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Ligand-Free Estrogen Receptor Alpha (ESR1) as Master Regulator for the Expression of CYP3A4 and other Cytochrome P450s (CYPs) in Human Liver*

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Running title: Ligand-free ESR1 as CYP3A4 master regulator

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Discussion: 1439

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; CYP, cytochrome P450s; ESR1, estrogen receptor alpha; FOXA2, forkhead box protein A2; gRNA, guide RNA; GTEx, Genotype-Tissue expression; HNF4A, hepatocyte nuclear factor 4 alpha; NR1I2, pregnane x receptor; NR1I3, constitutive androstane receptor; PGRMC1, progesterone receptor membrane component 1; PPARA, peroxisome proliferator activated receptor alpha; SSI, sobol's sensitivity Indices; TF, transcription factor

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Abstract:

CYP3A4 transcription is controlled by hepatic transcription factors (TFs), but how TFs dynamically interact remains uncertain. We hypothesize that several TFs form a regulatory network with nonlinear, dynamic, and hierarchical interactions. To resolve complex interactions, we have applied a computational approach for estimating Sobol's Sensitivity Indices (SSI) under generalized linear models, to existing liver RNA expression microarray data (GSE9588) and RNAseq data from Genotype-Tissue Expression (GTEx), generating robust importance ranking of TF effects and interactions. The SSI based analysis identified TFs and interacting TF pairs, triplets, and quadruplets involved in CYP3A4 expression. In addition to known CYP3A4 TFs, estrogen receptor alpha (ESR1) emerges as key TF with the strongest main effect and as the most frequently included TF interacting partner. Model predictions were validated using siRNA/shRNA gene knockdown and CRISPR-mediated transcriptional activation of *ESR1* in biliary epithelial Huh7 cells and human hepatocytes in the absence of estrogen. Moreover, ESR1 and known CYP3A4 TFs mutually regulate each other. Detectable in both male and female hepatocytes without added estrogen, the results demonstrate a role for unliganded ESR1 in CYP3A4 expression, consistent with unliganded ESR1 signaling reported in other cell types. Added estrogen further enhances ESR1 effects. We propose a hierarchical regulatory network for CYP3A4 expression, directed by ESR1 through self-regulation, cross regulation, and TF-TF interactions. We also demonstrate that ESR1 regulates the expression of other CYPs, suggesting broad influence of ESR1 on xenobiotics metabolism in human liver. Further studies are required to understand the mechanisms underlying role of ESR1 in CYP regulation.

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Significance Statement:

This study focuses on identifying key transcription factors and regulatory networks for CYP3A4, the main drug metabolizing enzymes in liver. We applied a new computational approach (Sobol's Sensitivity Analysis, SSI) to existing hepatic gene expression data to determine the role of transcription factors in regulating CYP3A4 expression and used molecular genetics methods (siRNA/shRNA gene knockdown and CRISPR-mediated transcriptional activation) to test these interactions in life cells. This approach reveals a robust network of TFs including their putative interactions, and the relative impact of each interaction. We find that ESR1 serves as a key transcription factor function in regulating CYP3A4, and it appears to be acting at least in part in a ligand-free fashion.

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Introduction:

Most abundant among all drug-metabolizing enzymes in the liver, CYP3A4 metabolizes 30-45% of commonly used drugs (Danielson, 2002). Large inter-person variability in both basal and induced CYP3A4 expression/activity (Achour et al., 2014; Rahmioglu et al., 2011) strongly influences optimal drug dosage, efficacy, and toxicity (Werk and Cascorbi, 2014), but the underlying causes remain largely unknown. Early studies had estimated that genetic factors constitute 80-90% of basal and 66% of induced CYP3A4 variability (Ozdemir et al., 2000; Penno et al., 1981; Rahmioglu et al., 2011), but genetic factors contributing to CYP3A4 variability remain poorly defined. The frequency of *CYP3A4* coding region variants is low (Wang and Sadee, 2012). We have identified a *cis*-acting regulatory intronic variant, named *CYP3A4*22* (minor allele frequency 5% in Europeans) , which reduces CYP3A4 expression *via* aberrant splicing (Wang and Sadee, 2016) and has been included in clinical biomarker panels predicting CYP3A4 activity (Wang et al., 2011). However, *CYP3A4*22* cannot account for a large portion of CYP3A4 variability that likely involves *trans*-acting transcription factors (TFs) and epigenetic processes.

Transcription of *CYP3A4* genes, located in a cluster of *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*, is regulated by dynamic interactions of numerous TFs (Istrate et al., 2010; Jover et al., 2001; Martínez-Jiménez et al., 2007; Ourlin et al., 1997; Rodríguez-Antona et al., 2003; Thomas et al., 2013; Tirona et al., 2003). While genetic TF variants can affect *CYP3A* expression in *trans*, the effect size of each single TF variant is limited by the complex interplay between several TFs and environmental conditions that impact TF expression. Thus, TF-TF interactions and epistasis between TF variants need to be considered to account for *CYP3A4* variability. A previous study has proposed that the combined effect of sex and genetic variants in ABCB1 and

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several TFs accounts for 24.6% of the variation in hepatic CYP3A4 expression, using multiple linear regression analysis (Lamba et al., 2010), but the p value increased with each predictor added to the model, and the final model is not significant (p=0.1). Another study (Yang et al., 2010), incorporating drug metabolizing activity, whole genome expression, and SNP data from 466 human livers, provides a comprehensive view of the functionality, genetic control and interactions of TFs and CYPs; yet, specific roles of each TF and dynamic interactions with other TFs remains unclear. Specifically, expression levels of ESR1 and other known CYP regulators (like NR1I2, NR1I3, HNF4A, etc.) were correlated with CYP3A4 and other measured enzyme activity levels, but these TFs failed to be included in higher order regulatory network (Yang et al., 2010). The possibly reason might be that conventional methods failed to control for the correlation between TFs (the input variables) that are known to be dependent on each other because of cross-expression regulation (Odom et al., 2004). This raises the question of the validity of traditional co-expression based methods to predict functional interactions. To overcome this issue, we have developed a novel computational approach for estimating Sobol's Sensitivity Indices (SSI) under generalized linear models (Lu et al., 2017), the potential utility of which was illustrated with computed network of TFs regulating CYP3A4 expression. SSI provides a unified way of quantifying output sensitivity with respect to any subset of the input variables, based on the variance decomposition of the response variable with applications in analyzing various complex systems (Chastaing et al., 2012; Chastaing et al., 2015; Li et al., 2010; Mara and Tarantola, 2012; Xu and Gertner, 2008). Unlike weighted co-expression network algorithm (Zhang and Horvath, 2005) that use the absolute value of pairwise expression correlation to define gene expression similarity, SSI analysis allows us to build the co-expression network using relatively smaller dataset (fewer parameters need to be estimated), and potentially

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avoid accumulating analytic biases introduced from piling up multiple different modelling techniques in a pipeline. Compared to other multivariate regression and p-value based analyses, our SSI based analysis has these features: (1) It can generate robust importance ranking of the input variables without requiring first to develop a model of the complete dynamic system. (2) It can tolerate irrelevant inputs without affecting the importance ranking of other relevant inputs. (3) It allows for dependence among input variables. Thus, SSI is well suited for analyzing TF-CYP3A4 interactions with an observational dataset of modest size. Moreover, our simulation studies show SSI based analysis outperforms ANOVA p-values and multivariate regression p-values in feature selection (Lu et al., 2017).

