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TITLE PAGE

Title:

Induced CYP3A4 expression in confluent Huh7 hepatoma cells as a result of decreased cell proliferation and subsequent PXR activation

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RUNNING TITLE PAGE

Running title:

PXR induced CYP3A4 expression in confluent Huh7 cells

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Abbreviations:

CDK2, cyclin-dependent kinase 2; CLEM4, far distal constitutive liver enhancer module; CYP, cytochrome P450; HNF, hepatocyte nuclear factor; PROX, proximal promoter region; PXR, pregnane X receptor; RXR, retinoid X receptor; XREM, xenobiotic-responsive enhancer module

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ABSTRACT

We have previously shown that confluent growth of the human hepatoma cell line Huh7 substantially induces the CYP3A4 mRNA, protein and activity levels. Here, the mechanisms behind were investigated and a transcriptome analysis revealed significant up-regulation of liver specific functions, whereas pathways related to proliferation and cell cycle were down-regulated in the confluent cells. Reporter analysis revealed that the CYP3A4 gene was transcriptionally activated during confluence in a process involving PXR. PXR expression was increased and PXR protein accumulated in the nuclei during confluent growth. The PXR ligand rifampicin further increased the expression of CYP3A4 and siRNA mediated knock-down of PXR in confluent cells resulted in decreased CYP3A4 expression. CDK2, a known modulator of the cell cycle and a negative regulator of PXR, was higher expressed in proliferating control cells. Trypsinization of the confluent cells and replating them subconfluent resulted in a decrease in CYP3A4 and PXR expression back to levels observed in subconfluent control cells whereas the CDK2 levels increased. Knock-down of CDK2 in proliferating control cells increased the CYP3A4 and PXR protein levels. Moreover, the CDK inhibitor roscovitine stimulated the expression of CYP3A4. A phosphorylation-deficient mutation (S350A) in the PXR protein significantly induced the CYP3A4 transcription. In conclusion, the data strongly suggest that the increased CYP3A4 expression in confluent Huh7 cells is caused by the endogenous induction of PXR as a result of cell-cell contact inhibited proliferation and subsequent decreased CDK2 activities indicating an endogenous, non-ligand dependent regulation of PXR and CYP3A4 possibly of physiological and pharmacological significance.

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INTRODUCTION

From a clinical aspect CYP3A4 is the most important CYP450 enzyme contributing to the metabolism of the largest number of therapeutically important drugs (Guengerich, 1999; Ingelman-Sundberg et al., 2007). CYP3A4 is a highly polymorphic enzyme with pronounced constitutive inter-individual expression in the human population. More than forty allelic variants have been described for CYP3A4 (http://www.cypalleles.ki.se), although none present at a high enough frequency as to explain the inter-individual variation in hepatic CYP3A4 expression (Ingelman-Sundberg et al., 2007). It is more likely that environmental (Burk and Wojnowski, 2004; Michalets, 1998) and epigenetic factors are important in this respect (Kacevska et al., 2011).

The *CYP3A4* 5'-promoter region has been well studied and three distinct regulatory elements have been shown to be of particular importance for the regulation of this gene: the proximal promoter region (PROX), the xenobiotic-responsive enhancer module (XREM) (Goodwin et al., 1999; Zhang et al., 2001) and the far distal constitutive liver enhancer module (CLEM4) (Matsumura et al., 2004) (schematically shown in Figure 3A). *CYP3A4* is regulated by various liver-enriched transcription factors, such as hepatocyte nuclear factors (HNF) and CCAAT/enhance-binding proteins (CEBP), important for the constitutive expression as well as induction (Martinez-Jimenez et al., 2007; Matsumura et al., 2004; Rodriguez-Antona et al., 2003; Tirona et al., 2003). *CYP3A4* is also regulated by the interaction of various transcription factors, like the nuclear receptors (NR) pregnane X receptor (PXR, NR1I2) (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998), constitutive androstane receptor (CAR, NR1I3) (Goodwin et al., 2002), vitamin D receptor (VDR, NR1I1) (Drocourt et al., 2002) and glucocorticoid receptor (GR, NR3C1) (Pascussi et al., 2001), which all bind to specific DNA motifs within the 5′-upstream region of the CYP3A4 gene.

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PXR, in particular, is an important transcriptional regulator of CYP3A4 (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). PXR has broad ligand specificity for both endogenous and exogenous substrates and modulates several key biochemical pathways including gluconeogenesis, beta-oxidation and cholesterol homeostasis (Ma et al., 2008). PXR is a xenobiotic-induced nuclear receptor and its central role in the regulation of CYP3A4 and other clinically important CYPs, like CYP2C9 (Chen et al., 2004), makes PXR crucial for the metabolism of many clinical drugs used today (Tirona and Kim, 2005). As a consequence PXR plays a part in many adverse drug reactions and is estimated to be the nuclear receptor responsible for about 60% of all undesirable clinically relevant drug-drug interactions involving CYP3A4 (Lehmann et al., 1998; Ma et al., 2008).

PXR regulation has been studied extensively but its precise role in *CYP3A4* regulation is far from being fully elucidated. PXR induction is considered ligand dependent and when activated it undergoes a cytosol-to-nuclear translocation where it binds to the promoter region of its target genes as a heterodimer with RXR (retinoid X receptor) (Wan et al., 2000). PXR regulates *CYP3A4* by binding to response elements composed of various direct (DR) and everted (ER) repeats of the consensus motif AG(G/T)TCA (Wang et al., 2012). The transcriptional activity of activated PXR also requires interaction with different co-activators such as HNF4α (Tirona et al., 2003), peroxisome proliferator-activated receptor-γ co-activator (PGC-1) and steroid receptor co-activtor-1 (SRC-1) (Li and Chiang, 2006). Several studies have reported on the post-transcriptional regulation of CYP3A4, both via direct targeting involving microRNA regulation of CYP3A4 mRNA (Pan et al., 2009) and indirect targeting through the regulation of transcription factors such as PXR (Takagi et al., 2008). Additionally, PXR is also subjected to post-translational regulation such as ubiquitination (Staudinger et al., 2011), acetylation (Biswas et al., 2011) and phosphorylation (Ding and Staudinger, 2005b; Lichti-Kaiser et al., 2009b; Lin et al., 2008).

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Previously, we have shown that when the human hepatoma cell line Huh7 is grown confluent the cells change phenotype, including significantly increased levels of CYP3A4. The elevation in CYP3A4 expression and activity is seen in the absence of added inducers. The Huh7 cell model thus constitutes an excellent system where the constitutive regulation of CYP3A4 transcription can be studied. Here we have evaluated mechanisms behind the confluence mediated increase in CYP3A4 expression and find that this gain is mediated by PXR, which in turn is regulated by CDK2 (cyclin-dependent kinase 2). Thus, the results indicate an endogenous non-ligand dependent regulation of PXR and CYP3A4 of possible importance for the explanation of the inter-individual differences in hepatic CYP3A4 expression.

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MATERIAL AND METHODS

Cell culture

The human hepatoma Huh7 cell line (Human Science Research Resources Bank, Tokyo, Japan) was routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37ºC in 5% CO_2. The cells were grown confluent up to 4 weeks or used when about 90% subconfluent as control cells. Medium was changed twice/week. When trypsinating the cells were washed twice with PBS and detached using 0.05% Trypsin-EDTA (Invitrogen, Carlsbad, CA).

RNA extraction and real-time polymerase chain reaction

Cells were collected at defined time points and total mRNA was extracted using RNEasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed using 0.5 µg total mRNA in a final volume of 10 µl reaction mix of oligo(dT) primer and the Moloney murine leukemia virus reverse transcriptase enzyme (Invitrogen, Carlsbad, CA). Real time PCR was carried out using ready-to-use TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative expression levels compared to control cells (relative expression value set to 1) were defined by the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (Livak and Schmittgen, 2001). TATA-box binding protein (TBP) was used as housekeeping gene.

Microarray experiments

An Affymetrix® Human Gene 1.1 ST Microarray (Santa Clara, CA) experiments were conducted on subconfluent control cells and 4 weeks confluent cells in triplicate. Quality

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control of the mRNA and cDNA followed by an analysis of the Array were performed by Bioinformatics and Expression analysis core facilities (BEA, Huddinge, Sweden). The reproducibility of the experiments was verified by hierarchical clustering of the global transcriptional dataset. The fold change (FC) values between the control and confluent grown cells were calculated and a 2-sided, unpaired t-test was applied to identify transcripts that were differentially expressed between the two cell types. A criterion of p-value < 0.05 was used to identify statistically different transcripts and was used as input into DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003; Huang da et al., 2009). A combined criterion of $p < 0.05$ and $FC > 2$ was used to analyze statistical and biological differences. To explore putative mechanistic effects of growing the Huh7 cells confluent, overrepresented up- and down-regulated biochemical pathways in confluent cells compared to control cells were analyzed using KEGG pathway mapping. The functional properties of the significantly up- and down-regulated genes were also investigated using a Gene Ontology (GO) enrichment analysis and significantly overrepresented GO annotations for biological process (BP), molecular function (MF) and cellular component (CC) were identified.

