Published in final edited form as:

Mol Cell Endocrinol. 2014 January 25; 382(1): 358–367. doi:10.1016/j.mce.2013.10.010.

COUP-TFII inhibits NFkappaB activation in endocrine-resistant breast cancer cells

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Abstract

Reduced COUP-TFII expression contributes to endocrine resistance in breast cancer cells. Endocrine-resistant breast cancer cells have higher NFkappa B (NFκB) activity and target gene expression. The goal of this study was to determine if COUP-TFII modulates NFκB activity. Endocrine-resistant LCC9 cells with low endogenous COUP-TFII displayed ~5-fold higher basal NFκB activity than parental endocrine-sensitive MCF-7 breast cancer cells. Transient transfection of LCC9 cells with COUP-TFII inhibited NFκB activation and reduced NFκB target gene expression. COUP-TFII and NFκB were inversely correlated in breast cancer patient samples. Endogenous COUP-TFII coimmunoprecipitated with NFκB subunits RelB and NFκB1 in MCF-7 cells. COUP-TFII inhibited NFκB-DNA binding *in vitro* and impaired coactivator induced NFκB transactivation. LCC9 cells were growth-inhibited by an NFκB inhibitor and 4-hydroxytamoxifen compared to MCF-7 cells. Together these data indicate a novel role for COUP-TFII in suppression of NFκB activity and explain, in part, why decreased COUP-TFII expression results in an endocrine-resistant phenotype.

Keywords

Antiestrogens; Tamoxifen; Drug resistance; Nuclear receptors; Transcription factors

1. Introduction

Selective estrogen receptor modulators (SERMs) such as tamoxifen (TAM) exert antiproliferative effects in breast cancer by competing with estrogens for binding to estrogen receptor α (ER α), leading to an inhibition of downstream genes, including those involved in breast cancer proliferation. Fulvestrant is a selective ER α downregulator (SERD) because it causes proteasomal degradation of ER α in addition to its SERM-like ability to antagonize estrogen-activated ER α transactivation (Wijayaratne et al., 1999). TAM inhibits cell cycle progression and induces apoptosis (Mandlekar and Kong, 2001; Salami and Karami-Tehrani, 2003). TAM has greatly increased the survival rate of breast cancer patients since its initial

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FDA approval in 1977, resulting in a 31% reduction in annual death rate (Early Breast Cancer Trialists' Collaborative, 2005; Jordan, 2009). Despite the initial efficacy of SERM and aromatase inhibitor treatment, approximately 40% of patients relapse and die from metastatic disease because the cancer cells become refractory to endocrine therapies (Ring and Dowsett, 2004).

The mechanisms for acquired endocrine resistance are complex and a better understanding of maintenance of endocrine sensitivity is clearly needed. Reduced expression of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) has a demonstrated role in acquired endocrine resistance in a breast cancer cell line model of human disease progression (Riggs et al., 2006). Restoration of COUP-TFII expression, which is reduced in endocrine-resistant breast cancer cell lines, inhibited cell proliferation and motility and increased apoptosis in TAM and fulvestrant-treated endocrine-resistant LCC9 and LY2 breast cancer cells (Riggs et al., 2006). Conversely, inhibition of COUP-TFII expression caused MCF-7 cells to become resistant to SERM and fulvestrant inhibition of proliferation (Riggs et al., 2006). A mechanism for the involvement of COUP-TFII in maintenance of endocrine sensitivity has not yet been determined. Since COUP-TFII acts as either a transcriptional activator or repressor in a gene- and cell-specific manner (Litchfield and Klinge, 2012), the mechanism of action may involve modulation of specific target genes including tumor suppressors and oncogenes.