In this study, we applied SSI analysis to existing liver gene expression microarray data (GSE9588) (Yang et al., 2010), as used in our preliminary SSI analysis (Lu et al., 2017), and RNA-seq data from Genotype-Tissue Expression (GTEx) (Battle et al., 2017), to test key TFs and their interactions critical for CYP3A4 expression. Then we used gene knockdown or CRISPR-cas9 mediated TF gene transcriptional activation to evaluate predicted effects of TFs on CYP3A4 expression. The results define TF interactions and support the hypothesis that unliganded ESR1 is a master regulator among several other TFs of CYP3A4. As these TFs also affect other CYP enzymes in human liver, we extended the analysis to determine other CYPs that are similarly affected by ESR1 and their TF network.

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Materials and Methods:

Sobol's sensitivity indices (SSI) analysis (Lu et al., 2017) of TF interactions with CYP3A4: The mRNA data sets used are published microarray data (GSE9588) from 427 livers (Yang et al., 2010) and RNAseq data from GTEx (153 livers) . We selected 45 liver enriched transcription factors (TF) (Yang et al., 2010). Seventy-eight probes were used in microarray data, since some of the TFs were measured by more than one probe. In the first order analysis, we estimated Sobol's indices of each single TF by fitting a univariate polynomial model of degree 3. The main effect Sobol's indices were estimated by empirical variances of the best fitting polynomial expressions. Ranking of these Sobol's indices can be interpreted as ranking the proportion of CYP3A4 (or other CYPs) variability that can be explained by individual candidate TFs. In the second-order analysis (test the interactions between two TFs), we estimated the main effect Sobol's indices of TF pairs by fitting full cubic polynomial model that contains all pairwise product terms in addition to the terms used in the first-order analysis. A similar method was used for third-order analysis (test the interactions between three TFs). For fourth-order analysis (test the interactions between four TFs), because the total number of possible TF quadruplets is too large (>1.4 million), we selected only TFs that appeared in the top 200 triplets for fourth-order analysis (total number of possible quadruplets is 194,580). For the detailed SSI method description, please refer to Lu et al *(Lu et al., 2017).* The relevant functions for SSI estimation under GLMs with identity, log, and logit links are implemented and available in the R package 'Sobol sensitivity' (CRAN repository, also see Supplemental Methods for the link to R script).

Cell culture conditions: Experiments were carried out in two cell types expressing CYP3A4, Huh7 from biliary epithelial cells and primary human hepatocytes. Primary human hepatocytes (Lonza, see Supplemental Table 1 for demographics) were incubated in serum-free William's E

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media supplemented with penicillin/streptomycin/fungizone (100 U/100 µg/0.25µg per ml), 100 nM dexamethasone, 2 mM L-glutamine, 15 mM HEPES and ITS (0.55 mg/ml human transferrin, 1 mg/ml bovine insulin and 0.5 µg/ml sodium selenite, from Sigma). Cells were on Matrigel Basement Membrane Matrix to create a 2D culture condition. Hepatocytes from all 6 donors were used for estrogen-stimulation experiments, while hepatocytes only from donor Hum-1, Hum-3 and Hum-6 were used for siRNA experiments. Huh7 cells were cultured at 37°C in a humidified incubator at 5% CO_2 in DMEM supplemented with 10% charcoal-stripped serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin.

Gene knock down of ESR1 and select TFs with shRNA and/or siRNA: shRNA targeting ESR1, NR1I3 and PGRMC1 were designed as reported (Huang and Sinicrope, 2010) (Supplemental Table 2). DNA fragments were inserted into Lentiviral based shRNA vector PSIH1 (Addgene, ID#26597) using BamH1 and EcoRI sites. Lentiviral particles were prepared as described (Wang et al., 2015) and transduced into Huh7 cells. Cells were harvested 72h later. Accell siRNAs targeting ESR1 was purchased from GE Healthcare Dharmacon (Accell siESR1 SMART pool) (Dharmacon, IL, USA). Silencer siRNAs targeting ESR1 (#4098), FOXA2 (#5941), NR1I2 (#6638), HNF4A (#290203), PGRMC1 (#108079), NR1I3 (#5535), PPARA (#5348), and non-targeting control were purchased from ThermoFisher (ThermoFisher, CA, USA). Accell siRNAs were introduced into Huh7 cells by incubating with 1 µM siRNA in the delivery medium. For Silencer siRNAs, lipofectamine RNAiMAX reagent was used. After incubation for 72h (Huh7 cells) or 6 days (hepatocytes), cells were harvested for total RNA preparation, reverse transcription and real-time PCR quantification. shESR1 stable cell lines were established using puromycin selection.

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CRISPR-mediated gene transcription activation: By fusing VP64, the universal transcription activator, with an inactive mutant cas9 protein, dcas9, the dcas9-VP64 fusion protein specifically activates gene transcription when tethered by gRNA to a target gene promotor (Konermann et al., 2015). We used lentiviral based vectors, Lentiviral-VP64-dcas9 (addgene #61425) and LentisgRNA vector (Addgene #61427), for VP64-dcas9 fusion protein and gRNA delivery. gRNA sequences were from (Konermann et al., 2015) (Supplemental Table 2), three gRNA for each promoter of the TFs listed in Supplemental Table 2. Three promoter domains were targeted for ESR1, isoforms 001 (NM_000125), 201(NM_001122742), and 202 (NM_001122740). These isoforms are ubiquitously expressed, share the same coding sequence but differ at their 5' UTR. For transient expression, lentiviral particles containing expression vectors for VP64-dcas9 and a mixture of three gRNA targeting a specific gene promoter were incubated with Huh7 in the presence of 8μg/ml SureEntry transduction reagent (Qiagen) for 24hrs. Cells were harvested 72hrs post transduction. Huh7 cell lines stably expressing VP64-dcas and gRNA targeting ESR1 promoters were established by blasticidin and zeocin double selection.

RNA preparation and RNA expression analysis: Total RNA was prepared using RNA mini prep kits from Zymos Research. RNA was reverse transcribed into cDNA using RTIII reverse transcriptase (Life Tech) and measured using real time PCR with specific primers (Supplemental Table 2) and SYBR Green PCR master mix (Life Technologies) using GAPDH as internal control (Wang et al., 2011). Human liver samples were obtained from Cooperative Human Tissue Network (CHTN) (see Supplemental Table 3 for demographics of liver donors).

CYP3A4 protein analyses using capillary western blot technology: Human hepatocytes in 6-well plates were lysed with 100 μl RIPA lysis buffer supplemented with protease inhibitor cocktail

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(Roche, South San Francisco, CA, USA). Total protein concentrations were measured using Bradford method (Thermofisher). Capillary Western analyses were performed using the Protein Simple Jess system (Biotechne, California, USA) according to manufacturer's protocol. Briefly, cell lysates were diluted with 0.1 x sample buffer to concentration of 1 mg/ml. Then 4 parts of diluted samples were combined with 1 part 5 x Fluorescent Master Mix (containing 5 x sample buffer, 5 x fluorescent standard and 200 mM DTT) and heated at 95°C for 5 min. Then the denatured samples, blocking reagent, mouse anti-CYP3A4 antibody (clone 946002, RD system, Minneapolis, MN, USA, at 1:50 dilution), rabbit anti-β-actin antibody (ab8227, abcam, Cambridge, MA, USA, 1: 50), HRP-conjugated anti-mouse secondary antibody (1:20), fluorescent-conjugated NIR anti-rabbit secondary antibody (1:20) and chemiluminescent substrate were dispensed into designated wells in an assay plate. A biotinylated ladder provided molecular weight standard for each assay. After plate loading, the separation, electrophoresis and immunodetection steps take place in the fully automated capillary system.