Plasmid constructs

Reporter constructs containing the three CYP3A4 5'-flanking regulatory regions PROX (P), XREM (X) and CLEM4 (C) and combinations thereof were generated by PCR: CYP3A4-P-luc (-362 to +53 bp) Fwd.: 5'-cattgctggctgaggtggtt and Rev.: 5'-catggatcctgttgctctttgctgggctatgtgc; CYP3A4-X-luc (-7836 to-7208 bp) Fwd.: 5'-ctagcccggggctggtttattctagagagatgg and Rev.: 5'-tagtagatctgatctcgtcaacaggttaaag; and CYP3A4-C-luc (-11,383 to -10,578 bp) Fwd.: 5'-cttggtagtagtcgttagaatctgaac and Rev.: 5'-tttccctcccaaaggagct. The -12.5 kb CYP3A4 reporter plasmid (Tegude et al., 2007) was used as a template. The regions were inserted in a pGL3 basic vector (Promega, Madison, WI)

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between Hind*III*/Bgl*II*, Sma*I*/Bgl*II* and Kpn*I*/Sac*I* respectively. All reporter plasmids were sequence verified. The human PXR expression vector was purchased from OriGene Technologies. The human RXR expression vector was generously provided by Dr. Richard B. Kim (The University of Western Ontario). A phosphorylation-deficient mutation in PXR at Ser^{350}, changing serine to alanine (S350A), was generated using the QuickChange site-directed mutagenesis kit (Agilent Technologies Inc., Rockville, MD) with the use of the mutagenesis primer: 5'-catctccctcttcttcgccccagaccgccc.

Transient transfections

Huh7 cells were seeded in 12 well plates at a density of 3 x 10^5 cells/well and used when cultured confluent for 4 weeks or when 90% subconfluent as control. The cells were transfected in medium without antibiotics using a transfection mixture of 1µg/well luciferase reporter plasmid, 0.2 µg pRT-TK (Promega, Madison, WI) and Lipofectamine™ LTX with PLUS™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. For transfection with pEF-RXRα (Tirona et al., 2003) and pCMV6-PXR (OriGene Technologies Inc., Rockville, MD) 1 µg/well of each expression plasmid was used and pEF6-V5His empty expression vector (Tirona et al., 2003) was used to normalize the amount of DNA. Luciferase activities were measured 48h post-transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI). In some experiments 16-24 hours post-transfection, cells were treated with 20 µM roscovitine (R stereoisomer, Cell Signaling Technology, Inc., Danvers, MA) 10µM rifampicin (Sigma, St Louis, MO) or 0.1 % DMSO (vehicle) and incubated for another 24 hours before activity was measured.

Short interfering RNA transfection

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PXR was knocked down using short interfering RNA (siRNA) in 4 weeks confluent Huh7 cells cultured in 12 well plates. The cells were treated with 200 nM ON-TARGETplus SMARTpool siRNA targeting PXR (L-003415, Thermo Fisher Scientific Inc., Waltham, MA) or Nontargeting Pool (D-001810, Thermo Scientific) together with 20µl/well DharmaFECT1 (Thermo Fisher Scientific Inc., Waltham, MA) diluted in 1ml medium with serum and without antibiotics. CDK2 was knocked down using siRNA in about 70% confluent control cells cultured in 6- or 12-well plates. The cells were treated with 50 nM ON-TARGETplus SMARTpool siRNA targeting CDK2 (L-003236, Thermo Fisher Scientific Inc., Waltham, MA) or Nontargeting Pool together with 5 or 16 µl/well DharmaFECT1 diluted in 1ml or 2 ml medium with serum and without antibiotics for 12- and 6–well plates, respectively. After 2-5 days incubation the cells were harvested and subjected to RT-PCR as described above or analyzed using western blot as described below. For protein analysis the cells were lysed in RIPA buffer pH 8.0 containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA and complete protease inhibitors (Roche Diagnostics, Mannheim, Germany), and centrifuged at 13,000×g and 4ºC for 10 min.

Isolation of cytoplasmic and nuclear extracts

Subconfluent control cells and 4 weeks confluent cells grown in 75 cm^2 flasks were washed with PBS and the cells were scraped from the flask in PBS. Cytoplasmic (CE) and nuclear extracts (NE) were prepared as previously described (Mwinyi et al., 2010). For the trypsinization experiments 4 weeks confluent cells were trypsinated and replated about 50% confluent. After 2 days in culture, when about 90% confluent, the cells were harvested and nuclear extracts prepared as described above. Protein concentration was determined according to Bradford (Bradford, 1976).

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Western blot analysis

The microsomal fraction from control and confluent Huh7 cells was isolated as previously described (Sivertsson et al., 2010). Equal amounts of protein (microsomal fractions, total-cell lysate, cytoplasmic extracts or nuclear extracts) were mixed with Laemmli sample buffer, subjected to SDS polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes as previously described (Sivertsson et al., 2010). The membranes were incubated with goat anti-CYP3A4 (Wang et al., 2009), mouse anti-CDK2 (610145, BD Biosciences, San Jose, California), rabbit-CDK2 (sc-163, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-PXR (ab85451, ABcam, Cambridge, UK) followed by conjugated horseradish peroxidase secondary antibodies (Dako Denmark A/S, Glostrup, Denmark). Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA).

Immunocytochemistry

Four-weeks confluent and subconfluent control cells, grown on glass cover slips, were fixed and stained as previously described (Sivertsson et al., 2010). The slides were incubated with goat anti-PXR (sc-9690, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-CDK2 (sc-163, Santa Cruz Biotechnology) antibodies followed by anti-goat Cy3 (Jackson Immunoresearch, West Grove, PA,) or anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA) conjugated antibodies. The coverslips were mounted with ProLong Gold anti-fade reagent containing 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA). The slides were examined and photographed at 40x magnification using a Nikon Eclipse E600 fluorescence microscope.

Statistical analysis

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Data were analyzed using unpaired t-test or one-way analysis of variance using Dunnett's multiple comparison test or Bonferroni's multiple comparison test.

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RESULTS

Differentially expressed genes – functional properties and affected pathways in confluent Huh7 cells

Previously we have shown that Huh7 cells grown confluent up to 4 weeks achieve a more differentiated phenotype more similar to primary hepatocytes (Sivertsson et al., 2010). To investigate the change in gene expression during this phenotypical switch, a global gene expression analysis was performed. In total the expression of 9,530 genes was significantly changed (p-value < 0.05) whereof 3,381 were up-regulated while 6,149 were down-regulated (Supplemental table 1). Genes with transcriptional differences less than 2-fold (FC > 2) were removed, resulting in 1,596 up-regulated and 2,980 down-regulated genes. Of these, 1,079 up-regulated and 2,228 down-regulated genes were annotated with an official gene symbol (Figure 1).

The functional properties of the differentially expressed genes were further explored with gene ontology (GO) annotation analysis. The results for the confluent cells showed an increase in annotations associated with more differentiated liver functions such as metabolism (Supplemental table 2) whereas genes associated with cell cycle and proliferation decreased in expression (Supplemental table 2). The effects on specific biochemical pathways between control and confluent cells were also investigated. With the input of the 4,576 genes (described above) and the criterion of a p-value < 0.05 and FC > 2, 21 KEGG pathways were identified as up-regulated (Table 1) and 15 KEGG pathways as down-regulated (Table 2) in confluent cells compared to control cells. Interestingly, the up-regulated pathways were highly involved in liver specific functions such as biotransformation and drug metabolism (Table 1). In contrast, many of the down-regulated genes are involved in cell cycle and proliferation processes (Table 2) indicating a more differentiated status of the confluent cells.

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CYP3A4 gene and protein expression is significantly increased in confluent cells

The relative gene expression of several cytochrome P450, such as CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6 and CYP3A4, and transcription factors, like CAR, VDR, PXR and HNF4α, were evaluated in control cells and 1-4 week confluent cells using quantitative RT-PCR analysis. The expression levels relative to control cells showed a significant increase in CYP3A4 and CYP2C9 mRNA expression after 4 weeks of confluence (Figure 2A). The increase in CYP3A4 expression levels is particular dramatic, resulting in mRNA up-regulation to about 1000-fold after 4 weeks of confluence (Figure 2A). The CYP3A4 protein expression is also increased in the same time-dependent manner (Figure 2B). In contrast, when the 4 weeks confluent cells were trypsinated and replated subconfluent again the increased *CYP3A4* gene expression was rapidly lost (Figure 2C). The same effect was seen on CYP3A4 protein levels (data not shown). The expression of the transcription factor PXR was significantly increased already after 1 week of confluence and remained sustained throughout time in confluent culture (Figure 2A). The expression levels of the other genes analyzed were not significantly affected by confluency (data not shown).

Transcriptional activation of CYP3A4 in confluent Huh7 cells

To study the transcriptional activation of the *CYP3A4* gene in the control and confluent Huh7 cells more in detail we created reporter constructs containing three CYP3A4 5'-flanking regions: PROX (P), XREM (X) and CLEM4 (C, Figure 3A), all known to be highly involved in the transcriptional regulation of the *CYP3A4* gene (Goodwin et al., 1999; Matsumura et al., 2004; Zhang et al., 2001). The activities of all different constructs were significantly higher in confluent cells compared to control cells (Figure 3B). The highest activity was observed using the CYP3A4-PXC-luc construct, indicating that all three regions are important for CYP3A4

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regulation in these confluent cells. The removal of one or more regions significantly reduced the promoter activity and the lowest activity was seen for the constructs containing only one of the sites. However, when combining two of the sites, the highest loss of activity was seen when either the PROX (CYP3A4-XC-luc) or the CLEM4 (CYP3A4-PX-luc) site was absent, indicating that these two regions are the most important for the regulation of CYP3A4 in the confluent cells (Figure 3B). The transcriptional activity of the *CYP3A4* gene was increased in a time-dependent manner during confluency as measured using the CYP3A4-PXC-luc construct (Figure 3C) and correlated well with the increase in CYP3A4 mRNA, protein (Figure 2A and B) and catalytic activity levels (Sivertsson et al., 2010). The relative luciferase activity gradually increased with time of confluence, becoming significant already after one week of confluence (Figure 3C) and confirmed that the mechanism behind the increased CYP3A4 mRNA and protein expression is indeed transcriptionally regulated.