The NF κ B family of dimeric transcription factors have important functions related to immune and inflammatory responses, and recent studies have demonstrated NF κ B activation plays a role in acquired endocrine-resistant breast cancer (Gu et al., 2002; Nehra et al., 2010; Riggs et al., 2005). Inactive NF κ B is located in the cytoplasm bound to I κ B, the inhibitor of NF κ B. Also present in the cytoplasm is the IKK complex containing the regulatory subunit NEMO (nuclear factor- κ B essential modulator or IKK γ) and the catalytic subunits IKK α and IKK β . Upon activation by a variety of stimuli, the IKK complex phosphorylates the inhibitor I κ B leading to its degradation, freeing the now activated NF κ B to translocate to the nucleus to regulate target gene transcription. Because NF κ B activation results in expression of genes that lead to antiapoptotic and pro-proliferative activities, increased NF κ B signaling promotes cancer cell survival (Perkins, 2007).

Dysregulation of the NF κ B subunits p65 (*RELA*), RelB (*RELB*), p50 (*NF κ B1*), p52 (*NF κ B2*), and c-Rel (*REL*) results in altered activation of the NF κ B pathway in breast cancer. Increased expression of p50/NF κ B1, p52/NF κ B2, and c-Rel was detected in breast tumors compared to adjacent normal tissue (Cogswell et al., 2000). Elevated p50/NF κ B1-DNA binding correlated with metastatic relapse and reduced disease-free survival in patients with ER α -positive tumors (Zhou et al., 2005a,b). An increase in NF κ B activity has been reported in endocrine-resistant breast cancer cells (Gu et al., 2002; Nehra et al., 2010; Zhou et al., 2007). p65/RelA level was also elevated (Nehra et al., 2010). The expression and DNA binding of p50 and p65 are also enhanced in LCC1 estrogen-independent, TAM-sensitive breast cancer cells compared to MCF-7 estrogen-dependent cells, further demonstrating a role for NF κ B in the pathway to endocrine resistance (Pratt et al., 2003).

Since COUP-TFII expression is decreased in endocrine-resistant breast cancer cells, we hypothesized COUP-TFII may play a part in the normal suppression of NF κ B activity in endocrine-sensitive breast cancer cells by regulating the transcription of components of the NF κ B pathway. In this study, we observed that COUP-TFII overexpression inhibited NF κ B activity in LCC9 endocrine-resistant breast cancer cells. This suppression of the NF κ B pathway resulted in reduced expression of downstream NF κ B target genes as well as NF κ B subunits and increased sensitivity to 4-hydroxytamoxifen (4-OHT) treatment. Our results provide a mechanism by which COUP-TFII maintains endocrine sensitivity by suppressing NF κ B expression and activity in breast cancer cells.

2. Materials and methods

2.1. Chemicals

4-hydroxytamoxifen (4-OHT) and BMS-345541 were purchased from Sigma–Aldrich (St. Louis, MO). Tumor necrosis factor α (TNF α) was purchased from PeproTech (Rocky Hill, NJ).

2.2. Antibodies

The following antibodies were purchased: monoclonal (mAb) anti-human COUP-TFII (R&D systems, Minneapolis, MN); polyclonal RelA/p65, polyclonal and mAb RelB, mAb NF κ B1 p105/p50, and polyclonal p-NF κ B p65/RelA (Ser 529) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal c-Rel, polyclonal NF κ B1 p105/p50, and polyclonal NF κ B2 p100/p52 (Cell Signaling, Danvers, MA), β -actin (Sigma–Aldrich). HRP-conjugated secondary antibodies were from GE Healthcare (Piscataway, NJ).

2.3. Cell culture and transient transfection

MCF-7 breast cancer cells were purchased from ATCC and used at passage <10. LCC9 are ER α /progesterone receptor (PR)+, multi-antiestrogen (tamoxifen and fulvestrant)-resistant breast cells derived from MCF-7 cells and were kindly provided by Dr. Robert Clarke (Brunner et al., 1997). MCF-7 and LCC9 cells were maintained as in (Riggs et al., 2006). Cells were transiently transfected as indicated in figure legends using FuGENE HD (Roche, Indianapolis, IN) as per the manufacturer's protocol.