Data analysis: Data are expressed as mean ± SD. Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA, USA).

University of Florida Biosafety Committee and IBR Committee approved the lentivirus study and human tissue study, respectively.

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Results:

1. Sobol's sensitivity indices (SSI) analysis identifies ESR1 as a main regulator of *CYP3A4* expression:

We used published microarray data (GSE9588) from 427 livers and selected 45 liver-enriched candidate TFs (Yang et al., 2010) for the SSI analysis. Some TFs were measured by more than one probe. First order analysis identifies 10 TFs with the highest SSI values (Fig1A&1B). As expected, these TFs are similar to previous results with regression analysis (Yang et al., 2010) using the same data set Two probes (both target coding regions) for ESR1 scored with the highest first order total effect index and the lowest p value (Fig 1A & 1B).

As the RNA datasets include liver tissues from both male and female donors, we tested whether the role of ESR1 was driven by female livers; however, ESR1 is the strongest predictor for CYP3A4 expression regardless of the sex of the donors. Subsequent higher order analysis, testing interactions between two, three or four TFs, identifies interacting pairs, triplets and quadruplets critical to CYP3A4 expression (Fig1C&D, Fig 2). Again, ESR1 appears most frequently in the top 1000 interacting pairs and triplets (Figure 1C & 1D) with the strongest main effects (Fig 2), suggesting ESR1 coordinates CYP3A4 expression by interacting with other TFs. Most of the TFs identified in first order analysis were consistently identified also in the higher order network analysis. This is in contrast to previous study (Yang et al., 2010) where TFs identified in regression analysis failed to be the top regulator or hub genes in network analysis. To replicate this result, we downloaded RNAseq data from GTEx. Because of the smaller sample size (153 livers), the full higher order SSI analysis is not feasible; we applied first order analysis to select the best predictors. Again, ESR1 emerges as a predictor for CYP3A4

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expression (Table 1), consistent with the result from microarray data. Moreover, NR1I3, NR1I2, and FOXA2 are also recognized as regulators of CYP3A4 in GTEx data.

2. Test SSI results using gene knockdown (KD) and CRISPR-mediated gene transcriptional activation (TA):

(a) ESR1 KD and TA: We used three different shRNAs to knockdown ESR1 in Huh7 cells (see Supplemental Table 2 for shRNA sequence). All three shRNAs reduced ESR1 expression to 17-40% of negative control levels, resulting in >60% reduction of CYP3A4 expression (Figure 3A). To replicate this result, we used siRNA. Similar to shRNA, siRNA from two different sources (Silencer and Accell siRNA) both decreased ESR1 mRNA levels ≥50%, leading to ~50% reduction in CYP3A4 expression (Supplemental Figure 1). To test the long-term effect of ESR1 KD on CYP3A4 expression, we established stably transfected cell lines using shRNA. Compared to negative controls (Huh7-shNC), two shESR1 Huh7 cell lines (Huh7-shESR1-C1 and Huh7-shESR-C2) expressing lower ESR1 levels also have substantially lower CYP3A4 expression (Fig3B). As a positive control, we also tested the expression level of RARA, a nuclear receptor previously shown to be responsive to ESR1 deletion in MCF7 cells (Caizzi et al., 2014). As expected, RARA expression decreased upon ESR1 KD.

For TA experiments, we used CRISPR-mediated gene transcriptional activation (TA) to increase ESR1 expression in Huh7 cells. We designed TA gRNA targeting three ESR1 promoters (Supplemental Table 2) that drive the ubiquitous expression of three ESR1 isoforms, ESR1-201 (ENST00000206249), ESR-207 (ENST00000440973) and ESR1-208 (ENST00000443427), respectively. These three ESR1 isoforms encode the same ESR1 protein but differ in their 5'UTR region. Compared to negative control (TA-NC), TA gRNA targeting different ESR1 promoters increased ESR1 expression from 3- to 67-fold (Fig 3C). All these conditions

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consistently increased CYP3A4 expression up to 3-fold regardless of the wide range of increases in ESR1 expression (Fig 3C). Similar results were obtained with two TA gRNA expressing stable cell lines (Huh7-TA-ESR1-C1 (ESR1-201) and Huh7-TA-ESR1-C2 (ESR1-208)), in which ESR1 mRNA levels increased 7- and 46-fold, while CYP3A4 expression increased 2- and 3-fold, respectively (Fig 3D). These results demonstrate a key role of ESR1 in CYP3A4 expression. Given the low expression of CYP3A4 mRNA in Huh7 compared to hepatocytes, it is possible that a 3-fold increase represents a ceiling level in Huh7 cells. Alternatively, ESR1 has many splice isoforms (Flouriot et al., 2000; Perlman et al., 2005; Poola et al., 2000), some of them displaying constitutive or dominant negative activities (Flouriot et al., 2000; Hattori et al., 2016). Different ESR1 expression profiles in Huh7 cells compared to human liver may limit its *trans*-activating effects on CYP3A4 expression. Indeed, Huh7 with TA-ESR1 expresses many ESR1 splice variants missing different coding exons, for example, exon 2, exon 4, exon5 and exon 6. These exon-missing variants encode either non-functional protein or C-terminal truncated ESR1 proteins (Hattori et al., 2016). Therefore, ESR1 TA may not generate sufficient levels of functional ESR1 protein. Moreover, it is unclear whether those C-terminal truncated isoforms have different *trans*-activity or dominant negative effects on full-length ESR1. The finding that expression of the positive control RARA also increased upon all TA experiments to a similar extent as CYP3A4 supports this notion. To test whether ESR1 regulates CYP3A4 expression in human primary culture hepatocytes, we treated hepatocytes cultured on Matrigel overlay (2D culture) with ESR1 siRNA for 6 days. This reduced ESR1 expression over 80%, and decreased CYP3A4 expression ~50% (Fig 4A), consistent with results from Huh7 cells. The expression of RARA mRNA also decreased to 65% with ESR1 siRNA (mean ± SD, 65.9 ±

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9.3%, $p<0.05$). Consistently, average CYP3A4 protein expression also reduced to 55% of control (mean ± SD, 54.8 ± 6.9%) after ESR1 KD (Fig 4B).

(b) KD and TA of other TFs: SSI analysis identifies PGRMC1 as potential TF in CYP3A4 expression, directing a small network independent of ESR1 in the microarray data set (Fig 2). KD of PGRMC1 using shRNA or siRNA (over 70% reduction) or TA of PGRMC1 expression (up to 8-fold) with TA gRNA did not change CYP3A4 expression (Fig 5), indicating PGRMC1 does not regulate CYP3A4 expression. Other TFs that repeatedly appear as CYP3A4 regulators in SSI analysis, for example NR1I3, NR1I2, and FOXA2, are known CYP3A4 transcriptional regulators. Manipulation by KD and TA of the expression of these TFs, all significantly changed the expression of CYP3A4 as expected (Fig 5). PPARA is a known CYP3A4 regulator, and KD PPARA in hepatocytes reduced CYP3A4 expression (Klein et al., 2012). Although not detected in the first order analysis, PPARA appears in network analyses (Fig2) and frequently interacts with ESR1 tripletes (Fig1d), indicating PPARA is part of the ESR1 network. Consistent with previous results, KD PPARA reduced CYP3A4 expression (Fig 5).

