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Title Page

Identification of a NFκB inhibition site on the proximal promoter region of human organic anion transporting polypeptide 1A2 coding gene *SLCO1A2*

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Running Title Page

Running title: *SLCO1A2 promoter region contains a NFκB inhibition site*

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Abbreviations: BCRP: breast cancer resistance protein; MRP2: multidrug resistance-associated proteins 2; NFκB: nuclear factor-κB; OATP: organic anion transporting polypeptide; TNFα: tumor necrosis factor-α

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Abstract

Organic anion-transporting polypeptides (OATPs; gene symbol *SLCO*) are membrane transporters that mediate the transport of wide ranges of compounds. Expression of different OATP members has been reported in the kidney, liver, placenta, brain, and intestine. Due to their broad substrate spectra and wide distribution within human body, these transporters have been proposed to play key roles in the influx transport of many oral drugs. Inflammation is known to regulate the expression and functions of many drug-metabolizing enzymes and drug transporters. As a proinflammatory cytokine, tumor necrosis factor-α (TNFα) has been shown to affect expression of different drug transporters including OATP family members. In the present study, a putative nuclear factor-κB (NFκB) binding site ranging from -1845~ -1836 was identified at the proximal promoter region of OATP1A2 coding gene *SLCO1A2*. Electrophoretic mobility shift assays and chromatin immunoprecipitation showed that nuclear extracts from both breast cancer cell MCF7 and liver cancer cell HepG2 interacted with oligonucleotide probe containing the putative NFκB binding site and the DNA-protein complexes contained both p65 and p50 subunits of NFκB. Further study revealed that the binding site may be in part responsible for the suppression effect of TNFα toward *SLCO1A2* expression. Because treatment of TNFα significantly increased formation of the DNA-protein complexes and that mutations at essential bases of the putative NFκB binding site abolished responsiveness to TNFα neutralizing antibody. In conclusion, the identified NFκB binding site may act as a functional negative regulatory element that participates in suppression of *SLCO1A2* expression in inflammation.

Introduction

The organic anion-transporting polypeptides (human OATPs; rodents Oatps; gene symbol *SLCO*) are membrane transporters that mediate sodium-independent transport of wide spectrum of structurally independent compounds (Hagenbuch and Gui, 2008). Substrates of OATPs include bile salts such as cholate, taurocholate, taurochenodeoxycholate, tauroursodeoxycholate and glycocholate (Kullak-Ublick et al., 1994; Eckhardt et al., 1999), bromosulfophthalein (BSP) (Kullak-Ublick et al., 1994), hormones and their conjugates (estrone-3-sulfate, estradiol-17b-glucuronide, dehydroepiandrosterone sulfate, aldosterone and cortisol) (Bossuyt et al., 1996a; Kanai et al., 1996; Eckhardt et al., 1999), thyroid hormones (T3, rT3and T4) (Abe et al., 1998; Pizzagalli et al., 2002) and eicosanoids (PGE2and LTC4) (Li et al., 1998; Cattori et al., 2001) as well as a broad range of drugs. Besides these charged compounds, they also transport uncharged drugs such as glycosides digoxin (Noé et al., 1997) and ouabain (Bossuyt et al., 1996b). Some OATP family members are expressed ubiquitously; while others, such as OATP1B1 and OATP1B3, are predominantly found in certain organs or tissues. In recent years, these transporter proteins have been proposed to play key roles in the influx transport of various oral drugs (Shitara et al., 2005; Poirier et al., 2007; Tami, 2012) and that regulation of OATPs functions may have a profound effect on the absorption and bioavailability of drugs. So far there are 12 members of the human OATP family discovered: OATP1A2, 1B1, 1B3, 1B7, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1 and 6A1 (Hagenbuch and Meier, 2003; Nakanishi and Tamai, 2012), though OATP1B7 is considered as non-functional and *SLCO1B7* was proposed as a pseudogene

(Stieger and Hagenbuch, 2014).

OATP1A2 was the first OATP family member cloned from human (Kullak-Ublick et al., 1995). It is widely distributed throughout the body and the highest mRNA expression was detected in the brain, liver, lung, kidney and testes (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004). OATP1A2 has a broad substrate specificity, studies on the substrates transported by other human OATPs demonstrated that most of them showed a substrate spectrum at least partially overlapping with that of OATP1A2 (König, 2011). Due to the extent of its substrate specificity and wide tissue distribution, OATP1A2 is believed to be a key determinant for drug absorption, distribution, and excretion (Lee et al., 2005).

Inflammation is known to regulate the expression and functions of many drug-metabolizing enzymes and drug transporters. Proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor-α (TNFα), and IL-6 have been shown to affect expression of rodent (Green et al., 1996; Siewert et al., 2004; Geier et al., 2003; 2005) and human hepatic drug transporters such as OATP1B1 and 1B3 (Le Vee et al., 2008; 2009). Transporters in extrahepatic tissue were also found to be regulated under inflammatory conditions (Morgan et al., 2008). However, compared with the hepatic OATP members, little is known about regulation of *SLCO1A2* in response to inflammation, though a minimal promoter region within 91 bp upstream to the transcription initiation site was identified, and a putative silencer element was suggested in the -662~-440 bp region (Kullak-Ublick et al., 1997). In the present study, we analyzed promoter sequence of *SLCO1A2* further upstream of the

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transcription initiation site and found a putative nuclear factor-κB (NFκB) binding site. NFκB is known to be induced by the proinflammatory cytokine TNFα (Fitzgerald et al., 2007) and is considered a major regulatory factor responsible for regulation of genes under inflammation conditions (Morgan et al., 2008). Further investigation with breast cancer cell line MCF7 and liver cancer cell line HepG2 revealed that this binding site interacts with NFκB and may be responsible for the suppressive effect of TNFα on *SLCO1A2* gene expression. In addition, inhibition of NFκB significantly elevated the suppressive effect of TNFα on *SLCO1A2* expression in multiple cancer cell lines.

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Materials and Methods

Materials - Reagents and enzymes for molecular biology and cell culture were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) except where otherwise stated.

Construction of plasmid DNA-The 2160 bp segment of the *SLCO1A2* promoter region was obtained by PCR amplification with the primer set: Forward 5' GGTACCCTGAAGCATTACCTGTTCCC 3' and Reverse 5' GAGCTCCCTGGAACGCTTTAATACAGA 3'. The underlined sequences are restriction sites for KpnI and SacI, which were used to ligate the fragment into the pGL3 basic vector (Promega, Fitchburg, WI, USA). Segments with other length were generated with omega-PCR technique (Chen et al., 2013); while site-directed mutagenesis was carried out with QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent (Santa Clara, CA, USA), using pGL3-2160 bp plasmid as the template. All mutant sequences were confirmed by full length sequencing.

Cell culture and transfection of plasmid constructs into cells – MCF7 and HepG2 cells (ATCC, Manassas, VA, USA) were grown at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS); while T-47D (ATCC, Manassas, VA, USA) cells were grown in RPMI1640 medium supplemented with 0.2 U/ml insulin and 10% FBS at 37 °C and 5 % CO_2. Confluent cells in 48-well were transfected with DNA plasmid using LipofectAMINE 2000 reagent (Thermo

Fisher Scientific) following manufacturer's instruction. Transfected cells were incubated for 48 hrs at 37 °C and then used for luciferase assay analysis.