PXR regulates CYP3A4 promoter activity in confluent cells

We have previously shown that the PXR ligand rifampicin further increased the CYP3A4 catalytical activity in confluent Huh7 cells (Sivertsson et al., 2010). To study the involvement of PXR in the transcriptional regulation of *CYP3A4* in more detail control and 4 weeks confluent cells were transfected with the CYP3A4-PXC-luc reporter construct followed by treatment with rifampicin or vehicle (0.1% DMSO). Treating the cells with rifampicin significantly induced the CYP3A4 promoter activity in confluent but not in control cells (Figure 4A) indicating that transcriptionally active PXR is present only in the confluent cells. Co-transfection of the CYP3A4-PXC-luc reporter construct together with the PXR expression plasmid only resulted in a modest increase in reporter activity in confluent cells. However, in the control cells, co-transfection with the PXR expression plasmid induced the relative luciferase activity about 10-fold resulting in similar levels as seen in untreated confluent cells

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(Figure 4B). When activated, PXR will heterodimerize with RXR and target PXR response elements in the CYP3A4 promoter (Goodwin et al., 1999; Savas et al., 1999). To investigate if the RXR levels are limiting, resulting in low CYP3A4 promoter activity in control cells, control and confluent cells were also co-transfected with the CYP3A4-PXC-luc reporter plasmid and a RXR expression plasmid. However, no effect on relative luciferase activity was seen when overexpressing RXR in either cell type either alone or in combination with PXR (Figure 4B) indicating sufficient endogenous RXR expression.

siRNA mediated down-regulation of PXR decreases the CYP3A4 levels in confluent cells

To further confirm the involvement of PXR in the CYP3A4 transcriptional regulation in the confluent cells, PXR was knocked down using siRNA. siPXR transfection significantly reduced the PXR mRNA expression by 60% as well as the PXR protein levels in confluent cells (Figure 5). Interestingly, the CYP3A4 mRNA expression levels were also significantly reduced by about 40% (Figure 5).

Nuclear localization of PXR in confluent cells

It is generally considered that transcriptionally active PXR is translocated from the cytoplasm to the nucleus where it binds to the CYP3A4 promoter region. The PXR levels in control cells were low and only small amounts could be detected in the nuclear fraction whereas in confluent cells the PXR levels were significantly higher and mainly were associated with the nuclear fraction (Figure 6A, upper panels). As illustrated by immunocytochemical staining, PXR is localized in the nuclei of confluent cells but not in control cells (Figure 6B, upper panels), confirming the western blot results (Figure 6A, upper panels). Notably, when the confluent cells are trypsinated and replated subconfluent for two days the PXR protein levels rapidly drop to levels comparable to those observed in control cells (Figure 7A and 7B, upper

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panels). Thus, the effect of confluent culture on the PXR levels in the Huh7 cells is reversible and mirrors the CYP3A4 expression data shown in figure 2C.

CDK2 is down-regulated in confluent cells

Previously it has been shown that CDK2 is able to inhibit PXR activity by direct phosphorylation of the receptor, resulting in attenuated *CYP3A4* gene expression (Lin et al., 2008). We found that the CDK2 protein is located in both the cytoplasm and the nucleus in both cell types but is present in much higher levels in control cells (Figure 6A, lower panels and Fig 7A, lower panel). This was confirmed by immunocytochemical staining where the CDK2 staining intensity generally was higher in control cells, especially the nuclear staining (Figure 6B, lower panels). Notably, CDK2 staining was particularly intense in cells undergoing meiosis confirming its role in cell division (Figure 6B, lower panel, enclosed picture). Interestingly, when 4 weeks confluent cells were trypsinated and replated subconfluent the CDK2 protein levels rapidly increased (Figure 7A and 7B, lower panel) in contrast to the effect seen on PXR protein levels (Figure 7A and 7B, upper panels) and CYP3A4 mRNA levels (Figure 2C).

Down-regulation of CDK2 increases the CYP3A4 levels in control cells

To determine the effect of CDK2 on PXR-mediated CYP3A4 transcriptional regulation, CDK2 was knocked down in subconfluent control cells using siRNA. Knock down of CDK2 resulted in increased levels of CYP3A4 both on RNA and protein level (Figure 8A). Additionally, the protein levels of PXR also increased significantly (Figure 8A, right panel). The role of CDK2 on the CYP3A4 transcriptional regulation was further confirmed by treating CYP3A4-PXC-luc transfected subconfluent control cells with roscovitine, a well-known CDK inhibitor (Lin et al., 2008; Sugatani et al., 2012). Roscovitine significantly

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increased the CYP3A4-PXC-luc activity by three-fold compared to vehicle treated cells (Figure 8B). Treatment with rifampicin in the presence of roscovitine did not have an additional effect on the CYP3A4-PXC-luc activity.

Phosphorylation-deficient mutation of PXR at Ser^{350} increased the transcriptional activation of CYP3A4

To provide further evidence that PXR is targeted by CDK2 in the proliferating control cells we generated a PXR construct with a mutation at a putative CDK phosphorylation site, Ser^{350} previously shown to be phosphorylated by CDK2 (Lichti-Kaiser et al., 2009a). Subconfluent control cells were transfected with the CYP3A4-PXC-luc reporter and co-transfected with either wild type PXR (PXR wt) or the PXR Ser^{350} mutant (PXR S350A). The results show that the S350A mutant caused a significantly higher luciferase activity compared to the wt PXR (Figure 9). In addition, the rifampicin-induced transcriptional activity was also significantly higher in the PXR S350A transfected cells (Figure 9).

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DISCUSSION

In a previous study we have shown that confluent culture of the human hepatoma cell line Huh7 has a more differentiated phenotype than subconfluent cells (Sivertsson et al., 2010). In particular, the expression of CYP3A4 was markedly increased. Here, we have investigated the mechanisms behind the increased CYP3A4 expression in confluent grown Huh7 cells. A transcriptome analysis revealed that cell proliferation was decreased and that the Huh7 cells achieved a more hepatocyte-like phenotype when grown confluent. These results are in compliance with the *in vivo* situation where primary hepatocytes are highly dependent on cellular architecture and tight cell-cell contacts for the maintenance of their organized tissue architecture, their differentiated functions and for the regulation of cell proliferation (Dvir-Ginzberg et al., 2003; Machide et al., 2006). This is also true regarding the expression of CYP3A4 which is reduced during liver regeneration (Favre et al., 1998). The importance of tight cell-cell contacts is further underscored when confluent cells are trypsinated and replated subconfluent which causes the increased CYP3A4 levels rapidly to drop back to levels observed in control cells.

In the CYP3A4 promoter, several binding sites for various transcription factors have been identified as being important for the CYP3A4 regulation (Goodwin et al., 1999; Hashimoto et al., 1993; Martinez-Jimenez et al., 2007; Matsumura et al., 2004). In order to study the CYP3A4 gene regulation in the confluent Huh7 cells we created several reporter constructs containing three CYP3A4 5'-flanking regulatory regions that are known to be important for the regulation of CYP3A4. In the confluent Huh7 cells the increase in CYP3A4 mRNA and protein expression levels correlate well which is in good agreement to what has been described for human liver (Lamba et al., 2010; Watanabe et al., 2004). When transfecting the cells with various CYP3A4 promoter constructs, the construct containing all three regions

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(CYP3A4-PXC-luc) displayed the highest activity and exhibited a stable and time dependent increase during time of confluence. Interestingly, even though all three regions were essential for maximal induction the XREM region seemed to have the least effect on activity in our cells. A possible explanation might be that the affinity of different transcription factors to different regulatory binding sites of the CYP3A4 promoter is dependent on the cellular mechanism regulating these interactions (Masuyama et al., 2003).

The hepatic expression of CYP3A4 is highly dependent on the activity of several transcription factors and nuclear receptors with PXR recognized to be a key regulator (Kliewer et al., 1998). In the Huh7 cells PXR mRNA and protein expression were increased during confluence and PXR protein accumulated in the nucleus. In addition to the increased CYP3A4 expression the expression of CYP2C9 which is also known to be regulated by PXR (Pondugula et al., 2009) was also significantly increased during confluence. Rifampicin, a known ligand and activator of PXR (Lehmann et al., 1998), significantly induced the transcription of CYP3A4 in the confluent cells but had no effect in the control cells which suggested that the low PXR levels found in the control cells are not transcriptionally active. In control cells overexpressing PXR, the CYP3A4-PXC-luc reporter activity was increased about 10-fold reaching the same level as observed in the confluent cells. In addition, in control cells overexpressing PXR, rifampicin was able to induce the CYP3A4 promoter activity even further demonstrating that when present, PXR is indeed able to induce CYP3A4 in control cells. The role of PXR in the CYP3A4 regulation in the confluent cells was further confirmed by siRNA-mediated down-regulation of PXR which resulted in significantly reduced CYP3A4 mRNA levels in the confluent cells.

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Although the molecular mechanism behind PXR activation has been studied extensively, the regulation of PXR activity is still not entirely clear. Much of what we know about PXR regulation is based on studies in mice or in cell systems using exogenous PXR ligands and PXR overexpression. The increased PXR activity seen in the confluent Huh7 cells did not require the addition of exogenous ligands and suggests the involvement of an endogenous ligand or signal transduction mediated regulation of PXR (Kliewer et al., 1998). PXR is known to be regulated by several cellular modulators and processes involving protein-protein interactions with different co-repressors (Istrate et al., 2010; Li and Chiang, 2006) and co-activators (Bhalla et al., 2004; Li and Chiang, 2006; Lim et al., 2009). In addition, post-transcriptional regulation of PXR by miRNA (miR-148a) has been reported (Takagi et al., 2008). Several post-translational modifications have been suggested to be involved in PXR regulation, like ubiquitination (Staudinger et al., 2011) and acetylation (Biswas et al., 2011). Even though PXR was present at low levels in the control cells, the CYP3A4 activity could not be induced by rifampicin treatment suggesting that PXR is not transcriptionally active possibly due to protein-protein binding with co-inhibitors or by post-translational modifications.