3. Cross-regulation between ESR1 and other key TFs: We then test whether changes in ESR1 expression affect the expression of TFs tested in this study or other known CYP3A4 regulators. KD of ESR1 in Huh7 (76% reduction in ESR1) decreased PGRMC1, FOXA2, NR1I2, and PPARA expression (Fig 6A). Similarly, KD of ESR1 in primary culture hepatocytes (82% reduction in ESR1) decreased expression of PGRMC1, FOXA2, NR1I2, PPARA, and HNF4A (Fig 6B), while it had no effect on NR1I3 in either cell type. Consistent with KD experiments, TA of ESR1 in Huh7 (15 fold increase in ESR1(ESR1-201 isoform)) increased the expression of PGRMC1, FOXA2, NR1I2, PPARA, and HNF4A (Fig 6C), but not NR1I3. On the other hand, KD or TA of PGRMC1, FOXA2, NR1I3, NR1I2 (see Fig 5 A&C for changes in the expression

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of these TFs) had no effects on ESR1 expression (Fig7). KD PPARA (68% reduction) reduced ESR1 expression, while TA PPARA or HNF4A (5 and 4-fold increase in PPARA and HNF4A, respectively) increased ESR1 expression, indicating cross regulation between ESR1 and other TFs.

4. Effects of estrogen on the expression of ESR1 and CYP3A4: One of the important features of master regulator (or core regulator) is self-regulation for sustainable expression. To test whether hepatocyte ESR1 can be regulated by its own ligand, estrogen, we treated hepatocytes with 1μM 17-beta-estradiol (E2) every 6 hr as reported (Choi et al., 2013) to maintain an effective level of E2 inside hepatocyte, since E2 is actively metabolized by CYP3A4. We measured a hepatic specific ESR1 isoform that is driven by a promoter ~150kb upstream of the canonical promoter for ESR1 (Flouriot et al., 1998). In all hepatocytes tested, hepatic specific ESR1 isoform mRNA increased after E2 treatment (Fig 8). The changes in ESR1 expression are more profound in hepatocytes from male than from female donors (two-way ANOVA, $P<0.001$). CYP3A4 mRNA increased substantially after ESR1 treatment only in cells from male donors (Fig 8). Different effects of estrogen treatment between male and female donors could have resulted from increased basal levels of ESR1 in females (Flouriot et al., 1998).

5. Regulation of other CYPs by ESR1: We then asked whether ESR1 regulates the expression of other CYPs. We used first order SSI analysis to scan the predictors for additional 11 CYPs using microarray and GTEx data sets. Shown in Table 1, ESR1 appears as regulator for CYP3A5, CYP3A7, CYP2C9, and CYP2C19, but not for CYP2D6, CYP1A1, CYP1A2 and CYP1B1 in both microarray and GTEx data sets. Consistently, KD of ESR1 (~80% reduction) in hepatocytes decreases expression of CYP3A5, CYP3A7, CYP2C9, and CYP2C19, while it did not change the expression of CYP2D6, CYP1A1, CYP1A2, and CYP1B1 (Fig 9). However, KD

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of ESR1 also reduced the expression of CYP2A6 even though SSI analysis failed to identify clear predictors for CYP2A6 in both data sets (Table 1 and Fig 9).

Consistent with the KD results, in a cohort of 84 livers, the expression levels of CYP3A4, 3A7, 2C9 and 2C19 are strongly correlated with that of liver specific ESR1, while the expression levels of CYP2D6 and CYP3A43 are not (Table 2). The expression level of CYP3A5 is not correlated with ESR1 in the whole cohort, probably due to the frequent genetic variant CYP3A5*3 which causes aberrant splicing and non-sense mediated RNA decay. After excluding CYP3A5 non-expressors (CYP3A5*3 homozygous carriers), the level of CYP3A5 is strongly correlated with ESR1 as expected (Table 2).

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Discussion:

Using Sobol's Sensitivity Indices (SSI) analysis, we have identified ESR1, as well as several TFs (FOXA2, NR1I3, NR1I2) as regulators for CYP3A4 expression in human adult liver, an organ with sparse studies of estrogen receptor functions. While previous multivariate regression analyses have similarly identified several overlapping TFs, including ESR1 (Yang et al., 2010), the SSI approach provides robust quantitative assessments of ranking order, placing ESR1 as a main regulator. While FOXA2, NR1I3 and NR1I2 are known CYP3A4 regulators, a role for ESR1 in CYP3A4 expression had not been studied in detail previously. Moreover, SSI enabled evaluation of dynamic interactions between TFs, also highlighting ESR1 as interactive partner listed most frequently in TF doublets, triplets, and quadruplets, placing it on top of a hierarchical TF network. These relationships appear to apply to both males and females, consistent with a role for unliganded ESR1, which has been shown to bind to numerous domains throughout the genome (Caizzi et al., 2014). The results were tested experimentally using gene knock down (KD) (with shRNA and/or siRNA) and CRISPR-mediated transcriptional activation (TA). KD or TA ESR1 not only changed CYP3A4 expression, but also changed the expression of other CYP3A4 TF regulators. In turn, ESR1 expression was regulated by its own ligand, estrogen, and by other CYP3A4 TFs, namely PPARA and HNF4A. These results support a hierarchical regulatory network for CYP3A4 expression, directed by ESR1 involving self-regulation, cross regulation and TF-TF interactions. Moreover, SSI analysis and gene KD in hepatocytes also support a regulatory role of ESR1 in expression of additional drug metabolizing CYPs, suggesting broad regulatory functions in hepatic CYP expression.

Unliganded ESR1 as a master regulator for CYP3A4 expression?

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While ESR1 is known to mediate estrogen-induced expression of several CYPs (Choi et al., 2013; Go et al., 2015), a role for unliganded ESR1 had not been reported. In this study, with no estrogen added in KD or TA experiments, we further support a role of unliganded ESR1 (without estrogen) in CYP3A4 expression in hepatic cells, and also in the expression of several other CYPs (2A6, 2C9, 2C19, 3A5, 3A7) (Fig. 9). The results are consistent with recent studies demonstrating unliganded ESR1 regulates gene expression (Stellato et al., 2016) in other cell types. This conclusion is strengthened by ChIP-seq data showing binding of unliganded ESR1 to a subset of ESR1 binding sites associated with developmental functions in MCF7 cells (Caizzi et al., 2014). Moreover, unliganded ESR1 binding is facilitated by other TFs (Caizzi et al., 2014), some of which known to regulate hepatic CYP3A4 expression in liver, for example FOXA, HNF4α, and PPAR. These TFs were also identified here as interactive ESR1 partners using SSI analysis in this study. We therefore propose a hierarchical regulatory network for CYP3A4 expression centered on ESR1. ESR1 is situated at a high hierarchical level, interacting directly with FOXA2, HNF4A, NR1I2, and NR1I3. In contrast, TFs at lower hierarchical order in this network, *e.g.*, PPARA, interact with upstream high order TFs but do not interact directly with downstream partners. This is consistent with PPARA appearing more frequently in interacting triplets than in pairs (Fig. 1 C&D). These multilayered interactions could account for limited observed effects of genetic variants of single TF on the expression of CYP3A4 (Wang and Sadee, 2012).

Unliganded ESR1 as regulator of multiple CYPs

In addition to CYP3A4, our results show that ESR1 also regulates other cytochrome P450 drug metabolizing enzymes CYP2C9, CYP2C19, CYP3A4, CYP3A5, CYP3A7 (Fig 9), which share common regulators with CYP3A4, such as NR1I2, NR1I3, FOXA2, themselves interacting with

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ESR1 (Achour et al., 2014). The result supports the notion that ESR1 is situated at higher hierarchical order of regulatory networks. The involvement of ESR1 in CYPs expression is further supported by reduced hepatic expression of many cyps in the cyp3a, cyp2a, cyp2b, cyp2c and cyp2d families in ESR1 knockout mice (Bryzgalova et al., 2006), supporting broad functions of ESR1 in liver maturation, differentiation and tissue specific gene expression including CYPs.