Luciferase assay- The luciferase activity was measured with the Dual-luciferase reporter assay system (Promega) according to the manufacturer's instruction. Briefly, forty-eight hours after transfection, cells in 48-well plate were lysed with the passive lysis buffer for 20 min at room temperature. Cell debris was then spinned down and the supernatant was transfer to a clean centrifuge tube. Twenty microliters of sample were added to 100 μl of Luciferase Assay Reagent II and then subjected to measurement with a GloMax®-Multi Jr Single Tube Multimode Reader (Promega). The Quenching of firefly luciferase luminescence and concomitant activation of *Renilla* luciferase are accomplished by adding 100 μl of Stop & Glo® Reagent.

Electrophoretic mobility shift assay- Oligonucleotides corresponding to the putative NFκB binding sequence or mutants were labeled with biotin with Beyotime EMSA Biotin-labeling kit according to the manufacturer's instruction (Beyotime Biotechnology Inc. Jiangsu, China). The incubations, which are consist of 100 nM of the labeled oligonucleotide and 3 μg of nuclear extracts in a final volume of 10 μl, were carried out with the Beyotime EMSA/Gel-shift kit. After incubation for 30 min at room temperature, loading buffer was added and the mixture was loaded onto a 4 % nondenaturing polyacrylamide gel, electrophoresed for 1.5 h at 4 °C at 90 V in 0.5×Tris/Borate/EDTA buffer and subsequently transferred to a positively charged nitrocellulose membrane. The membrane was cross-linked

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with UV at 254 nm, then blotted with blotting buffer and incubated with streptavidin-HRP conjugate for 30 min at room temperature. The membrane was washed, reacted with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA) and exposed to X-ray film for detection. In competition experiments, 1 to 100-fold excess of unlabeled oligonucleotide was added to the labeled oligonucleotide in buffer before the addition of nuclear extracts. Nuclear extracts were incubated in buffer with p65 or p50 antibody (1:10 dilution) for 2 h at room temperature before the labeled DNA was added in the supershift experiments. The oligonucleotide 5'- GCACACGGAATTTCCTGA TTTGGG-3' and its complement, which corresponds to -1850 to -1827 upstream region of the human *SLCO1A2* gene (Genbank Accession No. Y08062) as well as oligonucleotides for the mutants were synthesized by Thermo Fisher Scientific.

Chromatin immunoprecipitation-Chromatin immunoprecipitation (ChIP) was carried out with the ChIP assay kit (Beyotime Biotechnology Inc.) following manufacturers' instruction. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was then quenched with 0.125 M glycine, scraped down and collected in an eppendorf tube. Cells were washed with cold PBS and lysed with SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 % SDS with protease inhibitors phenylmethylsulfonyl fluoride, 500 μg/ml, and leupeptin, 3 μg/ml), subjected to sonication with a Branson S450-D digital sonifier (Branson Ultrasonic, Danbury, CT). The sonication procedure was as follows: 7 rounds of 5-second sonication on ice, with 10 seconds intervals in between at 50 amplitude microns power. Cell debris was removed by centrifugation and

the supernatant was transferred to a clean eppendorf tube, in which protein G agarose/salmon sperm DNA was added to pre-clean the sample for 1 h at 4 °C. Twenty microliter of the supernatant were taken out as input in the following PCR reaction and p65 or p50 NFκB antibody (1:100 dilution) was added to the remaining solution and incubated overnight at 4°C, then pulled down by protein G agarose/salmon sperm DNA. The agrose beads were washed sequentially with high salt wash buffer (0.1 % SDS, 0.1 % Triton X-100, 500 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.0) and TE buffer, released in elution buffer (0.1 % SDS, 0.1 M $NaHCO_3$, pH 8.0) and the cross-linking of DNA-protein was reversed by adding 200 mM NaCl and incubated at 65°C overnight. The DNA was purified with High pure PCR product purification kit (Roche Applied Science, Penzberg, Germany) and analyzed with PCR or real time quantitative polymerase chain reaction (qPCR) with the primers Forward: 5'-GCAGAAGTCACTGTGTAAAC-3', and reverse: 5'-GTCATTGTTGTCCATCTTGG -3'. For qPCR, the fluorescent dye SYBR Green methodology and an ABI Prism 7000 detector (Applied Biosystem, Foster City, CA, USA) were used as described previously (Jigorel et al., 2015). Specificity of amplification was verified at the end of each reaction by the analysis of dissociation curves of the PCR products.

Isolation of nuclear proteins and western blotting-Proteins were extracted with Cell nucleic and cytosolic proteins extraction kit (Beyotime Biotechnology Inc.) following manufacturer's instruction. Proteins were denatured with 4×Laemmli buffer and loaded onto a 7.5% SDS-polyacrylamide electrophoresis gel, then transferred electrophoretically to a

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polyvinylidene difluoride membrane (Millipore, Billerica, MA). Different subunits of NFκB were detected with corresponding antibodies (Beyotime Biotechnology Inc.).

Quantitative polymerase chain reaction to analyse SLCO1A2 expression- Total RNA was isolated from cells with TRIzol® reagent (Thermo Fisher Scientific). Same amount of RNA was subjected to reverse transcription reaction. The resulting cDNA was then used for qPCR analysis as described above with the primer set Forward: 5'-TTGTTGGCATCATTCTGTGC -3', and reverse: 5'-CGACCCAACGAGTGTCAGT-3'. Relative quantification of *SLCO1A2* mRNA level was calculated by normalizing the total cDNA amount tested to an *actin* endogenous control.

Data analysis- Statistical analysis was carried out with one-way analysis of variance with Bonferroni's *post hoc* test. Differences between means are regarded as significant if $p<0.05$.

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Results

Identification of a putative NFκB binding site at the promoter region of SLCO1A2

It has been reported that the *SLCO1A2* gene has a minimal promoter region of 91 base pair related to the transcription start site and that a putative suppressive element was localized in the -662~-440 region (Kullak-Ublick et al., 1997). In the study, though inhibitory effect was observed in the -662~-440 region and up to -1640bp upstream of the *SLCO1A2* transcription start site was analyzed, substantial amount of promoter activity was still observed. When we analyzed further upstream of the sequence, a region ranging from -1845~-1836 (Fig. 1) contains the sequence highly homologous to that of the consensus NFκB binding sequence described in literatures (Fig. 2). We therefore furthered our studies on this 10bp region.

The putative NFκB binding site interacts with different subunits of the transcription factor

It has been reported that *SLCO1A2* is expressed in breast cancer cell line MCF7 (Banerjee et al., 2012). In addition, our previous study on radiation-induced responses of OATP expression found out that OATP1A2 was the major transporter responded to low-dose, high LET irradiation in MCF7 cells (Zhou et al., 2017). The alteration of cytokines such as TNFα by radiation is well documented and our preliminary data suggested that low-dose, high LET irradiation may suppress the expression of TNFα (unpublished data). We hence speculate that radiation may affect expression of *SLCO1A2* through a TNFα-related pathway. Therefore, we utilized MCF7 as a cell model for the present study. To examine whether NFκB binds to the putative NFκB binding site at *SLCO1A2* promoter sequence, electrophoretic mobility shift assay (EMSA) was performed with biotin-labeled

oligonucleotides that contained the 10bp putative NFκB binding segment (-1845~-1836) plus 3bp at the 5' end and 6bp at the 3' end. As shown in Figure 3A, the labeled sequence formed two complexes (complex 1 and complex 2) with nuclear extracts from MCF7 cells. The intensity of both complex bands was reduced gradually when competed with increasing concentrations of the unlabeled specific NFκB binding segment. To further evaluate specificity of the interaction, we mutated two critical regions for NFκB binding within the sequence (Iber et al., 2000) as well as a mutant that contained mutation region outside the putative binding site (mu3). As shown in Figure 3B, addition of the unlabeled 5'GG (mu1) mutant partially affected the formation of DNA/protein complexes; while the presence of the unlabeled 3'CC (mu2) or double mutant showed no effect on the formation of the DNA/protein complexes. Mu3 exhibited a similar effect to that of the wild-type unlabeled oligonucleotides.