2.4. Luciferase assay

To analyze NF κ B activity, MCF-7 and LCC9 cells were transiently transfected with pGL4.32[luc2P/NF- κ B-RE/Hygro] (Promega, Madison, WI) containing five copies of a NF κ B response element, pGL4-hRluc-TK (*Renilla*, Promega), and pcDNA3.1 or pcDNA3.1-mCOUP-TFII (kindly provided by Drs. Sophia and Ming-Jer Tsai (Qiu et al., 1995)) for 48 h before performing dual luciferase assay (Promega). Where indicated, cotransfection was performed with plasmids for CBP (kindly provided by Dr. Margarita Hadzopoulou-Cladaras (Dell and Hadzopoulou-Cladaras, 1999)), SRC-1 (kindly provided by Dr. Bert W. O'Malley (Lanz et al., 1999)), GRIP1/SRC-2, or ACTR/SRC-3 (kindly provided by Dr. Michael Stallcup (Koh et al., 2001)).

2.5. NF κ B pathway PCR array

LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-mCOUP-TFII for 24 h before treating with 10 ng/ml TNF α for 6 h. RNA was isolated using RNeasy (Qiagen, Valencia, CA) and cDNA was prepared using RT² First Strand Kit (SABiosciences/Qiagen, Valencia, CA). Human NF κ B Signaling Pathway PCR Array (SABiosciences/Qiagen) was run according to manufacturer's instructions.

2.6. NF κ B family DNA-binding assays

MCF-7 or LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before preparing nuclear extract (NE). In brief, following centrifugation, cells were resuspended in 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT, Sigma–Aldrich), 1 $\times$ protease and phosphatase inhibitors (Roche). The cells were lysed with a 27 gauge syringe and centrifuged. The cytosolic extract was removed and the resulting nuclear pellet was resuspended in NE buffer (10 mM Tris HCl, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X 100, 12.5% glycerol, pH 7.9), 1 mM DTT, 1X protease and phosphatase inhibitor (Roche)), sonicated, and then sedimented by centrifugation with the resulting supernatant being the NE. Following protein concentration determination (as above), 15 μ g NE was used for TransAM NF κ B DNA binding ELISA assays (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Briefly, NE was added to 8 well strips containing immobilized NF κ B RE oligonucleotide. Primary antibodies specific for NF κ B subunits RelB, RelA, p50, p52, and c-Rel were used to identify subunit-specific NF κ B activation. Secondary HRP-conjugated antibody and provided buffers provided colorimetric quantitation via spectrophotometric analysis at 450 nm.

2.7. Co-immunoprecipitation

Nuclear extracts were prepared from MCF-7 cells as in (Litchfield et al., 2012). Dynabeads protein A (Life Technologies, Carlsbad, CA) were incubated with COUP-TFII antibody (Abcam, Cambridge, MA) or negative control IgG (Santa Cruz) for 30 min prior to the addition of 400 μ g MCF-7 NE for 4 h at 4 °C with rotation. Following washes, protein was eluted with Elution Buffer (Life Technologies, Carlsbad, CA) and pH was adjusted with 1 M TrisHCl.

2.8. Cell proliferation assay

MCF-7 and LCC9 cells were grown for 48 h in medium containing 5% charcoal-stripped fetal bovine serum (CSS) and transfected with pcDNA or pcDNA-mCOUP-TFII for 24 h before treatment with 0.01–5 μ M BMS-345541 in DMSO for 5 days. LCC9 cells were also treated with 1 μ M 4-OHT 10 ng/ml TNF α . Cell proliferation was assayed using CellTiter Aqueous One Solution Cell Proliferation Assay (MTS; Promega).

2.9. RNA isolation, RT-PCR and quantitative real-time-PCR (qPCR)

RNA isolation, RT-PCR, and qPCR were performed as in (Litchfield et al., 2012). The following SYBR green primers were used for qPCR analysis, relative to *GAPDH* control: *RELB* (Yun et al., 2011), *REL*, *NFKB1* and *NFKB2* (Panguluri et al., 2010), *IL6* (Sansone et al., 2007), *A20/TNFAIP3* (Vendrell et al., 2007), *ICAM1* (Silverman et al., 2001), *NR2F2*

and *GAPDH* (More et al., 2003), *Nr2f2* (Forward 5'-CCCCCATAGATATGGCAATGGTAGTCAGCACG-3', Reverse 5'-TTGATTATTATTGAATTGCCATATATGGCC-3'). p65/*RELA* was measured using TaqMan Gene Expression Assay (Applied Biosystems) relative to 18S control.