Regulation of ESR1 by estrogen in human liver

While estrogen downregulates ESR1 in breast cancer MCF cells and reproductive tissues (Flouriot et al., 1998; Shupnik et al., 1989), our results showed estrogen upregulates liver specific ESR1 isoform (Flouriot et al., 1998), driven by a promoter ~150kb upstream of canonical ESR1 promoter, in hepatocytes. This result is consistent with the presence of ESR1 binding sites at this distal promoter that was shown to be activated by estrogen (Flouriot et al., 1998). Estrogen mediated ESR1 up-regulation was highly variable, with stronger effects in hepatocytes from male donors than female donors, possibly caused by different levels of estrogen pre-exposure between male and female and among different individuals. This result suggests ESR1 is self-regulated by its own ligand in liver, which is a common feature for core regulators (Saint-André et al., 2016), an important mechanism for their sustainable expression. The result also suggests that ESR1 is upregulated by estrogen in female liver, consistent with higher levels of both ESR1 (Flouriot et al., 1998) and CYP3A4 in female livers (Wang et al., 2011; Wolbold et al., 2003).

Application of SSI analysis

SSI analysis was applied to select regulators for CYP3A4 and additional 11 drug metabolizing enzymes using both microarray and GTEx data. Of twelve CYPs analyzed (Table 1), ESR1 repeatedly showed up as a regulator for five CYPs (CYP2C9, CYP2C19, CYP3A4, CYP3A5,

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CYP3A7) in both microarray and GTEx data sets. ESR1 KD in hepatocytes validated the regulatory roles of ESR1 in the expression of these five CYPs. Similarly, SSI analysis showed no clear regulators for CYP2D6, CYP1A1, CYP1A2 and CYP1B1 expression in both data sets, consistent with a lack of effect on expression of these CYPs with ESR1 KD. These results support a highly predictive value of SSI analysis when results are consistent in the two data sets, with one exception. Whereas SSI analysis failed to show ESR1 effects on CYP2A6, KD of ESR1 reduced CYP2A6 expression in hepatocytes. This experimental result is consistent with a recent study showing regulation of CYP2A6 by ESR1 (Kao et al., 2017) and in ESR1 knockout mice (Bryzgalova et al., 2006). Possibly, the discrepancy may have been caused by confounding factors, for example, *CYP2A6* copy number variation (gene duplication and deletion) and strong upregulation by CYP2A6 inducers (Fukami et al., 2007; Mwenifumbo et al., 2008). While the expression of CYP1 family members is upregulated by estrogen treatment (Go et al., 2015), expression of these CYPs was not affected by ESR1 KD, suggesting different regulatory roles of unbound ESR1 and estrogen bound ESR1. These results also indicate that any possible residual estrogen in the cell culture serum cannot account for the observed ESR1 mediated effect under current study conditions. This is consistent with no differences in CYP3A4 and ESR1 expression in Huh7 cells cultured with normal fetal bovine serum (10%) or charcoal-stripped serum (data not shown). These results further support the unliganded ESR1 effects observed in this study.

SSI analysis of PGRMC1

SSI analysis of the CYP3A4 microarray data revealed a small TF network independent of ESR1, centered around PGRMC1 (Fig 2). PGRMC1 is known to bind directly to CYP proteins including CYP3A4 and to modulate CYP activity (Oda et al., 2011) with multiple functions in

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substrate binding, steroid signaling, and more (Li et al., 2016; Rohe et al., 2009); however, its role in CYP expression had not been reported. Our KD or TA results suggested PGRMC1 does not play a role in transcriptional *CYP3A4* regulation. Since PGRMC1 expression level correlates with that of ESR1, we consider the positive result of SSI analysis may have been caused by this co-expression. Alternatively, since SSI analysis using GTEx data did not reveal PGRMC1 as predictor for CYP3A4 and other CYPs, it is also possible that a positive prediction for PGRMC1 is confounded by some artifacts in the microarray data set.

Taken together, we have identified unliganded ESR1 as a master regulator for transcriptional regulation of CYP3A4. ESR1 appears to regulate transcription of several other CYPs. Moreover, we demonstrate the utility of SSI analysis for predictor selection and for higher order regulatory network construction. Since ESR1 expression is regulated by multiple factors, *e.g.* diet (Cordero et al., 2013; Gao et al., 2012), drugs (Killer et al., 2009), malignancy (Hishida et al., 2013), oxidative stress (Mahalingaiah et al., 2015), genetics, and DNA methylation (Maekawa et al., 2016), the network of interacting ESR1 and other TFs identified in this study may serve as a guide for integrating the combined effects of genetic, epigenetic and non-genetic factors in TF coding genes on expression of CYPs, and in particular CYP3A4. This approach has potential to account for a substantial portion of genetic variability in the expression of drug metabolizing CYPs. Future studies will focus on testing the association between *ESR1* genetic variants and expression of CYP enzymes, and understanding the mechanisms underlying ESR1 regulation in liver, including ESR1 expression profiles, ESR1 genomic occupancy, physical interactions between ESR1 and other TFs, and the involvement of lncRNA and miRNA in ESR1 regulatory networks.

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Disclosures declaration:

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Author contributions:

Participated in research design: Wang and Sadee

Conducted experiments: Wang

Contributed new analytic tools: Rempala and Lu

Performed data analysis: Lu and Wang

Wrote or contributed to the writing of the manuscript: Wang, Lu, and Sadee

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Footnotes:

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Figure legends:

Figure 1. Influence of the top-scoring liver-enriched transcription factors (from a total of 45 selected TFs) obtained from Sobol's sensitivity indices (SSI) analysis, using microarray gene expression data from 427 livers. A & B, results from first order analysis, total effect indices (A) and p value (B); C & D, results from higher order analysis, frequencies of TFs appearing in the top 1000 interacting pairs (C) and triplets (D). TFs measured by more than one microarray probe yielded similar results.

Figure 2. Key transcription factors and their interactions affecting CYP3A4 expression identified with Sobol's Sensitivity Indices (SSI) analysis. Dot sizes represent the main effect of each TF on CYP3A4 expression. The bigger the dot size, the greater regulatory effect of that TF has on CYP3A4 expression. The lines represent the interactions between the connected TFs. The distance of the line indicates the effect size of interactions. The shorter the distance the higher the SSI value of the interaction. The main quadruples are formed by different combinations of critical TFs: ESR1, HNF4A, PPARA, NR1I2, NR1I3, NFE2L2, NCOR and THRB. TFs measured by more than one microarray probe yielded similar results.

Figure 3. Effects of ESR1 knockdown (KD, A & B) or ESR1 transcriptional activation (TA, C & D) on the expression of CYP3A4 in Huh7 cells. A. Transient ESR1 knock down using shRNA; B. Two stable ESR1 knockdown Huh7 cell line (Huh7-shESR1-C1 and Huh7-shESR1-C2) established using shRNA; C. Transient expression of ESR1 TA guide RNA; D. ESR1 TA guide RNA expression stable Huh7 cell lines (Huh7-TA-ESR1-C1 (201) and Huh7-TA-ESR1-C2 (208)). RARA serves as positive control for ESR1 KD or TA. Data are mean ± SD,

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Compared to negative control (NC), * P<0.05; ** P<0.01; *** P<0.001, ANOVA with Bonferroni: compare selected pairs post test

Figure 4. Effects of ESR1 knock down with silencer siRNA or Accell siRNA on the expression of CYP3A4 in primary culture hepatocytes form three donors. A. ESR1 and CYP3A4 mRNA expression. Compared to negative control (siNC), [a] P<0.001; [b] P<0.01; [c] P<0.05, ANOVA with Bonferroni: compare selected pairs post test. B. Image of CYP3A4 protein expression from capillary Western Blotting.