To investigate the participation of NFκB subunits in the observed complexes, antibodies for p50 and p65 NF-κB subunits were used for the supershift experiments. Unexpectedly, when nuclear extracts were incubated with the p65 and p50 subunit antibodies, we could not observe any band that was shifted to a lower mobility. However, the addition of p65 or p50 antibodies prior to addition of the probe significantly reduced the intensity of both bands; while addition of the biotin-labeled probe before the antibodies disrupted formation of these complexes. The addition p50 antibody appeared to exert a more significant effect than the p65 antibody (Fig. 3C). To confirm the interaction of the putative NFκB binding sequence with different NFκB subunits exits in endogenous DNA, chromatin immunoprecipitation

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assay was performed. Figure 3D showed that both p65 and p50 antibodies could precipitate the corresponding fragment that contains the NFκB binding sequence.

To rule out the possibility that interactions between the putative binding site and NFκB subunits were cell-type specific, we also analyzed the above-mentioned effects with liver cancer cell line HepG2, another cell line that was demonstrated to express *SLCO1A2* (Kullak-Ublick et al., 1996). As shown in Figure 4A, the putative NFκB binding site at *SLCO1A2* promoter sequence also specifically formed DNA/protein complexes with the nuclear extracts from HepG2 cells. In addition, ChIP analysis demonstrated that the putative NFκB binding sequence interacts with both subunits of NF-κB in HepG2 cells as well (Fig. 4B).

The putative NFκB binding site has a suppressive effect on SLCO1A2 expression.

Next we wanted to see whether this putative NFκB binding sequence has effect on the expression of *SLCO1A2*. For such a purpose, we further generated three additional promoter constructs that contained the -293bp, -710bp, and -1826bp regions relative to *SLCO1A2* transcription start site and expressed them in MCF7 cells (Fig. 1). Consistent to the previous report (Kullak-Ublick et al., 1997), the construct with -293bp region exhibited significant promoter activity, while that of -710bp and -1826bp showed reduced function (Fig. 5A). Interesting, promoter function of the construct that contained the -1845~-1836 segment was further suppressed, suggesting the presence of an inhibitory element. To evaluate the effect of NFκB binding with the -1845~-1836 region on *SLCO1A2* promoter activity, we analyzed

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the *SLCO1A2* promoter activity in the 5'GG or 3'CC mutants as well. Figure 5B showed that both mutations partially increased promoter function. Double mutation of both sites did not seem to release the suppressive effect more significantly, suggesting these two sites are important for the inhibitory effect, and that mutation of either one interferes with NF-κB inhibition of *SLCO1A2* promoter activity. When cells were pre-treated with NF-κB inhibitor BAY117082 (Pierce et al., 1997), suppression of the promoter activity was partially relieved, while those of the mutants remained unchanged (Fig. 5C). These results suggested the -1845~-1836 region interacts with NFκB and that such an interaction has a suppressive effect on *SLCO1A2* expression. We thus named this element as *neg*-NFκB.

SLCO1A2 responses to TNFα treatment is mediated through neg-NFκB

Since TNFα is a well known cytokine that affects NFκB translocation and activation (Schütze et al., 1995), and that cytokines have been demonstrated to inhibit expression of OATPs (Geier et al., 2003; Le Vee et al., 2009), we next want to see whether TNFα affects expression of *SLCO1A2* through the interaction of NFκB with *neg*-NFκB. To first test the effect of TNFα on NFκB activation, MCF7 cells were treated with TNFα or TNFα neutralizing antibody and the nuclear fraction of NFκB level was analyzed. As shown in Figure 6A&B, both p65 and p50 subunits of NFκB increased in the nuclear fraction after TNFα treatment; while reduced with the treatment of neutralizing antibody. A similar effect exerted by TNFα modulators was observed in HepG2 cells (Fig. 6C). These results suggested that NFκB is activated by TNFα and that both p65 and p50 subunits were translocated into the nuclear in MCF7 and HepG2 cells.

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To see the responses of cells toward TNFα, EMSA was carried out after TNFα treatment. As shown in Figure 7A, when the nuclear extracts of MCF7 cells were incubated with the biotin-labeled probe that corresponds to *neg*-NFκB, a more intense shifted band (50% increase, $p<0.05$) was observed in the TNFα-treated sample compared with that of the untreated control. In addition, when cells were pre-treated with TNFα neutralizing antibody, suppressive effect of *SLCO1A2* expression was partially released (Fig. 7B). On the other hand, when the critical regions of *neg*-NFκB was mutated (mu1 and mu2 and double mu), the addition of TNFα neutralizing antibody exerted no effect on promoter activity of *SLCO1A2*, implicating that the binding of NFκB with *neg*-NFκB is involved in *SLCO1A2* responses to TNFα treatment. In addition, quantitative PCR analysis coupled with ChIP showed that treatment of TNFα increased the formation of endogenous DNA/protein complexes (Fig. 7C).

Similar results were obtained from HepG2 cells. A more intense shifted band compose of *neg*-NFκB / NFκB subunits (two fold increase, $p<0.05$) was observed under TNFα treatment; while the signal was reduced when the cells were treated with TNFα neutralizing antibody (Fig. 8A). In addition, both TNFα neutralizing antibody and NFκB inhibitor BAY117082 partially relieved the suppressive effect on *SLCO1A2* promoter activity in HepG2 cells, while those of the mutants remained unchanged (Fig. 8B). Quantitative PCR analysis coupled with ChIP also demonstrated that the amount of endogenous DNA/protein complexes increased after TNFα treatment (Fig. 8C).

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Finally, to see whether the NFκB-mediated TNFα suppression occurs *in vivo,* we treated MCF7, HepG2 and another breast cancer cell line T47-D, which has been reported to show high level of *SLCO1A2* (Obaidat et al., 2012) with TNFα and BAY117082. As shown in Figure 9, TNFα significantly decreased expression of *SLCO1A*2 in all three cells lines; while the inhibition of NFκB relieved such a suppression.

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Discussion

Inflammation cytokines have been reported to inhibit expression of OATP members, and NFκB is an important mediator in response to cytokines such as TNFα. However, whether NFκB affects promoter activity of OATP family members remains unclear. In the present study, we showed that NFκB can bind to the promoter region of *SLCO1A2* and exert inhibitory effect on transcriptional activity of the gene in breast cancer cell line MCF7 and hepatocarcinoma cell line HepG2. In addition, our results demonstrated that TNFα, a well-known proinflammatory cytokine, may suppress *SLCO1A2* expression through a negative regulation element.