As most nuclear receptor superfamily members, PXR is a phosphoprotein and is dynamically regulated by its phosphorylation status which affects the expression levels, protein stability, localization, ligand and DNA binding and interaction with other modulators which indirectly regulates CYP3A4 (Ghose et al., 2004; Gu et al., 2006; Rochette-Egly, 2003). Kinases, like PKA (Ding and Staudinger, 2005a; Lichti-Kaiser et al., 2009b) and PKC (Ding and Staudinger, 2005b), are known to regulate PXR by phosphorylation at several putative serine and threonine phosphorylation sites that have been identified within the human PXR protein (Lichti-Kaiser et al., 2009a). Lately, several reports have shown that CDK2, a key regulator of

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cell cycle progression, is also involved in the regulation of PXR (Lin et al., 2008; Sugatani et al., 2012). It was further shown that PXR-mediated CYP3A4 expression is lower in S phase where CDK2 has its maximal activity (Lin et al., 2008). Our global gene array data indicate that the cell cycle is arrested when the Huh7 cells are grow confluent subsequently leading to increased PXR protein levels with CYP3A4 induction as a result. Hence, we hypothesize that when the cells are grown confluent proliferation is inhibited resulting in decreased CDK2 levels (illustrated in Figure 10). Indeed, the total levels of CDK2 are significantly higher in the proliferating control cells compared to confluent cells as shown by western blot and by immunocytochemical staining. Moreover, when the confluent cells were trypsinized and replated at low density, the cells started proliferating again with increased CDK2 levels and decreased PXR and CYP3A4 levels as a result. A previous study conducted in HepG2 cells showed that siRNA-mediated down-regulation of CDK2 resulted in increased expressed levels of CYP3A4 (Sugatani et al., 2010). Moreover, HepG2 cells treated with an antimitogenic factor resulted in decreased activity of CDK2 and increased expression of UGT1A1, CYP2B6 and CYP3A4 (Sugatani et al., 2010) which are all regulated via PXR (Gardner-Stephen et al., 2004; Goodwin et al., 2001; Lehmann et al., 1998). In a more recent study, the same group showed that UGT1A1 expression was increased in HepG2 cells treated with the CDK inhibitor roscovitine in a process that involved PXR activation (Sugatani et al., 2012). Another study by Lin *et al.* shows that treatment with the CDK2 inhibitor roscovitine lead to PXR-mediated CYP3A4 promoter activity in HepG2 cells (Lin et al., 2008). To determine the role of CDK2 on the PXR-regulated transcriptional activation of CYP3A4 in our Huh7 cells, CDK2 was knocked-down in subconfluent control cells which indeed resulted in increased PXR and CYP3A4 protein levels. Moreover, roscovitine treatment of CYP3A4-PXC-luc transfected subconfluent control cells resulted in markedly increased resistance to the repressive CDK2 effects in the subconfluent cells. Roscovitine treatment did, however,

not make the subconfluent cells more permissive to CYP3A4 induction by rifampicin possibly due to competitive binding of roscovitine to the PXR ligand-binding domain (Lin et al., 2008). Several studies have suggested that PXR is phosphorylated at Ser^{350}, modulating it's transcriptionally activity (Lin et al., 2008; Sugatani et al., 2012). Co-transfection of the CYP3A4-PXC-luc reporter with the phosphorylation deficient mutant PXR S350A in subconfluent control cells resulted in significantly higher transcriptional activity compared to the wild type PXR co-transfected cells. In addition, the rifampicin-induced transcriptional activity was also significantly higher in the PXR S350A mutant transfected cells. Together these results suggest that CDK2 affects the CYP3A4 expression levels in the Huh7 cells by modulation of the PXR protein levels and subsequently the transcriptional activity, most likely by phosphorylation of PXR at Ser^{350}.

In summary, we have shown that CYP3A4 mRNA, protein and catalytic activity is increased in confluent grown Huh7 cells. We conclude that the expression of CYP3A4 is regulated by the endogenous activation of PXR as a result of decreased CDK2 activity which is linked to the reduced cell proliferation in the confluent cell culture (Figure 10). The high constitutive expression of CYP3A4 in the confluent Huh7 cells makes this cell system useful for mechanistic studies of regulation of PXR and the *CYP3A4* gene. Additionally, the data indicate endogenous regulation of PXR and CYP3A4 which is ligand independent. This may contribute to better understanding of the large inter-individual differences in hepatic CYP3A4 expression, thus being of possible physiological and pharmacological significance.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Sivertsson, Edebert, Ingelman-Sundberg, Neve

Conducted experiments: Sivertsson, Porsmyr-Palmertz, Neve

Contributed new reagents or analytic tools:

Performed data analysis: Sivertsson, Porsmyr-Palmertz, Neve

Wrote or contributed to the writing of the manuscript: Sivertsson, Edebert, Ingelman-Sundberg, Neve

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FOOTNOTES

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This work will be reused in a thesis/dissertation from Karolinska Institutet entitled “Novel human *in vitro* systems for studies of drug induced hepatotoxicity” by Louise Sivertsson.

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FIGURE LEGENDS

Figure 1. Differentially expressed genes in 4 weeks confluent Huh7 cells compared to subconfluent control cells as analyzed by an Affymetrix human whole transcript array.

A two-sided, unpaired t-test for 3 experiments identified 9,530 transcripts with p-value < 0.05 that were differentially expressed in 4 weeks confluent cells compared to subconfluent control cells. To increase the biological relevance of our results only genes with transcriptional differences more than 2-fold change (FC > 2) were further analyzed resulting in total of 4,576 genes where 1,596 were up-regulated and 2,980 were down-regulated in confluent cells compared to control cells. Of these, 1,079 up-regulated and 2,228 down-regulated genes were annotated with an official gene symbol.

Figure 2. Expression of CYP3A4, CYP2C9 and PXR in confluent Huh7 cells.

A) The relative gene expression of CYP3A4, CYP2C9 and PXR as quantified by TaqMan real-time PCR in 1-4 weeks confluent cells. The relative gene expression levels in the confluent cells were normalized to the levels detected in subconfluent control cells and TBP was used as reference gene. The results are from 3 replicates and are expressed as mean ± S.D., *p < 0.05 and **p < 0.01 compared to control cells (Ctrl). B) The CYP3A4 protein levels were analyzed by western blot using the microsomal fractions isolated from the Huh7 cells at different times of confluence. C) Four weeks confluent Huh7 cells were trypsinized, replated at low density and grown subconfluent for 4 days. The relative CYP3A4 gene expression levels were measured directly after trypsinization (day 0) or after 1-4 days. Relative CYP3A4 gene expression was normalized to the levels detected in subconfluent control cells and TBP was used as reference gene. The results are from 3 replicates and are expressed as mean ± S.D., **p < 0.01 and ***p < 0.001 compared to 4 weeks confluent cells (day 0).

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Figure 3. The transcriptional activity of CYP3A4 in confluent Huh7 cells.

A) Schematic representation of the CYP3A4 promoter containing the three important regulatory regions: the proximal promoter region (PROX, P), the xenobiotic-responsive element module (XREM, X) and the far distal constitutive liver enhancer module (CLEM4, C). B) The transcriptional activity of the different CYP3A4 promoter constructs containing the PROX, XREM and/or CLEM site in 4 weeks confluent cells (closed bars) and subconfluent control cells (open bars). The relative luciferase activities are shown for 3 individual experiments and are expressed as mean ± S.D., *$p < 0.05$ and ***$p < 0.001$ compared to the transcriptional activity of the full length CYP3A4-PXC-luc construct. In the figure, only the significant differences for the confluent cells are shown. C) The transcriptional activity of the full length CYP3A4-PXC-luc construct was increased during time of confluent culture. The relative luciferase activity levels are from 5 individual experiments and are shown as mean ± S.D., ***$p < 0.001$ compared to subconfluent control cells (Ctrl).

Figure 4. The role of PXR in the regulation of CYP3A4 in confluent Huh7 cells.

A) Subconfluent control cells (open bars) and 4 weeks confluent cells (closed bars) were transfected with the CYP3A4-PXC-luc reporter construct and treated with (+) rifampicin (Rif) or vehicle (-). The relative luciferase activity levels are shown as mean ± S.D for 3 experiments, **$p < 0.01$ compared to the untreated cells. B) Subconfluent control cells (open bars) and 4 weeks confluent cells (closed bars) were co-transfected with the CYP3A4-PXC-luc reporter construct and with the PXR and RXR expression plasmids or the empty vector as indicated. The relative luciferase activity levels are from 3 individual experiments and are

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expressed as mean ± S.D., *p < 0.05 and ***p < 0.001 compared to empty vector transfected cells.

Figure 5. siRNA-mediated down-regulation of PXR modulates the CYP3A4 expression in confluent Huh7 cells.

Four weeks confluent cells were transfected with PXR siRNA (siPXR, closed bars) or nontargeting siRNA (siCtrl, open bars) and cells were harvested 48h post-transfection and subjected to RT-PCR analysis to determine the gene expression of PXR and CYP3A4. Down-regulation of PXR was also verified by western blot. The results are from 5 replicates and are shown as mean ± S.D., *p < 0.05 and ***p < 0.001 compared to siCtrl transfected cells.

Figure 6. Protein levels and intracellular localization of PXR and CDK2.

A) PXR and CDK2 protein levels were determined by western blot in the nuclear extracts (NE) and cytoplasmic extracts (CE) isolated from subconfluent control and 4 weeks confluent Huh7 cells. B) Intracellular distribution of PXR (red) and CDK2 (green) in subconfluent control and confluent Huh7 cells. A cell undergoing meiosis is shown in the enclosed picture for the control cells. Nuclei were visualized by DAPI staining (blue). Bar indicates 10 µm.