Figure 5. Effects of gene knock down (KD using shRNA or siRNA) (panel A & B) or CRISPR-mediated gene transcription activation (TA) (panel C & D) of indicated transcription factors on CYP3A4 expression in Huh7 cells. A & C, mRNA levels of indicated transcription factors; B & D, mRNA levels of CYP3A4. Compared to negative control (NC), * P<0.05, ** P<0.01, *** P<0.001, ANOVA with Bonferroni: compare selected pairs post test.

Figure 6. Effects of ESR1 knock down (KD) (A B) or CRISPR-mediated gene transcription activation (TA) (C) on the expression of transcription factors as indicated in Huh7 cells (A & C) or hepatocytes (B). Compared to negative control (NC), * <0.05, ** P<0.01, *** P<0.001, ANOVA with Bonferroni: compare selected pairs post test.

Figure 7. Effects of gene knock down (A) or CRISPR-mediated gene transcription activation (B) of indicated transcription factors on the expression of ESR1 in Huh7 cells. Compared to negative control (NC), * P<0.05, ** P<0.01, ANOVA with Dunnett: compare to control post test.

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Figure 8. Effects of estrogen treatment on the expression of ESR1 and CYP3A4 in hepatocytes from male (A) and female donors (B). Hepatocytes were treated with 1 μM 17β estradiol (E2) for 48 hours and mRNA levels of liver specific ESR1 isoform and CYP3A4 were measured. Data represent results of hepatocytes from 6 donors (HUM1 to 6, three females and three males), each in triplicates. Compared to DMSO, *P<0.05, *** P<0.001, ANOVA with Bonferroni: compare selected pairs post test.

Figure 9. Effects of ESR1 knock down on the expression of CYPs as indicated in primary culture hepatocytes. Hepatocytes culture with matrigel overlay were treated with siRNA for 6 days, then the mRNA levels of indicated CYPs were measured using real-time PCR with CYBR green. Compared to negative control (NC), * P<0.05, ** P<0.01, *** P<0.001, ANOVA with Bonferroni: compare selected pairs post test.

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Table 1. Gene expression regulators for drug metabolizing CYPs predicted by Sobol's sensitivity indices analysis using microarray and GTEx data.

Gene	Microarray data	GTEx data
CYP3A4	ESR1, NR1I3, PGRMC1, FOXA2, NR1I2	ESR1, NR1I3, FOXA2, NR1I2
CYP3A5	ESR1, NR1I3, PGRMC1	ESR1, NR1I2, NR1I3, NR1H3
CYP3A7	ESR1, NR1I3, PGRMC1	ESR1, NR1I2, NR1I3, NR3C1
CYP3A43	ESR1, NR1I3, PGRMC1	no clear predictors
CYP2B6	ESR1, NR1I3, PGRMC1	NR1I2, NR1I3
CYP2C9	NR1I3, PGRMC1, ESR1	NR1I3, ESR1, PGRMC1, NR1H3, PPARD
CYP2C19	NR1I3, PGRMC1, ESR1, NCOR2	ESR1, NR3C1, NR1I2
CYP2A6	no clear predictors	no clear predictors
CYP2D6	no clear predictors	no clear predictors
CYP1A1	no clear predictors	no clear predictors
CYP1A2	no clear predictors	no clear predictors
CYP1B1	no clear predictors	no clear predictors

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Table 2. Correlation between mRNA expression levels of several CYPs and liver specific ESR1 isoform in 83 human livers.

Gene	**Correlation coefficient**	**P value**
CYP3A4	0.446	<0.001
CYP3A5	0.108	0.409
CYP3A7	0.347	0.007
CYP3A43	0.012	0.931
CYP2C9	0.327	0.005
CYP2C19	0.485	<0.001
CYP2D6	-0.012	0.929
CYP3A5 # n=13	0.693	0.009

Excluding CYP3A5*3 homozygous carriers

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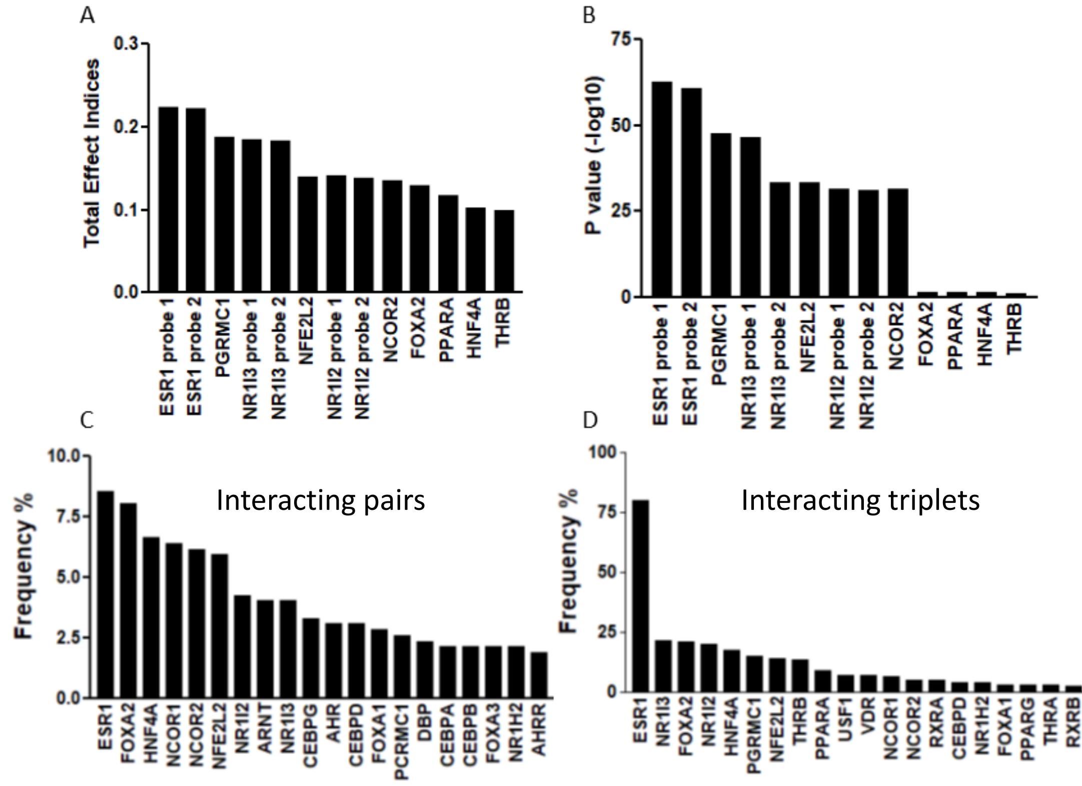