2.10. Microarray analysis

Published microarray data was obtained from Gene Expression Omnibus (GEO) (dataset GSE17705) on primary breast tumors from 298 ER α + patients treated with tamoxifen for 5 years to correlate genomic markers to relapse free survival (Symmans et al., 2010). We analyzed this dataset to assess the correlation between COUP-TFII (*NR2F2*) expression and NF κ B subunit (*NFKB1*, *NFKB2*, *REL*, *RELA*, *RELB*) and target gene (*ICAM1*, *IL6*, *TNFAIP3*) expression. Gene expression data was log2 transformed and quantile normalized prior to analysis. Correlation analysis was performed using the Spearman Rho (ρ S) statistic to assess whether *NR2F2* expression was inversely correlated with NF κ B subunit/target genes.

2.11. Statistical analysis

Statistical analyses were performed as in (Litchfield et al., 2012).

3. Results

3.1. COUP-TFII suppresses NF κ B activity

To determine if NF κ B activity is differentially regulated by COUP-TFII in endocrine-sensitive vs. -resistant cells, MCF-7 (sensitive) and LCC9 (resistant) cells were transfected with a luciferase reporter containing five tandem repeats of a NF κ B responsive element as well as pcDNA3.1 parental plasmid or pcDNA3.1-COUP-TFII (Fig. 1A). Transfected LCC9 cells showed ~5-fold higher COUP-TFII expression compared with similarly transfected MCF-7 cells (Supplemental Fig. 1). Basal NF κ B activity in LCC9 is ~5-fold higher than MCF-7 cells. When treated with TNF α , a large increase in NF κ B activity is observed in LCC9 but not MCF-7 cells. A statistically significant, dose-dependent decrease in the TNF α -induced NF κ B activation occurs in LCC9 cells upon overexpression of COUP-TFII. These data demonstrate that COUP-TFII suppresses NF κ B activity in endocrine-resistant breast cancer cells. The expression of NF κ B subunits *NFKB2*, *REL*, and *RELB* was higher in LCC9 compared to MCF-7 cells (Fig. 1B), accounting for the higher NF κ B activity in LCC9 cells.

3.2. Identification of effects of COUP-TFII on the expression of genes related to the NF κ B-regulated signaling pathway

An NF κ B signaling pathway PCR array was used to determine the effect of COUP-TFII on the expression of 84 genes related to NF κ B-mediated signaling in LCC9 endocrine-resistant cells treated with TNF α using a 3-fold cutoff (Supplemental Fig. 2A). As displayed in the resulting heatmap, a general decrease in the expression of NF κ B pathway genes was detected with COUP-TFII overexpression (Supplemental Fig. 2B, increased COUP-TFII protein is shown in Supplemental Fig. 2). Among the genes suppressed >3-fold by COUP-TFII were NF κ B -responsive genes, *i.e.*, *CCL2*, *IL6*, *LTA*, *ICAM1*, and *TNFAIP3*, as well as

NF κ B subunits (*RELB*), reflecting a possible role for COUP-TFII in downregulation of the NF κ B pathway.