Figure 7. PXR and CDK2 protein levels in proliferating and confluent Huh7 cells.

A) PXR and CDK2 protein levels in nuclear extracts prepared from control (Ctrl) and 4 weeks confluent (Conf) Huh7 cells. B) The 4 weeks confluent Huh7 cells were trypsinized, replated at low density and grown subconfluent for 2 days. The PXR and CDK2 protein levels present in the nuclear extracts from 4 weeks confluent cells (Conf) and the trypsinized replated cells (Conf tryps.) were determined by western blot analyses.

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Figure 8. Involvement of CDK2 in the regulation of PXR and CYP3A4 in subconfluent Huh7 cells.

A) Subconfluent control cells were transfected with CDK2 siRNA (siCDK2, closed bars) and nontargeting siRNA (siCtrl, open bars). The cells were harvested 72h post-transfection and subjected to RT-PCR analysis (left panels) and western blot analysis (right panel). The results are shown as mean ± S.D. for 4 experiments, ***$p < 0.001$ compared to siCtrl transfected cells. ERp29 was used as a loading control for the western blot analysis. B) Subconfluent control cells were transfected with the CYP3A4-PXC-luc reporter construct and treated with 10μM rifampicin (Rif.), 20μM roscovitine (Rosc.) or DMSO vehicle as indicated. Roscovitine or vehicle was added to the cells 16h post-transfection and rifampicin or vehicle was added 6h later. The CYP3A4 promoter activity levels were analyzed 24h after the addition of rifampicin. The results are shown as mean ± S.D. for 3 experiments, ***$p < 0.001$ compared to vehicle treated cells.

Figure 9. A phosphorylation-deficient mutation of PXR increases CYP3A4 promoter activity.

Subconfluent control cells were co-transfected with the CYP3A4-PXC-luc reporter construct and wild type PXR (PXR wt, open bars) or a phosphorylation-deficient PXR variant (PXR S350A, closed bars). 24h post-transfection the cells were treated with 10μM rifampicin (Rif.) or DMSO vehicle. The CYP3A4 promoter activity levels were analyzed 24h after the addition of rifampicin. The results are shown as mean ± S.D. for 3 experiments. Significant differences in activity levels between wild type PXR and PXR S350A are shown as †† $p < 0.01$. The rifampicin treated cells (+) were compared to vehicle treated cells (-), ***$p < 0.001$.

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Figure 10. Proposed mechanism for the involvement of CDK2 in the PXR dependent regulation of CYP3A4 in Huh7 cells.

In the non-confluent proliferating Huh7 cells the levels of active CDK2 are increased, which results in increased levels of phosphorylated PXR. Phosphorylation of PXR affects ligand binding and cytosol-nucleus translocation. Phosphorylation may also target PXR to be degraded and, thus, subsequently the transcriptional activation of CYP3A4 is reduced. In the confluent cells, cellular proliferation is inhibited and cell cycle is arrested, resulting in decreased levels of active CDK2. Unphosphorylated PXR can now be activated, possibly by an endogenous ligand or by other processes, and translocated to the nucleus. Here it heterodimerizes with RXR and bind with cofactors to the regulatory elements of the *CYP3A4* gene, resulting in increased expression of CYP3A4. For further details see text.

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TABLES

Table 1. Up-regulated pathways in confluent cells. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significantly expressed ($p < 0.05$) genes that were up-regulated in 4 weeks confluent cells compared with control cells. The percentage of involved genes compared to total genes is also shown for every pathway.

KEGG Id	Pathway	Count	%	P-Value
hsa00982	Drug metabolism	25	2.2	1.38E-15
hsa00980	Metabolism of xenobiotics by cytochrome P450	22	1.9	9.11E-13
hsa00830	Retinol metabolism	18	1.6	6.81E-10
hsa00983	Drug metabolism	16	1.4	1.74E-09
hsa00140	Steroid hormone biosynthesis	16	1.4	5.25E-09
hsa04610	Complement and coagulation cascades	17	1.5	4.81E-07
hsa00500	Starch and sucrose metabolism	12	1	6.87E-06
hsa00053	Ascorbate and aldarate metabolism	8	0.7	9.30E-06
hsa00040	Pentose and glucuronate interconversions	8	0.7	1.51E-05
hsa00150	Androgen and estrogen metabolism	10	0.9	8.84E-05
hsa04742	Taste transduction	11	1	3.17E-04
hsa02010	ABC transporters	10	0.9	4.55E-04
hsa00591	Linoleic acid metabolism	8	0.7	5.49E-04
hsa00860	Porphyrin and chlorophyll metabolism	8	0.7	1.30E-03
hsa00590	Arachidonic acid metabolism	10	0.9	2.75E-03
hsa04612	Antigen processing and presentation	11	1	9.42E-03
hsa03320	PPAR signaling pathway	10	0.9	1.12E-02
hsa05332	Graft-versus-host disease	7	0.6	1.16E-02
hsa04512	ECM-receptor interaction	11	1	1.43E-02
hsa00380	Tryptophan metabolism	7	0.6	1.91E-02
hsa05330	Allograft rejection	6	0.5	3.03E-02

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Table 2. Down-regulated pathways in confluent cells. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significantly expressed ($p < 0.05$) genes that were down-regulated in 4 weeks confluent cells compared with control cells. The percentage of involved genes compared to total genes is also shown for every pathway.

KEGG Id	Pathway	Count	%	P-Value
hsa03010	Ribosome	62	2.7	3.91E-37
hsa03040	Spliceosome	60	2.6	1.11E-22
hsa03018	RNA degradation	26	1.1	1.72E-09
hsa04120	Ubiquitin mediated proteolysis	36	1.6	4.55E-06
hsa03050	Proteasome	17	0.7	2.81E-05
hsa03020	RNA polymerase	13	0.6	4.34E-05
hsa00230	Purine metabolism	37	1.6	4.86E-05
hsa04114	Oocyte meiosis	29	1.3	5.51E-05
hsa04110	Cell cycle	31	1.3	8.77E-05
hsa00970	Aminoacyl-tRNA biosynthesis	14	0.6	7.40E-04
hsa00240	Pyrimidine metabolism	23	1.0	1.76E-03
hsa03022	Basal transcription factors	12	0.5	2.03E-03
hsa03410	Base excision repair	10	0.4	1.70E-02
hsa00030	Pentose phosphate pathway	8	0.3	2.51E-02
hsa00450	Selenoamino acid metabolism	8	0.3	3.09E-02

Figure 1.

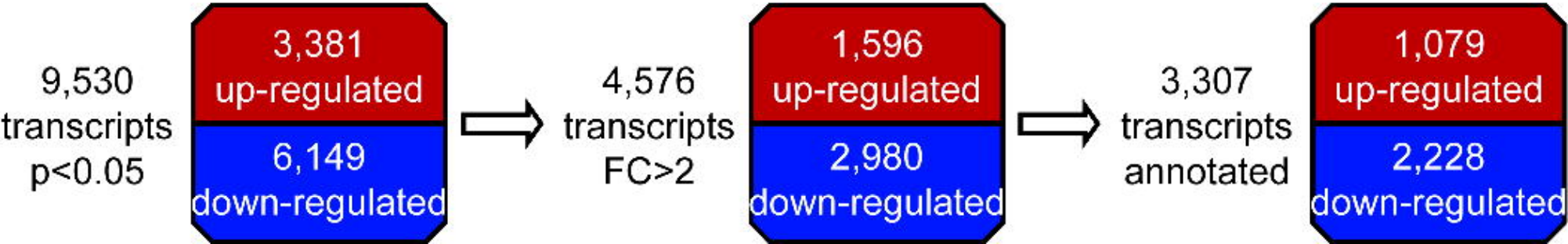
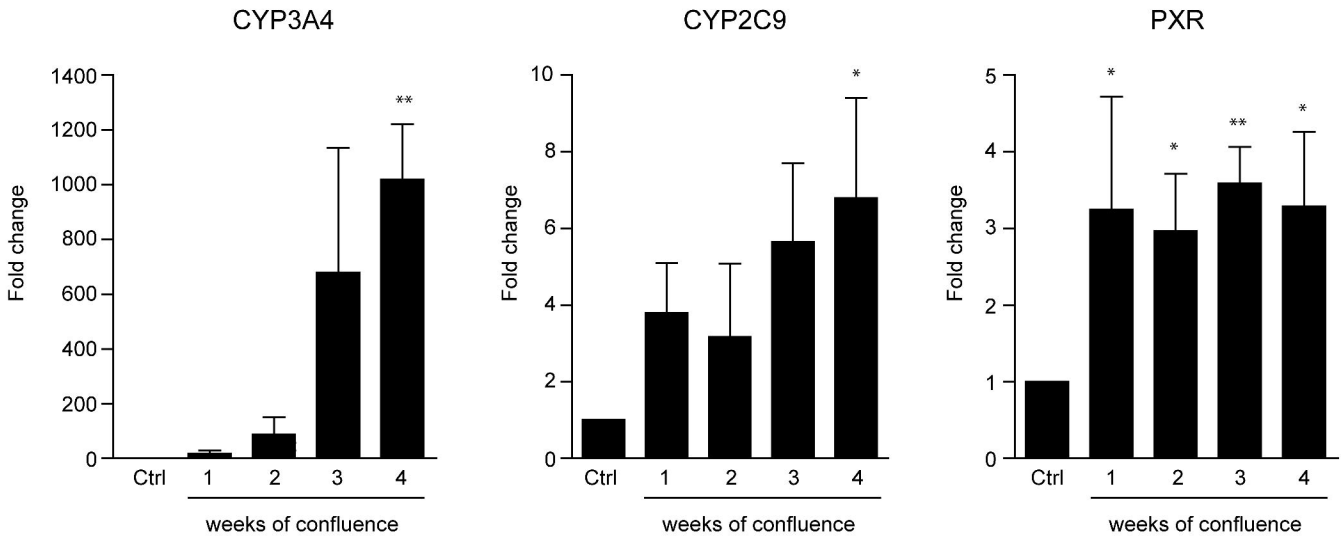
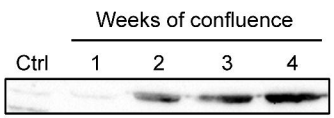

9,530
transcripts
p<0.05
3,381
up-regulated
6,149
down-regulated
4,576
transcripts
FC>2
1,596
up-regulated
2,980
down-regulated
3,307
transcripts
annotated
1,079
up-regulated
2,228
down-regulated

Figure 2.