Figure 1

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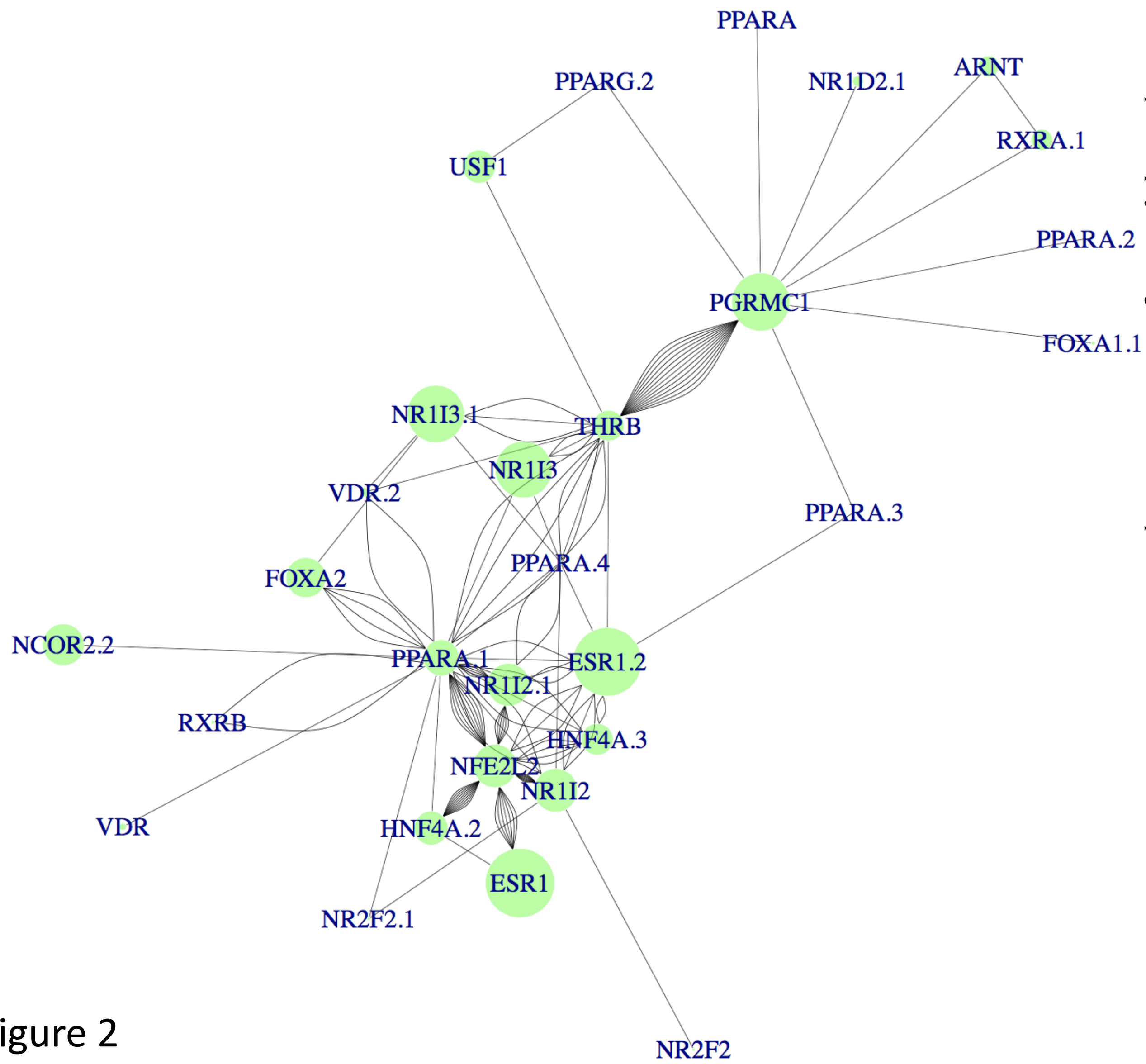

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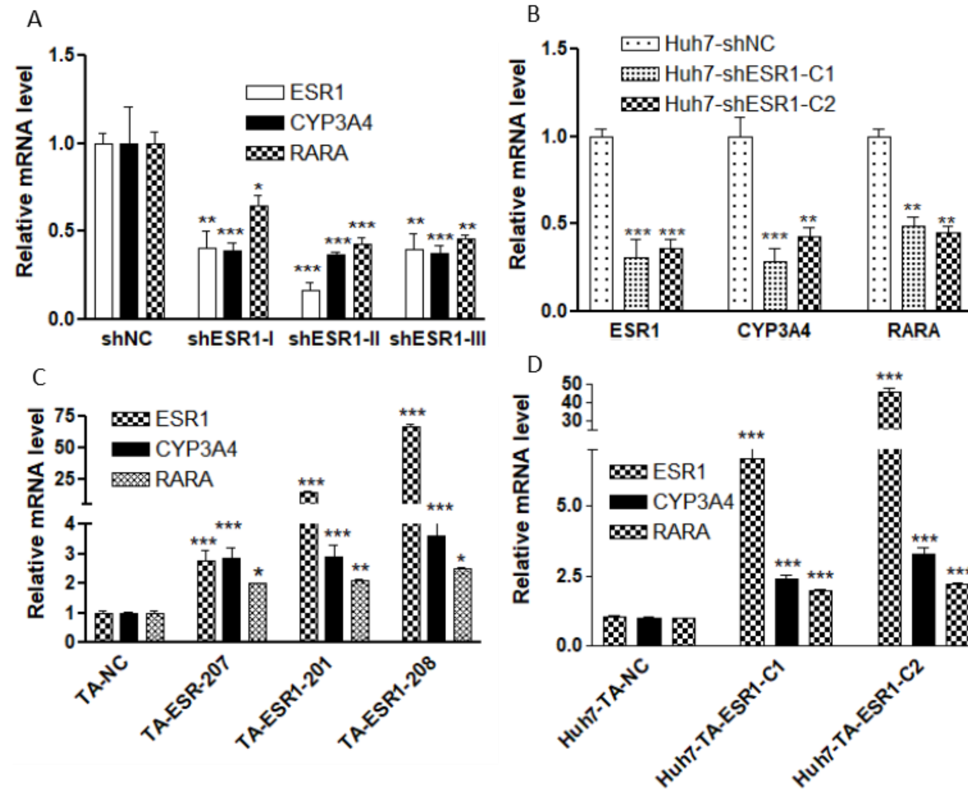

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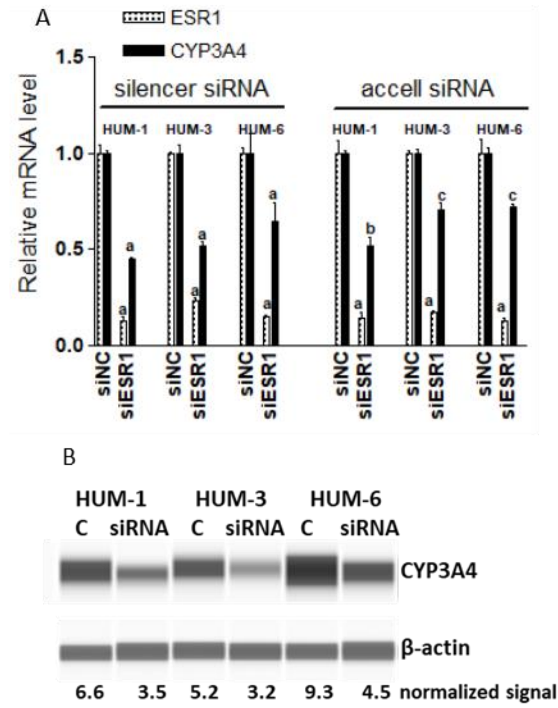