3.3. COUP-TFII suppresses endogenous NF κ B target gene expression

To extend and confirm the results from the NF κ B pathway PCR array, MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII and treated with TNF α to determine the effect of COUP-TFII on endogenous NF κ B target gene expression. COUP-TFII expression following transient transfection was verified via western blot (Supplemental Fig. 1). Three NF κ B target genes with known roles in breast cancer progression were examined through qPCR to validate the results from the NF κ B pathway PCR array: interleukin 6 (*IL6*), intercellular adhesion molecule 1 (*ICAM1*), and TNF α -induced protein 3 (A20, *TNFAIP3*) (Fig. 2). Higher basal levels of *IL6* and *TNFAIP3* transcripts were observed in LCC9 compared to MCF-7 cells (Supplemental Fig. 3). TNF α increased the expression of *IL6*, *ICAM1*, and *TNFAIP3* in both cell lines (Fig. 2A–C). COUP-TFII overexpression reduced the basal expression of *IL6* in LCC9 cells (Fig. 2A) and *ICAM1* and *TNFAIP3* in both MCF-7 and LCC9 cells (Fig. 2B and C). The TNF α -induction of *IL6* and *ICAM1* was reduced by COUP-TFII in LCC9 cells (Fig. 2A and B), while the TNF α -induction of *TNFAIP3* was reduced in MCF-7 cells (Fig. 2C). The suppression of *IL6*, *ICAM1*, and *TNFAIP3* expression by COUP-TFII is in agreement with the data from the NF κ B PCR array.

3.4. COUP-TFII selectively suppresses NF κ B subunit gene and protein expression

RELB expression was decreased by COUP-TFII overexpression in the NF κ B pathway PCR array. Increased NF κ B subunit expression has been reported in several lineages of endocrine-resistant breast cancer cells (Nehra et al., 2010; Riggins et al. 2005; Yde et al., 2012a,b), but possible correlation with COUP-TFII expression has not yet been examined. Consistent with these reports, we observed higher *NFKB2*, *REL*, and *RELB* expression in LCC9 vs. MCF-7 cells (Fig. 1B). To compare the effect of COUP-TFII on basal and TNF α -induced increases in NF κ B subunit expression, we set the parental vector (pcDNA) transfected relative expression of each gene to 1 in both MCF-7 and LCC9 cells (Fig. 3). TNF α increased the expression of *NFKB1*, *NFKB2*, *REL*, and *RELB*, but not *RELA* in both MCF-7 and LCC9 cells (Fig. 3A–E). COUP-TFII overexpression inhibited the basal expression of *REL* and *RELA* in LCC9 cells (Fig. 3C and E). The TNF α -induced increase in *NFKB1*, *REL*, and *RELB* expression was significantly reduced by COUP-TFII in LCC9 cells, but not in MCF-7 cells (Fig. 3A, C, and D).

Next, we examined the effect of COUP-TFII on basal and TNF α -alterations in NF κ B subunit protein expression. We set the parental vector (pcDNA) transfected relative expression of protein to 1 in MCF-7 to compare MCF-7 vs. LCC9 expression of the NF κ B subunits (Fig. 4). LCC9 cells have higher basal expression of NF κ B1 p105 and p50; NF κ B2, RelB, and cRel compared to MCF-7 cells. RelA/p65 was lower in LCC9 than MCF-7 cells. Our data appear to differ from a report by Riggins et al. (2005) showing higher RelA/p65 and equal NF κ B1 p50 protein levels in LCC9 and LCC1 cells, but we note that we are comparing LCC9 with MCF-7, not LCC1 cells. At the protein level, COUP-TFII overexpression decreased basal NF κ B1 p105, NF κ B2 p100, and RelB in MCF-7 cells and

basal NF κ B2 p100 in LCC9 cells (Fig. 4). COUP-TFII inhibited the TNF α -induced expression of NF κ B2 p100, RelA, RelB, and c-Rel in MCF-7 cells and NF κ B1 p105, RelA, and RelB in LCC9 cells (Fig. 4). Thus, COUP-TFII inhibits TNF α -induced increases in NF κ B1 and RelB at both the mRNA and protein levels in LCC9 cells. Differences between protein and mRNA results for NF κ B subunit expression may result from treatment conditions used for the experiment. Future plans include dissecting the time course of COUP-TFII's effect on mRNA and protein expression.

3.5. COUP-TFII and NF κ B expression are inversely correlated in breast cancer patient samples

To examine the relationship between COUP-TFII and NF κ B in breast cancer patients, publically available microarray gene expression data from 298 ER α + patients treated with tamoxifen for 5 years was analyzed using the Spearman Rho (ρ S) statistic determine if there was a correlation between COUP-TFII expression and the expression of NF κ B subunits and target genes. COUP-TFII (*NR2F2*) expression was inversely correlated with NF κ B subunit genes *NFKB2*, *REL*, *RELA*, and *RELB* and NF κ B target genes *ICAM1*, *IL6*, and *TNFAIP3* (Table 1).