Previous study demonstrated that constructs with up to -1640bp upstream of the *SLCO1A2* transcription start site exhibited substantial promoter activity (Kullak-Ublick et al., 1997). However, when a construct containing the putative NFκB binding site located within -1845~-1836 was generated and expressed within MCF7 cells, significant suppressive effect on transcription activity of *SLCO1A2* was observed, which implicated that binding of NFκB to this element may negatively regulate expression of the gene. EMSA analysis showed the presence of two shifted bands, both of which displayed lower intensity in the presence of unlabeled probes, suggesting there is more than one DNA/protein complex formed during the incubation with nuclear extracts. Addition of p50 or p65 antibody revealed that the putative NFκB binding segment interacts with both subunits because the presence of either antibody significantly reduced intensity of both shifted bands. Interestingly, the reduction of band intensity was more dramatic for the p50 subunit, because it only required 0.5 h

incubation with the antibody to obtain a similar result as that of 2 h incubation with the p65 antibody. These results suggested that p50 may be more readily interact with neg-NFκB than the p65 subunits. The binding between NFκB subunits with *neg*-NFκB was further confirmed using a system containing whole genomic DNA through the ChIP assay, a technique that is used to investigate association of regulatory molecules to specific DNA regions *in vivo*. However, we could not rule out the possibility that other subunits of NFκB interact with the sequence as well. The binding of p50 and/or p65 subunits of NFκB to *neg*-NFκB was also observed in HepG2 cells, suggesting such interactions is not cell-type specific.

The result of the EMSA-supershift experiment is different from analysis of many other transcription factors, in which binding of the antibody increases size of the DNA/protein complex and display a classic super-shift band (Furuya et al., 2013). In our experiments, the addition of either p65 or p50 antibody before incubation with the biotin-labelled probe did not resulted in size change of the DNA/protein complex but showed intensity change instead. On the other hand, when the labelled probe was incubated with nuclear extracts before antibodies were added, the disruption of DNA/protein complex formation was abolished. Such a phenomenon was also observed in synovial cells (Juarranz et al., 2005), NIH 3T3 fibroblasts (Balasubramanian et al., 2011) as well as in the study of *slc2a4* interaction with NFκB in white adipose tissue and 3T3-L1 adipocytes (Furuya et al., 2013). It was suggested that p50 or p65 antibody binds to their respective proteins in a location that is close to the DNA binding site and hence hinder the formation of the DNA/protein complex. On the other

hand, formation of the protein/DNA binding may lead to conformational changes that impair further antibody binding to the protein (Furuya et al., 2013).

Inflammation occurs in many acute and chronic disease states and it has been shown to modulate pharmacokinetic by changing expression of metabolizing enzymes and membrane-associated drug transporters. Many key transcription factors including NFκB are believed to be involved in this kind of expression regulation (Cressman et al., 2012). NFκB has been shown to increase expression of transporters such as P-glycoprotein (Bentires-Alj et al., 2003), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins 2 (MRP2) (Ke et al., 2013) but also can be an inhibitory factor for metabolizing enzymes such as cytochrome P450 enzyme CYP2C11 (Iber et al., 2000) and transporters like organic anion transporter 3 (Phatchawan et al., 2014). OATP family members OATP1B1 and 1B3 as well as OATP2B1 were shown to be down-regulated by TNFα and interleukin-6 (Le Vee et al., 2009). In the present study, although a reliable suppressive effect of *SLCO1A2* by TNFα treatment was hard to obtain due to low activity of the untreated luciferase construct control, both MCF7 and HepG2 cells treated with TNFα neutralizing antibody showed significantly increased promoter activity. The mutation of essential positions within *neg*- NFκB abolished such an effect, and that TNFα treatment increased the amount of DNA/protein complexes formed between *neg*- NFκB and the corresponding NFκB subunits suggested that the putative binding site of NFκB may in part responsible for the suppressive effect of TNFα on *SLCO1A2* expression. Our qPCR results showing that NFκB inhibitor BAY117082 relieved the suppressive effect of TNFα on *SLCO1A2*

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expression in different cancer cell lines seemed to support such a speculation.

In summary, we identified a functional NFκB binding site located at -1845~ -1836 bp upstream of the transcription start site of *SLCO1A2*. Such an element exerts repressive effect on *SLCO1A2* expression and may mediate the gene responses toward inflammation. To our knowledge, this is the first report demonstrating that expression of OATP family members can be directly regulated by the transcription factor NFκB. Such a regulatory mechanism may also be found in inflammation-related regulation of other OATP members.

Authorship Contributions

Participated in research design: Yi and Hong.

Conducted experiments: Xiang, Li, Wang, and Chen.

Performed data analysis: Yi and Hong.

Wrote or contributed to the writing of the manuscript: Hong

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Footnotes

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Legends for Figures

Figure 1 Putative NFκB binding sequence at the promoter region of *SLCO1A2*. The promoter region of *SLCO1A2* was extracted from NCBI Genbank and analyzed with MatInspector for the corresponding motifs. The putative NFκB binding site from -1845 to -1836 was underlined. Start positions for promoter regions with different length and transcription start site were indicated and marked in bold.

Figure 2 Consensus sequences of NFκB binding site. The 10 bp putative NFκB binding segment was compared to NFkB binding sites of metabolizing enzymes and transporters. A consensus sequence derived from these sequences was also shown.

Figure 3 Putative NFκB binding site at *SLCO1A2* promoter interacts with NFκB in MCF7 cells. **A**. Nuclear extracts specifically binds to the putative NFκB binding site. EMSA was performed as described in the Methods and Materials section. Nuclear extracts from MCF7 cells were incubated with 0.1 μM of biotin-labeled oligonucleotides. Increasing concentrations of the unlabeled probe, which was 1-, 10- and 100-fold molar excess over the labeled probe was added into the incubation system. **B**. Wild-type or mutated unlabeled oligonucleotides that were 10-fold molar excess over the labeled probe were incubated with the labeled NFκB binding segment. The mutations were underlined. **C**. Supershift analysis of the DNA/protein complexes. Antibodies to the p50 and p65 subunits of NFkB was incubated with the nuclear extracts for 0.5 h and 2 h, respectively, before the labeled oligonucleotides were added; or the biotin-labeled segments were added 30 min before the

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antibodies were added into the incubation system. **D.** ChIP analysis for the interaction of NFkB p50 and p65 subunits with the putative binding sequence. The cross-linked DNA/protein complexes were precipitated with p50 or p65 antibodies, with rabbit IgG (IgG) as the non-specific binding control, pulled down by protein G agarose/salmon sperm DNA, and subjected to PCR for detection of the *neg*- NFκB region. For all the analysis, three independent experiments were performed and a representative figure was presented.

Figure 4 Putative NFκB binding site at *SLCO1A2* promoter interacts with NFκB in HepG2 cells. **A**. Wild-type or mutated unlabeled probe that were 10-fold molar excess over the labeled NFκB binding segment compete for the binding with NFκB. **B.** Analysis for the interaction of NFkB subunits with the putative binding sequence with ChIP assay. The cross-linked DNA/protein complexes were precipitated with p50 or p65 antibodies and subjected to PCR for detection of the *neg*- NFκB region as described in Figure 3. For all the analysis, three independent experiments were performed and a representative figure was presented.

Figure 5 The putative NF-κB binding site has a suppressive effect on *SLCO1A2* expression. **A**. Analysis of promoter function of *SLCO1A2* constructs containing different lengths of the promoter region relative to transcription start site. Ratio of the firefly luciferase activity to *Renilla* luciferase activity was measured and calculated to represent promoter activity. The results were expressed as fold changes compared to that of pGL3 empty vector. Three independent experiments were performed, each with triplicate measurements. The data were expressed as mean ± S.D. (n = 3). **B.** Interference of NFκB binding partially released the

inhibitory effect on *SLCO1A2* promoter function. Fold changes of mutants promoter activity compared to wild-type was shown. Different letters indicate significant difference between treatments ($p<0.05$). The results shown are mean ± S.D. ($n = 3$). **C**. NFκB inhibitor relieved the suppressive effect of wild-type promoter but showed no effect on mutants. Cells were pre-treated with 5 μM of BAY117082 for 16 h before luciferase activity was measured. Fold changes of promoter activity compared with untreated control was shown. Asterisks indicate significant difference compared to untreated control ($p<0.05$). The results shown are means ± S.D. ($n = 3$).