Figure 4

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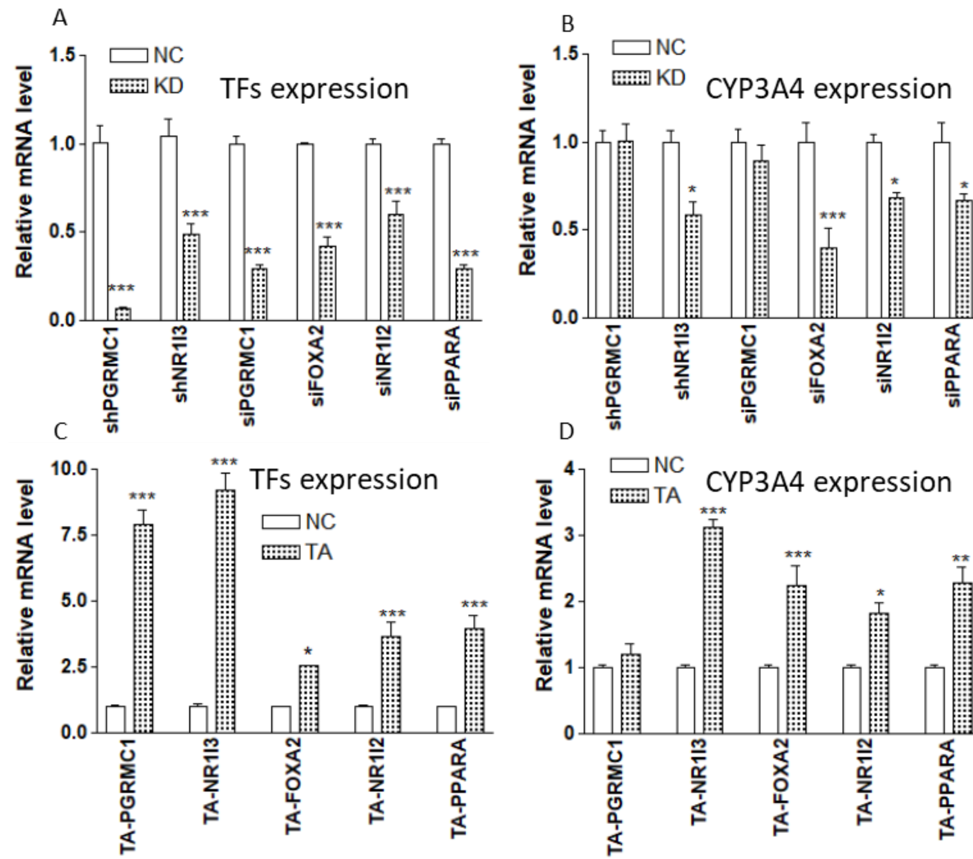

Figure 5

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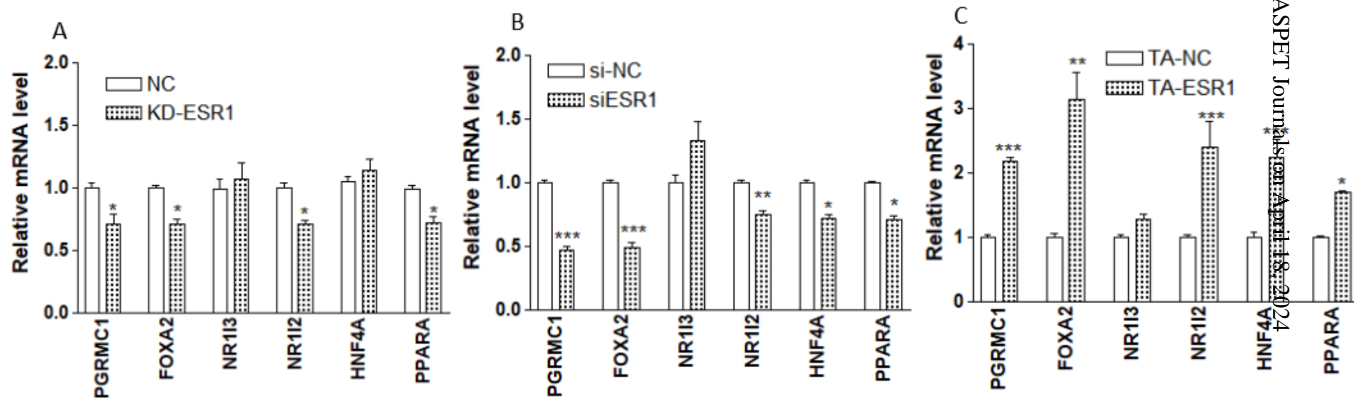

Figure 6

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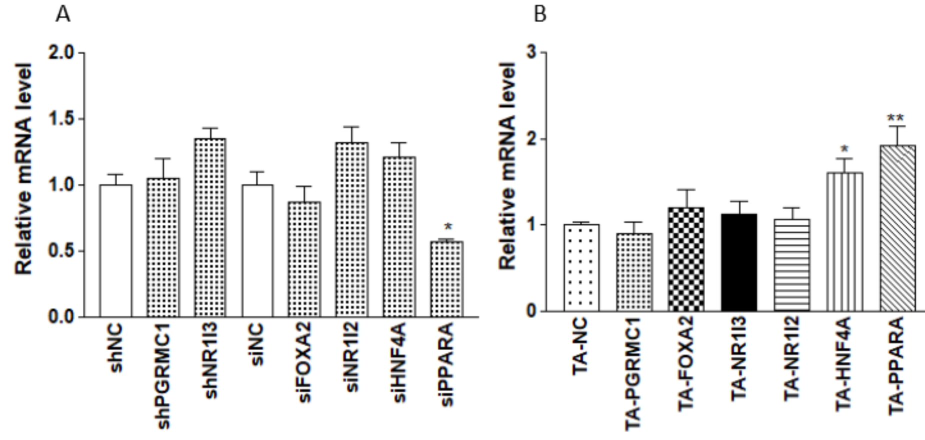

Figure 7

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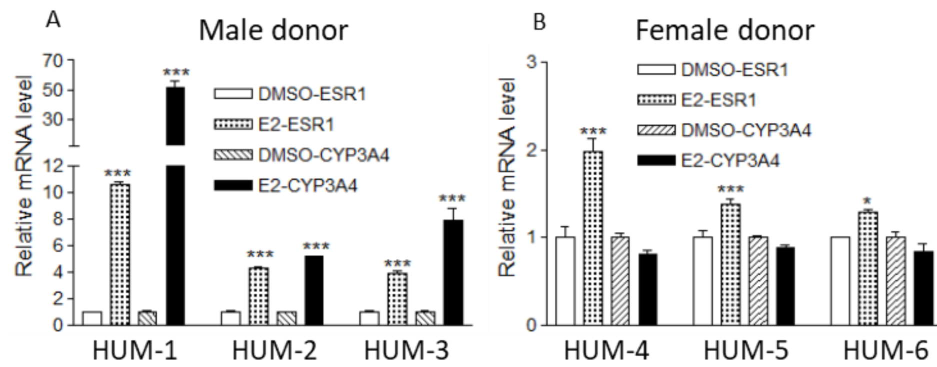

Figure 8

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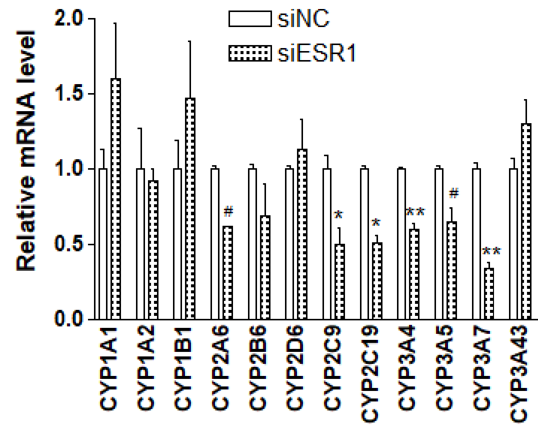

Figure 9