3.6. COUP-TFII inhibits RelB, RelA, and p50 NF κ B-DNA binding

To examine the effect of COUP-TFII on specific NF κ B subunit interaction with the NF κ B RE, TransAM NF κ B family assays were performed (Fig. 5). This assay quantitates specific protein-DNA binding in an ELISA format. The higher RelB-DNA binding activity detected in MCF-7 compared to LCC9 cells contrasts with the higher RELB mRNA (Fig. 1B) and protein (Fig. 4) detected in LCC9 cells. The lack of increase in RelB-DNA binding in TNF α -treated MCF-7 cells in the ELISA was also surprising since we detected increased RELB mRNA in TNF α -treated MCF-7 cells (Fig. 3D); although, TNF α did not increase total RelB protein in MCF-7 cells (Fig. 4). The precise reason for this difference is unknown. It is possible that RELB, while higher in expression, might not be activated in LCC9 and thus, would not be expected to enter the nucleus. Basal RelA and cRel activation were not statistically different between the two cell lines, in agreement with mRNA and protein expression levels shown in Figs. 1B and 4. NF κ B2 p52 activation was low in MCF-7 or LCC9 (data not shown), consistent with the lack of p52 protein expression seen in western blots (Fig. 4).

In agreement with the higher level of NF κ B1 p50 protein in LCC9 compared to MCF-7 cells (Fig. 4), NF κ B1 p50 DNA binding activity was greater in NE from LCC9 than MCF-7 cells. While TNF α increased NF κ B1 p50 activation in MCF-7 cells, no increase was detected in LCC9 cells. These data agree with the TNF α -induced increase in *NFKB1* mRNA expression in MCF-7, but not the similar fold increase in *NFKB1* mRNA in LCC9 cells (Fig. 3A). COUP-TFII had no effect on basal NF κ B1 p50 DNA binding in MCF-7 cells, but inhibited basal NF κ B1 p50 DNA binding in LCC9 cells. These data are opposite of the effect on NF κ B1 p50 protein levels in MCF-7 where COUP-TFII reduced basal NF κ B1 p50 protein, but had no effect on the amount of NF κ B1 p50 in TNF α -treated MCF-7 cells (Fig. 4). These data likely reflect differences in the type of cell extracts used in these assays, *i.e.*, whole cell extracts in Fig. 4 would include active and inactive proteins vs. the NE in Fig. 5 which

would contain only active proteins. While no further increase in NF κ B1 p50 DNA binding was detected with TNF α treatment of LCC9 cells, COUP-TFII reduced the level of activated NF κ B1 p50 DNA binding detected.

RelB activation was significantly decreased by COUP-TFII-transfection in TNF α -treated MCF-7 cells. These data reflect the decrease in RelB protein with COUP-TFII transfection of MCF-7 cells (Fig. 4). In contrast, there was no significant effect of COUP-TFII on TNF α -induced *RELB* mRNA expression in MCF-7 cells (Fig. 3D). Activation of RelA was inhibited by COUP-TFII in both MCF-7 and LCC9 TNF α -treated cells as well as untreated LCC9 cells. COUP-TFII inhibited NF κ B1/p50 more than RelA. The inhibition of RelA-DNA binding agrees with the decrease in RelA protein in COUP-TFII-transfected, TNF α treated MCF-7 and LCC9 cells (Fig. 4).

Together, these data suggest that COUP-TFII has the most impact on inhibiting the DNA binding of NF κ B subunit NF κ B1 p50 to the NF κ B RE *in vitro* and these results reflect the ability of COUP-TFII to reduce NF κ B1 mRNA in LCC9 cells and NF κ B1 p50 protein in both MCF-7 and LCC9 cells.