Figure 6 TNFα affects nuclear translocation of NF-κB. **A**. MCF7 cells were pre-treated with TNFα (20 ng/ml) or TNFα neutralizing antibody (50 ng/ml) for the indicated time periods and subjected to nuclear protein extraction. **B.** MCF7 cells were pre-treated with TNFα (0.5 h) or TNFα neutralizing antibody (1 h) for the indicated concentrations and subjected to nuclear protein extraction. **C.** HepG2 cells were pre-treated with TNFα (20 ng/ml) for 0.5 h or TNFα neutralizing antibody (50 ng/ml) for 1 h and subjected to nuclear protein extraction. Fifteen micrograms of proteins were loaded on a 7.5% SDS-PAGE gel, followed by western blotting with anti-p50 or anti-p65 antibody (1:1000 dilution). Same blot was probed with anti-Histone H3 antibody (1:1000 dilution) as loading control. Three independent experiments were carried out and a representative blot was shown.

Figure 7 Effect of TNFα on *SLCO1A2* promoter function is mediated by NFκB in MCF7 cells. **A.** Treatment of TNFα neutralizing antibody or TNFα affect the binding of NFκB with

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neg-NFκB segment. Cells were treated with 50 ng/ml TNFα neutralizing antibody for 1 h or 20 ng/ml TNFα for 0.5 h before EMSA was performed. Three independent experiments were performed and a representative figure was shown. Different letters indicate significant difference between treatments ($p<0.05$). The results shown are means ± S.D. ($n = 3$). **B**. TNFα neutralization antibody partially released the inhibition of *SLCO1A2* promoter activity but showed no effect on *neg*-NFκB mutants. Cells were pre-treated with 50 ng/ml TNF neutralization antibody for 16h and luciferase activity was measured as described in Figure 5. Fold changes of promoter activity compared with untreated control was shown. Asterisks indicate significant difference compared to untreated control ($p<0.05$). The results shown are means ± S.D. ($n = 3$). **C**. ChIP analysis for the interaction of p50 and p65 subunits with *neg*-NFκB after cells were subjected to TNFα treatment. Cells were treated with 20 ng/ml TNFα for 0.5 h and ChIP analysis was carried out as described in Figure 3. The DNA pull-downed by p65 or p50 antibody was evaluated by qPCR. Three independent experiments were performed. Asterisks indicate significant difference compared to untreated control ($p<0.05$). The results shown are means ± S.D. ($n = 3$).

Figure 8 Effect of TNFα on *SLCO1A2* promoter function is mediated by NFκB in HepG2 cells. **A.** TNFα neutralizing antibody or TNFα affect the binding of NFκB with *neg*-NFκB segment. Cells were treated and analyzed as described in Figure 7. Three independent experiments were performed and a representative figure was shown. Different letters indicate significant difference between treatments ($p<0.05$). The results shown are means ± S.D. ($n =$

3). **B**. TNFα neutralization antibody or NFκB inhibitor BAY117082 partially released the inhibition of *SLCO1A2* promoter activity but showed no effect on *neg*-NFκB mutants. Cells were pre-treated with 50 ng/ml TNF neutralization antibody or 5 μM of BAY117082 for 16h and luciferase activity was measured as described in Figure 5. Fold changes of promoter activity compared with untreated control was shown. Different letters indicate significant difference between treatments of the same construct ($p<0.05$). The results shown are means ± S.D. ($n = 3$). **C**. ChIP analysis for the interaction of p50 and p65 subunits with *neg*- NFκB after cells were subjected to TNFα treatment. Cells were treated with 20 ng/ml TNFα for 0.5 h and ChIP analysis was carried out as described in Figure 3. The DNA pull-downed by p65 or p50 antibody was evaluated by qPCR. Three independent experiments were performed. Asterisks indicate significant difference compared to untreated control ($p<0.05$). The results shown are means ± S.D. ($n = 3$).

Figure 9 NFκB inhibitor relieved suppressive effect of *SLCO1A2* expression by TNFα. Cells were pre-treated with 20 ng/ml TNFα or 5 μM BAY117082 or both for 16h.Total RNA was isolated from cells and subjected to reverse transcription reaction. The resulting cDNA was then used for qPCR analysis. Expression of *SLCO1A2* after treatment was compared relative to the untreated control. Three independent experiments were performed. Different letters indicate significant difference between treatments for the same cell line ($p<0.05$). The results shown are means ± S.D. ($n = 3$).

Figure 1

-2087
CTGAAGCATTACCTGTTCCCATTTGAATGCTGAGAAAGAGTTAGGGAAATAACATGATTCTCCACTTAATAAAACTTCAGCATG

TCTTTTTCATTAGAAGTGATGATTGACAGGGATTTGTTGGTGACACTGTGTGGTCCTGGGAGCAGAAGTCACTGTGTAAACAAC

ACTGTGCTAACAAAAGAGGACTGTTCACATGGCTGGCACTAGCACAGCCTCGAGAAGATGAAAAGACGGCACACGGAATTTCCT
NFκB binding site

-1826 -1845 -1836
GATTTGGGGCCAAGATGGACAACAATGACTATGATATTAACCGTTGGATTTTATGGATTTTTATTGAATAAATGGAATAGATTT

TGTTAATTTGTTTATTTTCCTCCATGAAGATGTTTGAAATGTATCTGAAAATATAAAATATTTTCATCATATAAGTTGGAAAAC

TAGGGAAAGTTTAGGGTTAATTTAATCCCATGTAATTCTTTTCAAGTGCTTAGCCTTAAATATTACCTCTTACTTCAGTTAAGA

TAATAGTTTTTTAACCCCTTTTTTCAATATGTTAAAACATTTTGAAGCAAATCTTCCAGACATAGTGAAAAAACCTGCTCTAGA

TGGTTACTTAGTGTCACAATTCATGGAGGGAACTATATAGAATTCCCCTATATGGTGATTTTCTGTGGCACTACCTCCAGCTTT

TATGCCCTCTGGCTATATATGAAAAAATAGACATTTAATATAGTAATGTTTTTAGCCACAAAGAAGAGTGAAGGCAATTTTGAC

GTGGGAGAAGAATAAGTTCTCTCCCTTAGTGTTTGATACGGTGACTATATTGGGCTAAGATAATGGTTAGGAGAGAGAGAAGAG

AAAAGGGGAGAGAGATTGGGTTCTAAATGCACGAGAAGAAAGAAATGAGGGCAGTTCAGAACAATAACCTGTGAATTTTTTCAC

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-710
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-293
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AAAAAGATTTTTGAGCCTTGGTAGGGTTAGTGAAGCAGGTTTTTAGTACAAACACAACTCAAAGTATGTGTGTTATTAAAAGTA