A


CYP3A4
CYP2C9
PXR
Fold change
1400
1200
1000
800
600
400
200
0
10
8
6
4
2
5
3
1
**
*
Ctrl
1
2
3
4
weeks of confluence

B


Weeks of confluence
Ctrl
1
2
3
4

C

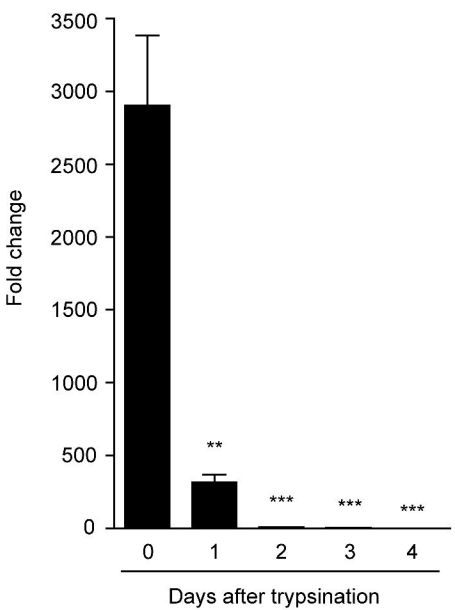

Fold change
3500
3000
2500
2000
1500
1000
500
0
**
***
0
1
2
3
4
Days after trypsination

Figure 3.

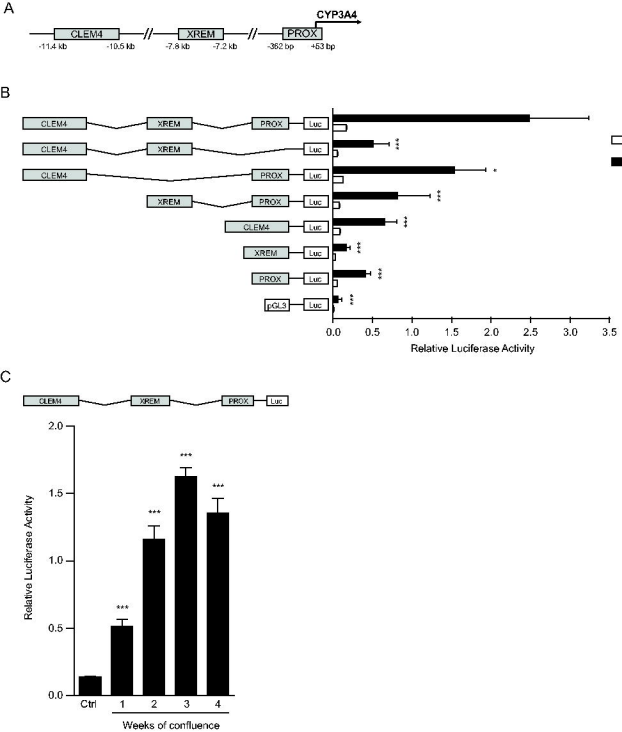

A
CYP3A4
CLEM4
XREM
PROX
-11.4 kb
-10.5 kb
-7.8 kb
-7.2 kb
-362 bp
+53 bp
B
CLEM4
XREM
PROX
Luc
pGL3
Control
Confluent
Relative Luciferase Activity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
C
Relative Luciferase Activity
Ctrl
1
2
3
4
Weeks of confluence

Figure 4.

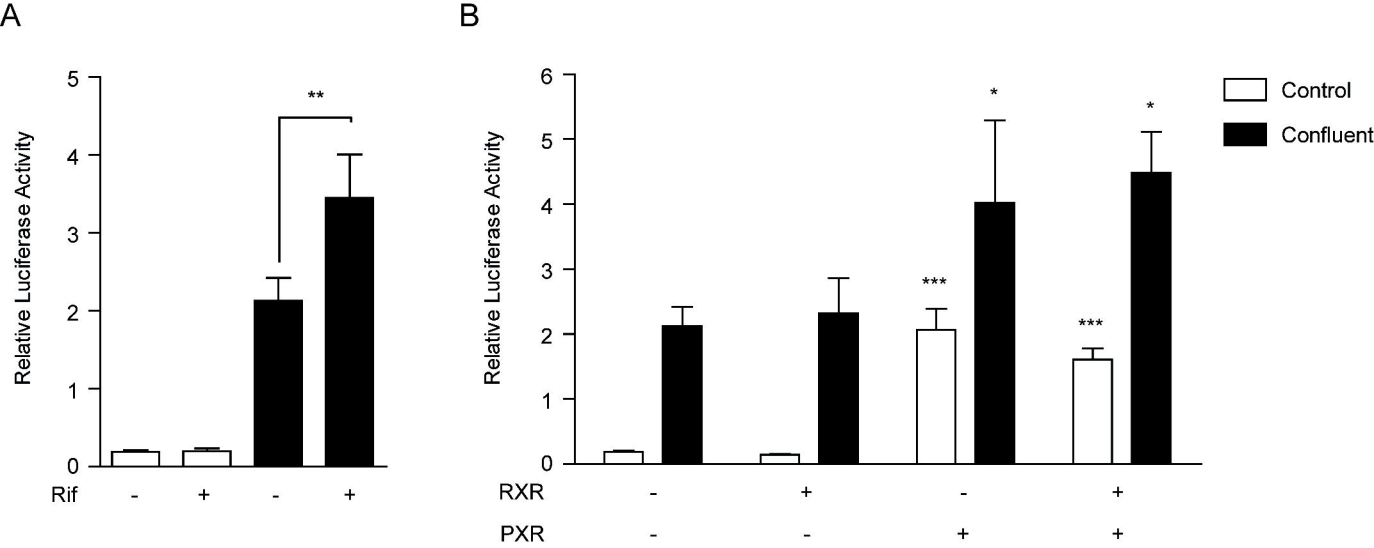

A
Relative Luciferase Activity
5
4
3
2
1
0
**
Rif
-
+
-
+
B
Relative Luciferase Activity
6
5
4
3
2
1
0
*
*
***
***
Control
Confluent
RXR
-
+
-
+
PXR
-
-
+
+

Figure 5.

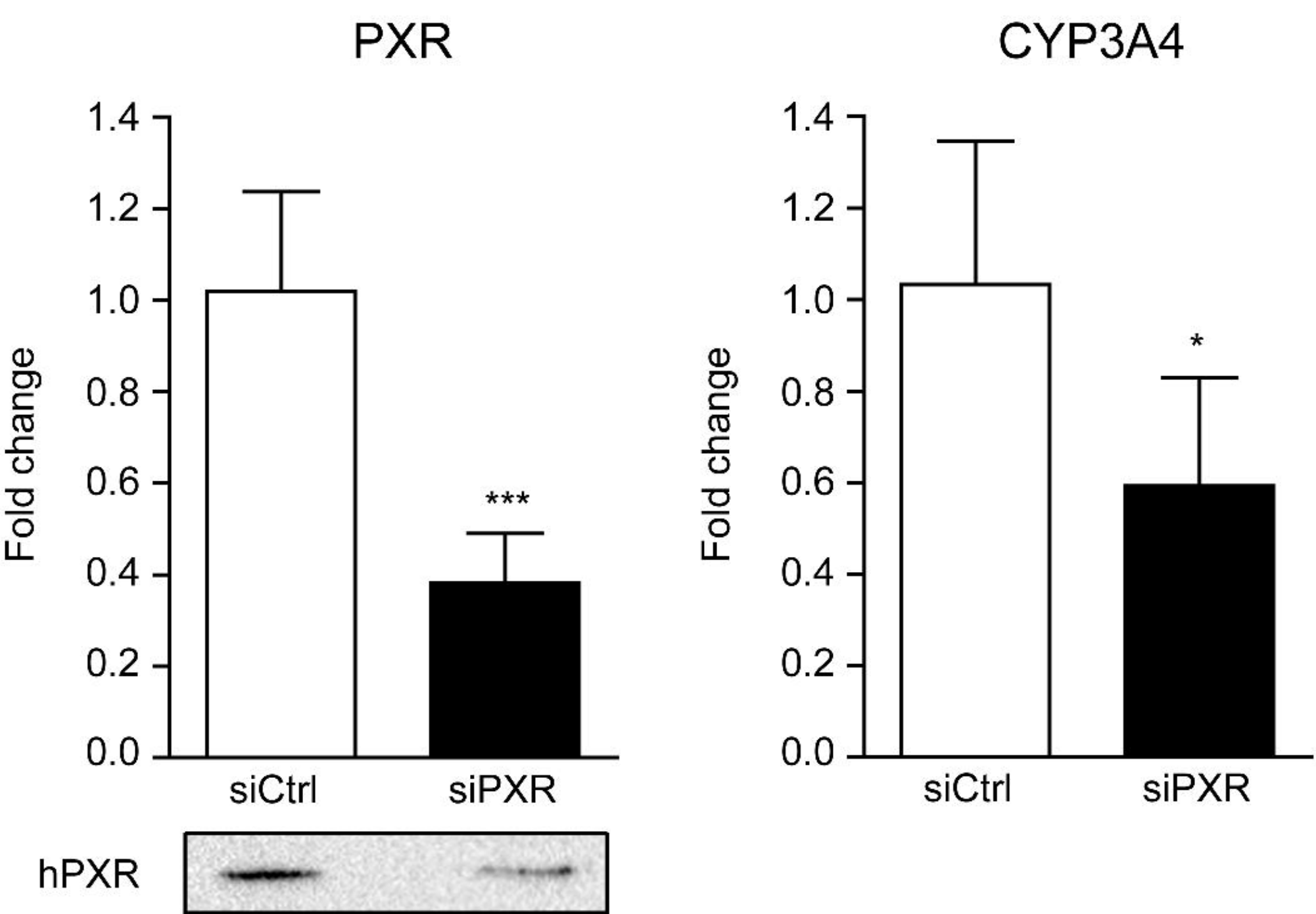
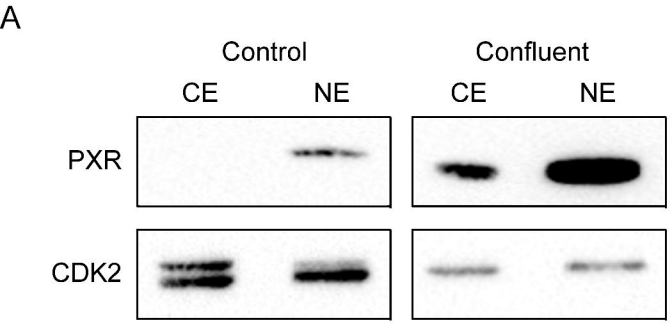