3.7. COUP-TFII interacts with NF κ B subunits

As COUP-TFII overexpression reduced NF κ B1 p50 and RelB activation in TNF α -treated MCF-7 cells (Fig. 5), and inhibited target gene expression (Fig. 2A–D), co-immunoprecipitation was used to determine if endogenous COUP-TFII interacts with NF κ B subunits in NE from TNF α -treated MCF-7 cells. COUP-TFII basal expression is higher in MCF-7 than LCC9 cells progression (Riggs et al., 2006) (see also Supplemental Fig. 1). Immunoprecipitation (IP) of COUP-TFII revealed interaction between endogenous COUP-TFII and the NF κ B subunit RelB and NF κ B1 p50 in the NE (Fig. 6A). While interaction with RelB appears robust, we note that p50 is not detectable in the input NE, but a faint band is seen in the COUP-TFII-IP.

3.8. Inhibition of coactivator function by COUP-TFII reduces NF κ B transcriptional activity

COUP-TFII was reported to inhibit the activity of androgen receptor (AR) by competing with transcriptional coactivators for AR interaction (Song et al., 2012). SRC-1, SRC-2, SRC-3, and CBP function as NF κ B coactivators (Gao et al., 2005). Each of these coactivators also have known interactions with COUP-TFII (Litchfield and Klinge, 2012). To determine if COUP-TFII represses NF κ B signaling by interfering with coactivator function, LCC9 cells, with low endogenous COUP-TFII ((Riggs et al. 2006) and Supplemental Fig. 1) and high NF κ B activity (Fig. 1A) were cotransfected with an NF κ B luciferase reporter, with either pcDNA3.1 (empty vector negative control) or pcDNA3.1-COUP-TFII plasmids (as in Fig. 1A), and expression plasmids for SRC-1, SRC-2, SRC-3, or CBP. The cells were treated with 10 ng/ml TNF α for 6 h to activate NF κ B. TNF α increased Firefly luciferase activity ~10-fold (data not shown). The TNF α -induced luciferase reporter activity in the pcDNA-transfected cells was set to one to allow comparison of the relative effect of each transfected expression vector. SRC-1, SRC-3, and CBP increased NF κ B activity in LCC9 cells compared to parental vector pcDNA (Fig. 6B). SRC-2 transfection had no effect on NF κ B activity. Cotransfection with COUP-TFII decreased the TNF α -

activated luciferase activity ~50%, similar to the data in Fig. 1A. COUP-TFII significantly inhibited the increase in NF κ B activity by the coactivators SRC-1, SRC-3, and CBP (Fig. 6B), implicating COUP-TFII inhibition of coactivator function, *e.g.*, competing with NF κ B for coactivator interaction, as part of the mechanism of NF κ B suppression.

3.9. Inhibition of p65/RelA phosphorylation by COUP-TFII

Phosphorylation of p65/RelA at Serine 529 is necessary for transcriptional activation (Wang and Baldwin, 1998; Zhao et al., 2008). To determine if COUP-TFII has an effect on p65/RelA phosphorylation, MCF-7 and LCC9 cells were transfected with COUP-TFII and treated with TNF α , and p65/RelA phosphorylation at Serine 529 was compared to total p65/RelA and β -actin (Fig. 7). Two p-RelA Ser 529 bands were detected and quantified relative to the β -actin loading control. Two p-RelA/p65 Ser 529 bands were seen previously (Kim et al., 2007; Yde et al., 2012a,b). COUP-TFII decreased both basal and TNF α -induced phosphorylation of p65/RelA in MCF-7 and TNF α -induced phosphorylation in LCC9 cells, suggesting this may be part of the mechanism of inhibition of NF κ B activation by COUP-TFII.