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+1
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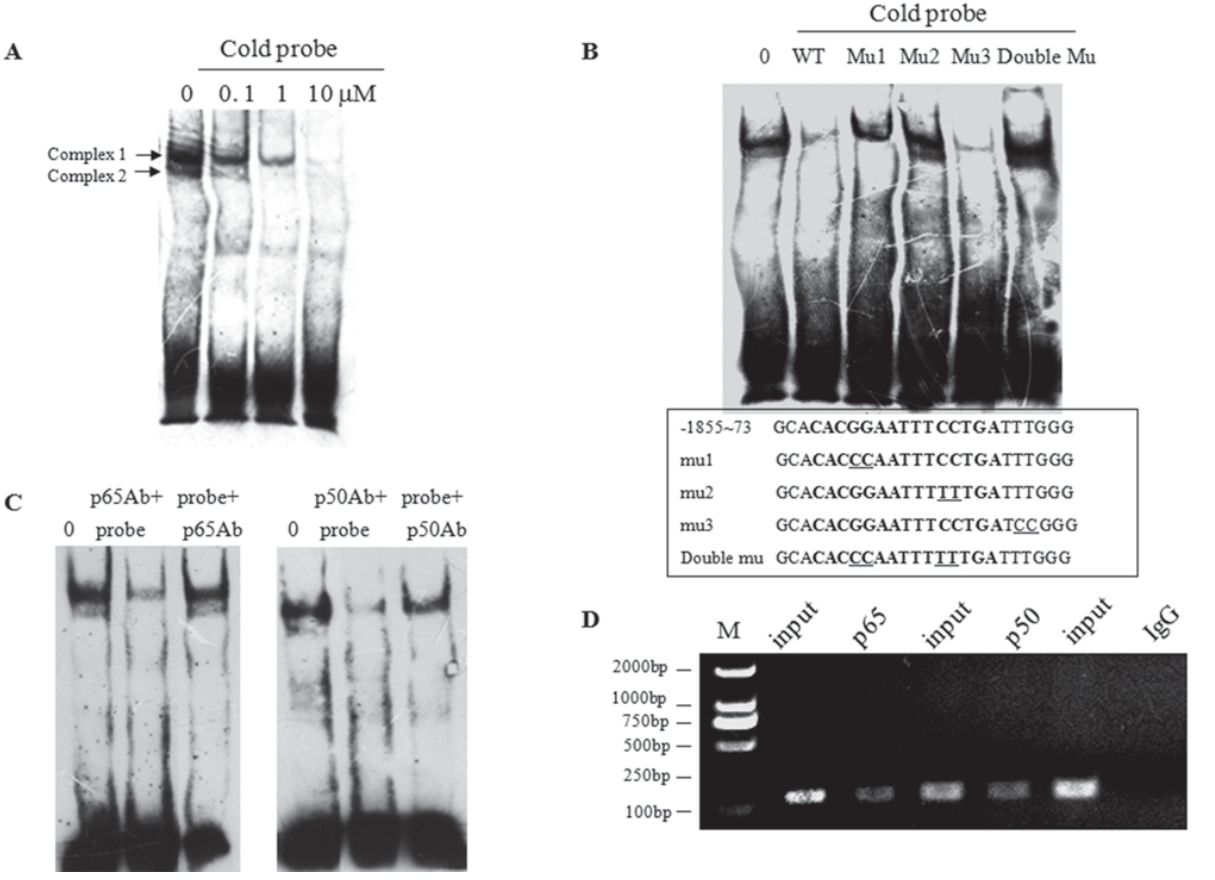
ACCAACAACTAAATCTTCTCTAACACCATGATTTTCTAATCTGTATTAAAGCGTTCCAGG

Figure 2

Mouse slc2a4	GGGCGTGTCC	(Furuya et al., 2013)
	GGGCGTGGCC	
ABCC6	GGGACCCCCC	(Jiang *et al.*, 2006)
ABCG2	TGGGGAAACC	(Pradhan *et al.*, 2010)
Rat mdr1b	GGGGAATTCC	（Yu et al., 2008）
CYP2C11	AGGAGTCTCC	(Iber et al., 2000)
Consensus NF-κB binding sequence	NGGAMTNYCC	
SLCO1A2	GCACACGGAATTTCCTGATTT	

(N=any nucleotide, M=A or T; Y=C or T)

Figure 3


A
Cold probe
0 0.1 1 10 μM
Complex 1
Complex 2
B
Cold probe
0 WT Mu1 Mu2 Mu3 Double Mu
-1855~73 GCACACGGAATTTCCTGATTTGGG
mu1 GCACACCCAATTTCCTGATTTGGG
mu2 GCACACGGAATTTTTTGATTTGGG
mu3 GCACACGGAATTTCCTGATCCGGG
Double mu GCACACCCAATTTTTTGATTTGGG
C
p65Ab+ probe+
0 probe p65Ab
p50Ab+ probe+
0 probe p50Ab
D
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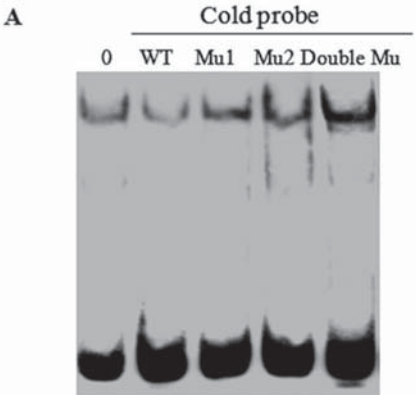
2000bp
1000bp
750bp
500bp
250bp
100bp

Figure 4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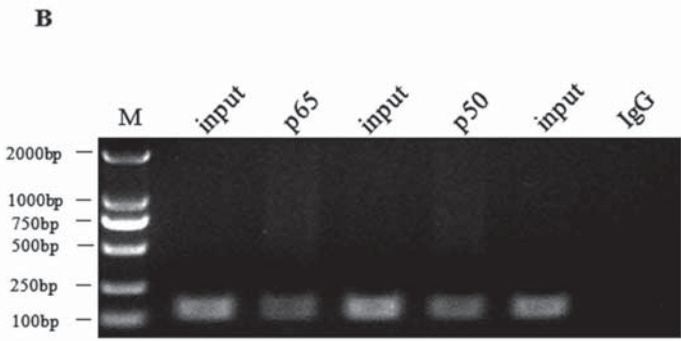
Cold probe
0
WT
Mu1
Mu2
Double Mu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M
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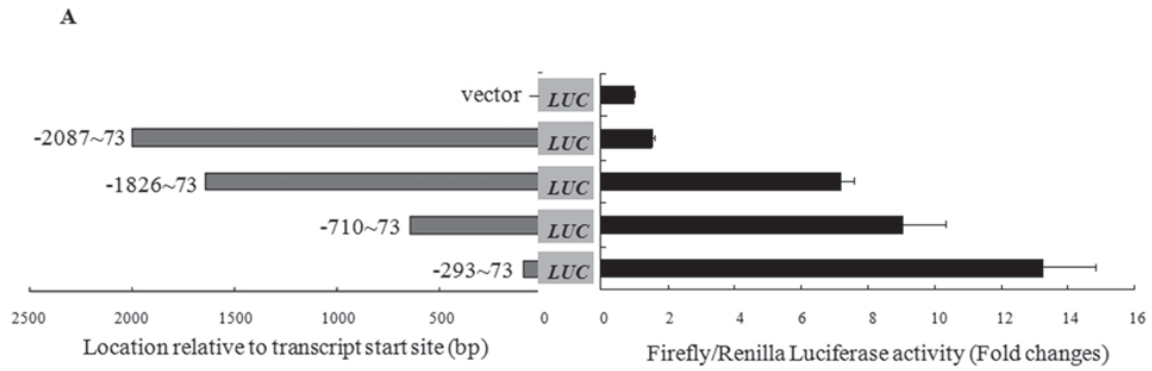
p65
input
p50
input
IgG
2000bp
1000bp
750bp
500bp
250bp
100bp