PXR
CYP3A4
Fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
*
siCtrl
siPXR
hPXR

Figure 6.


A
Control
Confluent
CE
NE
CE
NE
PXR
CDK2

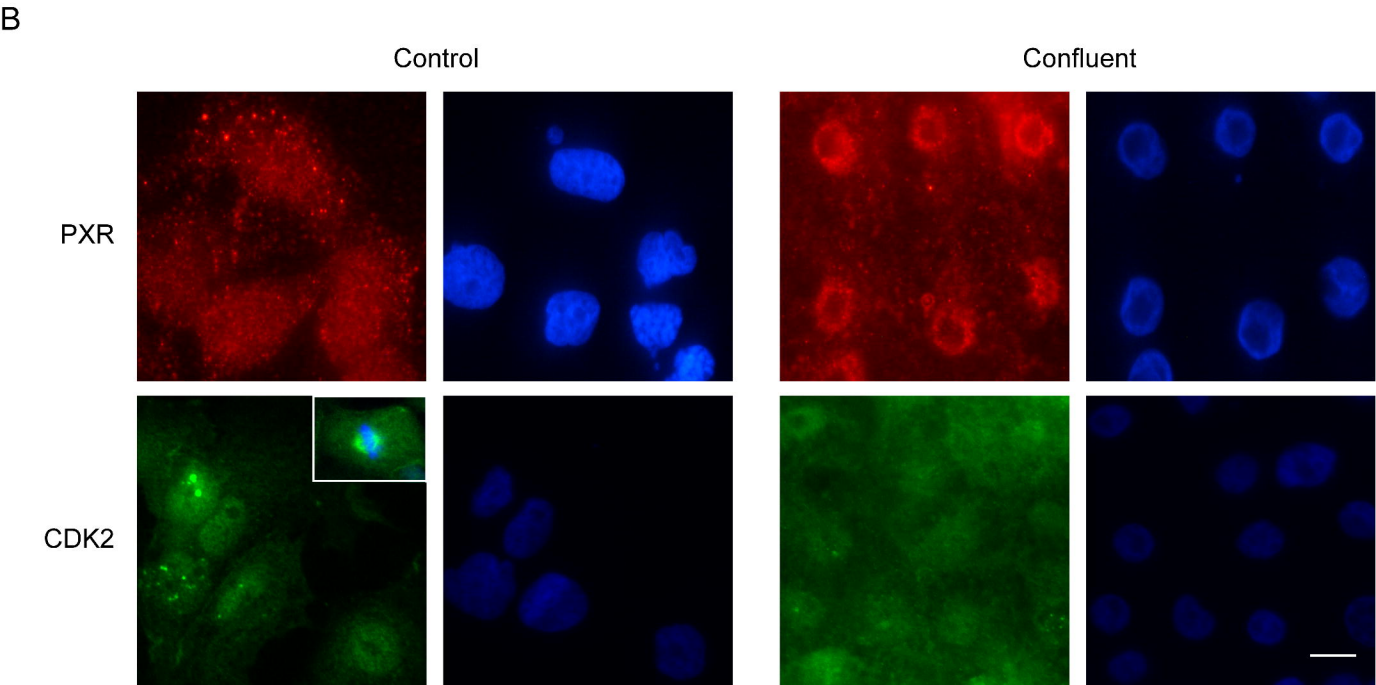

B
Control
Confluent
PXR
CDK2

Figure 7.

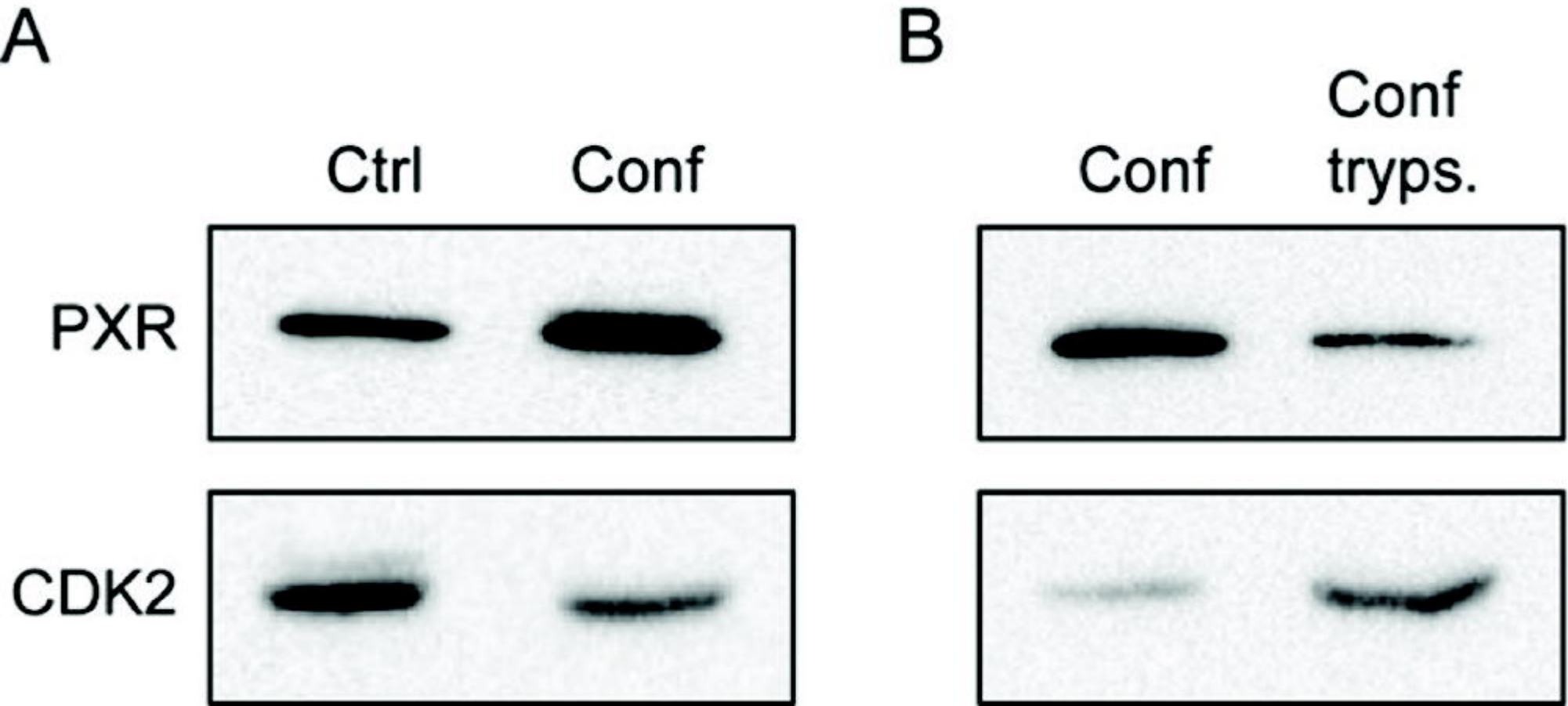
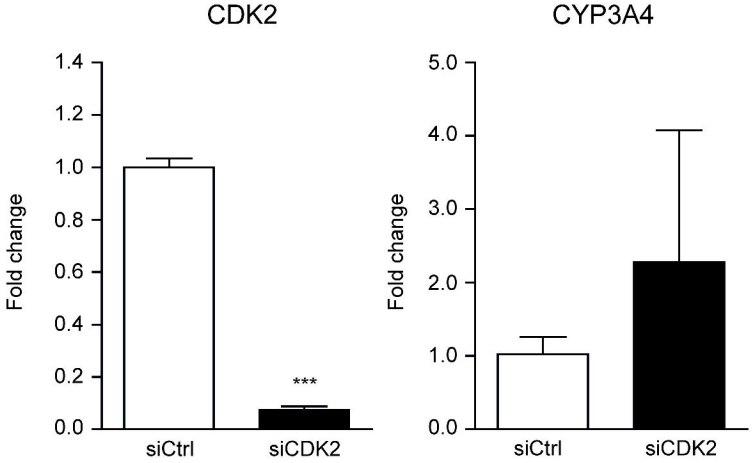
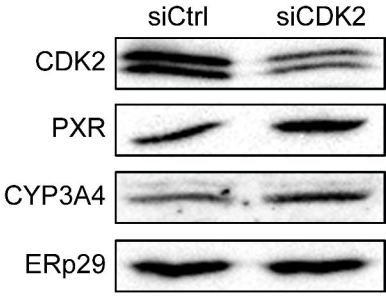


Figure 8.

