) AsPC-1 cells were transfected with either pooled *NAA10* siRNAs or a single *NAA10* siRNA for 48 h. Wholecell lysates were probed with antibodies as indicated.

P values were determined with Student *t* test. *****P* < 0.0001, *n* = 3. Results are expressed as the mean ± SE.

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FIGURE 8:

Graphs represent the knockdown efficiency of *NAA10* and *HNF4A* with *NAA10* siRNA #1 and *HNF4A* pooled siRNA respectively in AsPC-1 cells (upper panels) and HPAF-II cells (lower panels).

FIGURE 9:

NAA10 functions in complex with HNF4α. *NAA10* and *HNF4A* were individually or concomitantly knocked down in AsPC-1 cells (A) and HPAF-II cells (B). *PXR* mRNA was assessed 48 h after transfection. (C) AsPC-1 and HPAF-II cell lysates were immunoprecipitated with an anti-NAA10 antibody followed by immunoblotting with the indicated antibodies. IgG was used as a control. The data presented are based on three independent experiments, and *P* values were determined by ANOVA with Tukey honest significant difference test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

FIGURE 10:

PXR does not affect apoptosis or drug response in AsPC-1 cells. (A) Caspase 3/7 activity was measured by CaspaseGlo in AsPC-1 cells. AsPC-1 cells were transfected with nontargeting or *PXR* siRNAs. CaspaseGlo reagent was added to lyse cells to detect Caspase3/7 activity at 48 h after transfection. (B) Graph represents the efficiency of *PXR* knockdown in AsPC-1 cells. (C) A collection of 12 commonly used chemotherapeutic drugs were screened for differential responses in AsPC-1 cells with (+dox) and without (–dox) shRNA-mediated *PXR* knockdown (shPXR) or (D) with (+dox) and without (–dox) *PXR* overexpression (PXR OE). Both knockdown and overexpression of *PXR* were doxycycline (dox) inducible.

FIGURE 11:

CYP3A5 protein levels are not correlated with those of PXR in AsPC-1 cells treated with indicated compounds. AsPC-1 shPXR (shPXR) or AsPC-1 PXR OE (PXR OE) cells with (+dox, 24 h) or without (–dox) induction were treated with bortezomib (Bor, top panel) or capecitabine (Cap, bottom panel) for 72 h as described in section 2.11 and the legend of Figure 10. Representative Western blot shows the protein levels of CYP3A5, and the numbers (the ratio between CYP3A5 and β -actin, multiplied by 10) below the protein bands indicate their relative intensity.

FIGURE 12:

(A) HPAF-II cells transfected with nontargeting, *PXR* pooled siRNA, or *NAA10* siRNA#1 were subjected to migration assays. Graphs represent fold differences in the number of migrated cells toward chemoattractant (complete media). Knockdown efficiency is shown for *PXR* (B) and *NAA10* (C) in HPAF-II cells. (D) Graph represents the knockdown efficiency of *PXR* in AsPC-1 cells with doxycycline (dox)-inducible shRNA-mediated *PXR* knockdown. (E) Graph represents the overexpression of *PXR* in AsPC-1 cells with dox-inducible *PXR* overexpression.

FIGURE 13:

PXR negatively regulates AsPC-1 cell migration. (A) AsPC-1 cells were transfected with nontargeting (NT), *PXR*, or *NAA10* siRNA for 48 h. Images are representative of the observed phenotype. (B) AsPC-1 cells transfected with NT, *PXR*, or *NAA10* siRNA were subjected to migration assays. Graphs represent fold differences in the number of migrated cells toward chemoattractant (complete media). (C) AsPC-1 cells with shRNA-mediated *PXR* knockdown or (D) *PXR* overexpression treated with or without doxycycline (dox) were subjected to migration assays. Graphs represent fold differences in the number of migrated cells toward chemoattractant. The data presented are based on three independent experiments, and *P* values were determined by ANOVA with Tukey honest significant difference test. *****P* < 0.0001.

TABLE 1:

Primers used for ChIP-qPCR.

Promoter regions amplified	Forward primer	Reverse Primer	Catalog #
PXR -200 region	5'-CAGCTCTCTTTATAACAATT-3'	5'-TTGGACTAGAAATGAGAAAA-3'	
PXR -1200 region	5'-AAATTTATGTGTTAGAATTT-3'	5'-TAACCTGAATTCTACTTGA-3'	
PXR -2200 region	5'-TATTATTGGTTTATGAAGTC-3'	5'-CTAGAATTAGACATGAGAAA-3'	
E-cadherin promoter	5'-CCAGGCTAGAGGGTCACCGC-3'	5'-CGCCACTGAGAGGGGTGCG-3'	
C-myc promoter	5'-GCTCTCCACTTGCCCCTTTTA-3'	5'-GTTCCCAATTTCTCAGCC-3'	
PXR coding region			Hs01114267_m1
GAPDH coding region			Hs02786624_g1