Figure 5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vector
-2087~73
-1826~73
-710~73
-293~73
LUC
2500
2000
1500
1000
500
0
0
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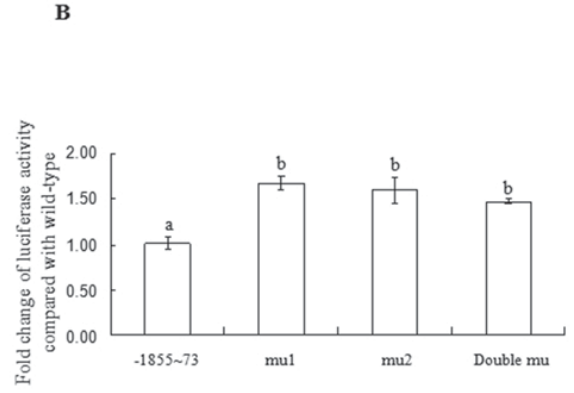
4
6
8
10
12
14
16
Location relative to transcript start site (bp)
Firefly/Renilla Luciferase activity (Fold changes)


B
Fold change of luciferase activity compared with wild-type
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1.50
1.00
0.50
0.00
a
b
b
b
-1855~73
mu1
mu2
Double mu

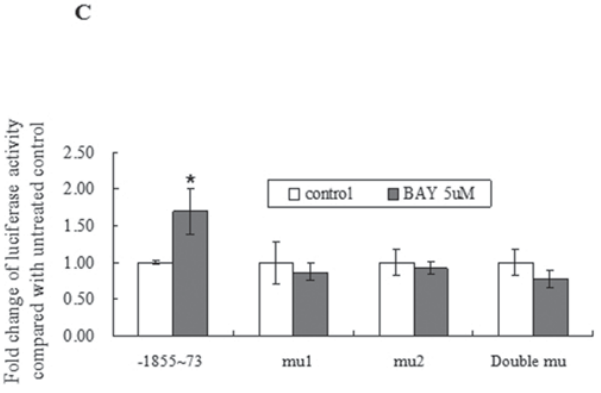
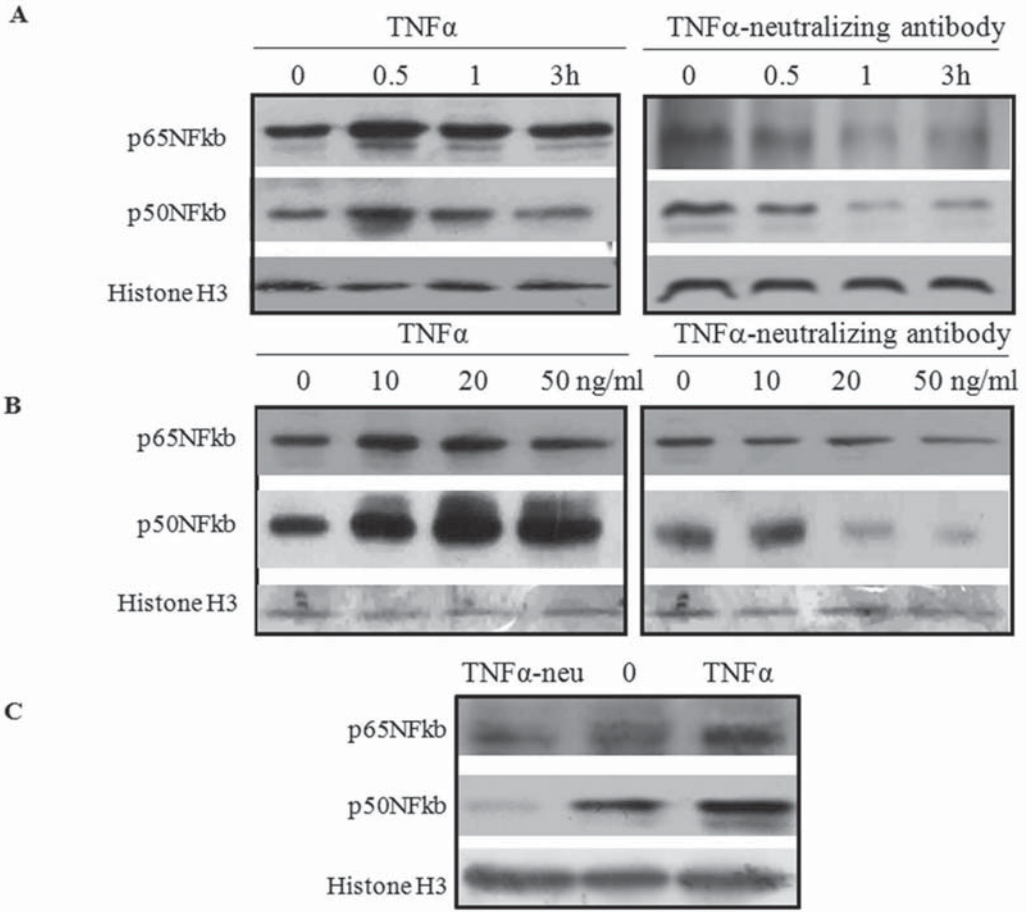

C
Fold change of luciferase activity compared with untreated control
2.50
2.00
1.50
1.00
0.50
0.00
*
control
BAY 5uM
-1855~73
mu1
mu2
Double mu

Figure 6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TNFα
TNFα-neutralizing antibody
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0.5
1
3h
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0.5
1
3h
p65NFkb
p50NFkb
Histone H3
B
TNFα
TNFα-neutralizing antibody
0
10
20
50 ng/ml
0
10
20
50 ng/ml
p65NFkb
p50NFkb
Histone H3
C
TNFα-neu
0
TNFα
p65NFkb
p50NFkb
Histone H3