A


CDK2
CYP3A4
Fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
siCtrl
siCDK2
5.0
4.0
3.0
2.0
1.0
0.0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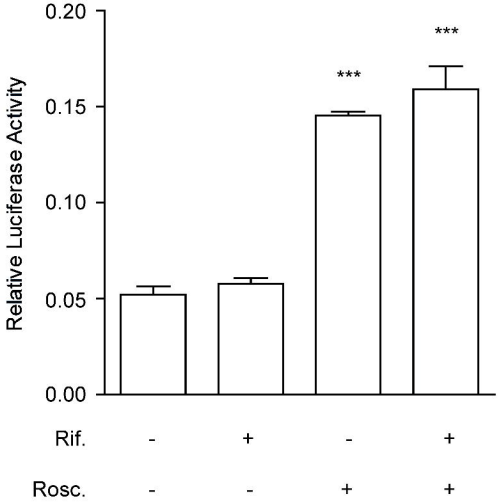
siCDK2
CDK2
PXR
CYP3A4
ERp29

B


Relative Luciferase Activity
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0.15
0.10
0.05
0.00
***
***
Rif.
Rosc.

Figure 9.

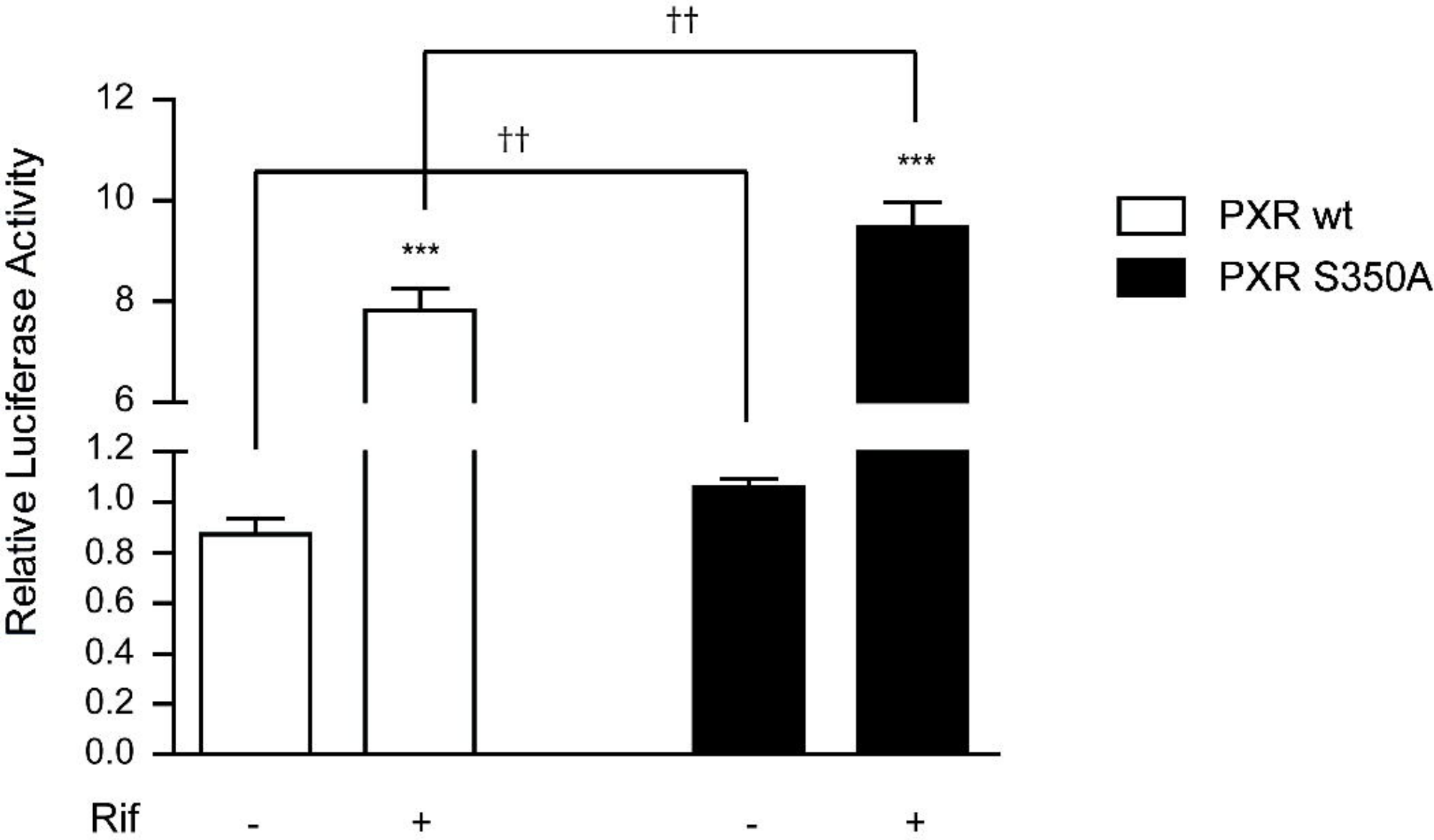
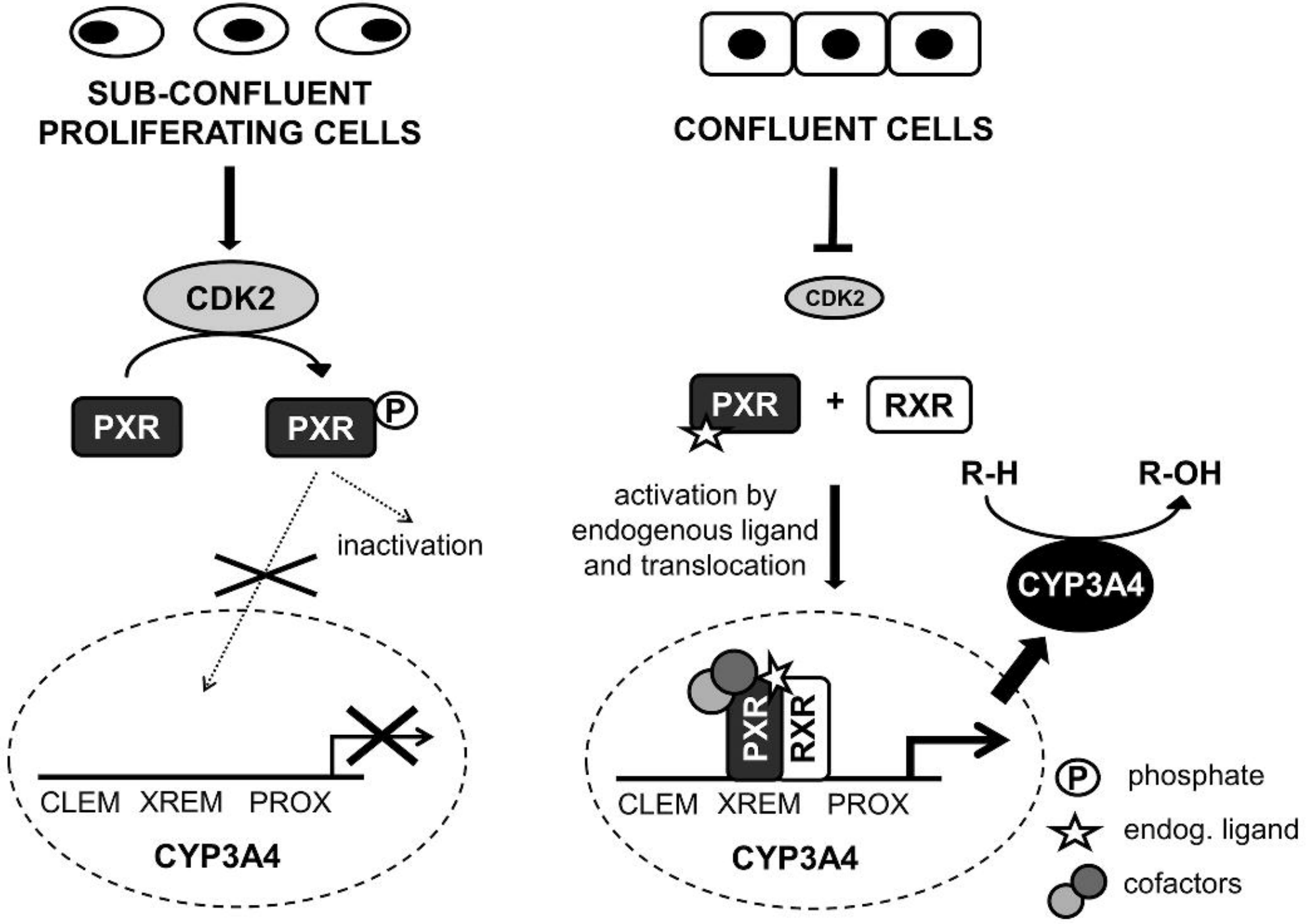

††
††
***
***
12
10
8
6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Luciferase Activity
PXR wt
PXR S350A
Rif
-
+
-
+

Figure 10.


SUB-CONFLUENT
PROLIFERATING CELLS
CDK2
PXR
PXR
P
inactivation
CLEM XREM PROX
CYP3A4
CONFLUENT CELLS
CDK2
PXR
+
RXR
activation by
endogenous ligand
and translocation
R-H
R-OH
CYP3A4
PXR
RXR
CLEM XREM PROX
CYP3A4
P
phosphate
endog. ligand
cofactors