Figure 7

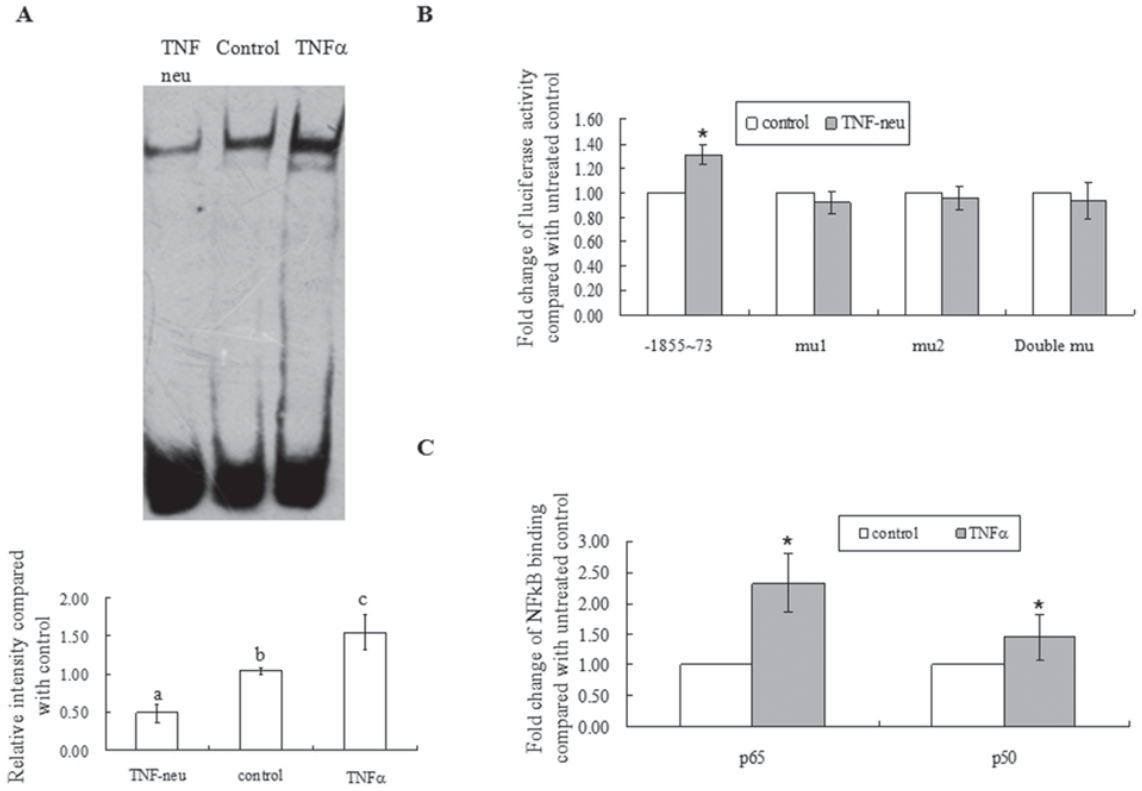

A
TNF neu
Control
TNFα
Relative intensity compared with control
2.00
1.50
1.00
0.50
0.00
a
b
c
TNF-neu
control
TNFα
B
Fold change of luciferase activity compared with untreated control
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
control
TNF-neu
*
-1855~73
mu1
mu2
Double mu
C
Fold change of NFkB binding compared with untreated control
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
TNFα
*
*
p65
p50

Figure 8

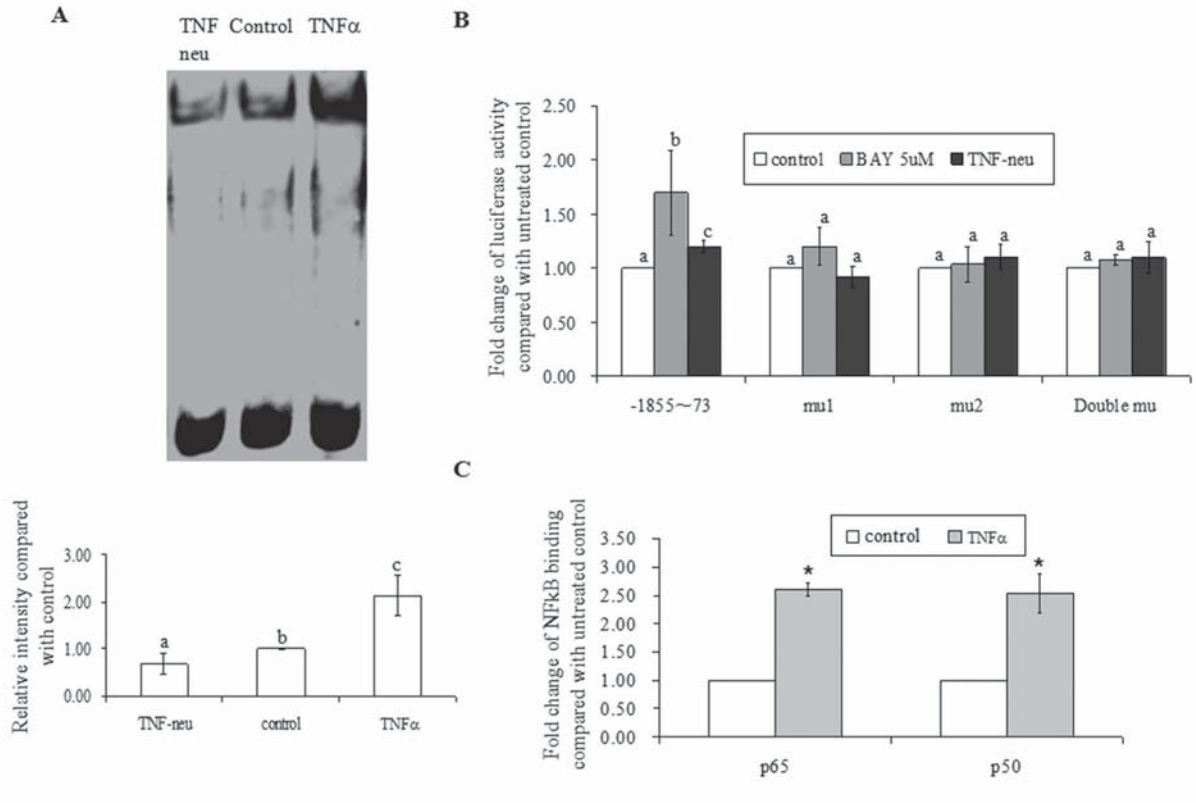
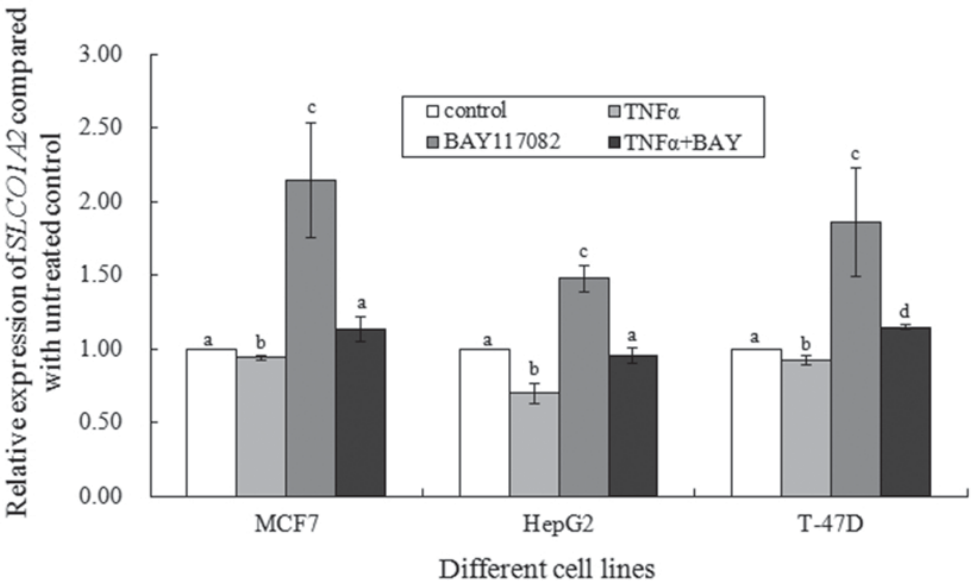

A
TNF neu
Control
TNFα
B
Fold change of luciferase activity compared with untreated control
2.50
2.00
1.50
1.00
0.50
0.00
control
BAY 5uM
TNF-neu
a
b
c
a
a
a
a
a
a
a
a
a
-1855~73
mu1
mu2
Double mu
C
Relative intensity compared with control
3.00
2.00
1.00
0.00
a
b
c
TNF-neu
control
TNFα
Fold change of NFkB binding compared with untreated control
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
TNFα
*
*
p65
p50

Figure 9


Relative expression of SLCO1A2 compared with untreated control
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
TNFα
BAY117082
TNFα+BAY
a
b
c
a
a
b
c
a
a
b
c
d
MCF7
HepG2
T-47D
Different cell lines