3.10. Endocrine-resistant cells are sensitive to NF κ B-inhibition

Cell proliferation assays were performed to determine phenotypic effects of suppression of NF κ B activity by COUP-TFII and to determine whether inhibition of NF κ B by BMS-345541, an NF κ B inhibitor that functions by inhibiting I κ B kinase and subsequent downstream NF κ B activation (Burke et al., 2003), would affect sensitivity of cells to 4-OHT. MCF-7 and LCC9 cells were treated with BMS-345541 in a range of concentrations (0.01–5 μ M) determined to be effective in inhibiting NF κ B activity in serum-starved MCF-7 and LCC9 cells (Supplemental Fig. 4). MCF-7 were unaffected by BMS-345541 (Fig. 8A), commensurate with the low activity of NF κ B in this cell line (see Fig. 1A). In contrast, the viability of LCC9 cells was inhibited in a concentration-dependent manner by BMS-345541 (Fig. 8B), reflecting LCC9's increased reliance on a functional NF κ B pathway for cell growth as endocrine-resistant cells (Nehra et al., 2010; Riggins et al., 2005).

Reduced LCC9 cell viability was observed upon combined transfection with COUP-TFII and concomitant treatment with 4-OHT (Fig. 9A, white bars), reflecting COUP-TFII's role in enhancing sensitivity of LCC9 to antiestrogens, as we previously reported (Riggs et al., 2006). LCC9 cells transfected with COUP-TFII and treated with a combination of 4-OHT, and BMS-345541 (Fig. 9A, white bars) exhibited a reduction in the higher range of BMS-345541 concentrations (Fig. 9A). Interestingly, when LCC9 cells were treated with TNF α , 4-OHT increased cell viability (Fig. 9B, dark grey bars). This effect was not observed in MCF-7 cells (Supplemental Fig. 5 or data not shown). This finding could have important translational implications if tamoxifen therapy causes similar effects in patients with high levels of inflammation and NF κ B activation. This observed induction of LCC9 cell proliferation with TNF α and 4-OHT treatment was blocked by transfection with COUP-TFII (Fig. 9B, light grey bars) and/or treatment with higher concentrations of BMS-345541 (Fig. 9B, white bars).

4. Discussion

The NF κ B pathway is constitutively activated in many types of cancer, including breast, gastrointestinal, genitourinary, hematological, and gynecological cancers, through a variety of mechanisms (Prasad et al., 2010). NF κ B target genes have roles in oncogenic transformation, including apoptosis resistance (Opipari et al., 1992; Vendrell et al., 2007), invasion (Soria and Ben-Baruch, 2008), and epithelial to mesenchymal transition (Huber et al., 2004). Here we demonstrate that COUP-TFII inhibits TNF α -induced NF κ B activity in LCC9 endocrine-resistant breast cancer cells. Consistent with previous reports that the NF κ B pathway is activated during progression to breast cancer endocrine-resistance (Nakshatri et al., 1997; Nehra et al., 2010; Pratt et al., 2003; Riggins et al., 2005; Sovak et al., 1997; Yde et al., 2012a,b), LCC9 cells exhibited higher basal and TNF α -induced NF κ B activity than MCF-7 parental, endocrine-sensitive breast cancer cells. This is the first demonstration that COUP-TFII inhibits NF κ B activity. This inhibition led to downregulation of TNF α -induced NF κ B target gene expression of *IL6*, *ICAM1*, and *TNFAIP3*, which had reduced basal and/or TNF α -induced expression upon COUP-TFII overexpression. The inflammatory cytokine IL6 is a negative prognostic indicator for breast cancer patients (Knupfer and Preiss, 2007), and may have a role in trastuzumab resistance (Korkaya et al., 2012). Downregulation of the glycoprotein adhesion molecule ICAM1 at the mRNA and protein levels was reported to suppress human breast cell invasion (Rosette et al., 2005). The ubiquitin-editing enzyme A20/TNFAIP3 protects MCF-7 cells from TNF α -induced apoptosis, and cells overexpressing TNFAIP3 are resistant to tamoxifen (Krikos et al., 1992; Opipari et al., 1992; Vendrell et al., 2007). Thus, we suggest the ability of COUP-TFII to inhibit NF κ B-stimulated TNFAIP3 may play a role in the observed ability of COUP-TFII to resensitize LCC9 cells to 4-OHT.

NF κ B subunit expression is regulated by the NF κ B pathway in a positive feedback loop. Specifically, RelA activates expression of RelB (Bren et al., 2001) and NF κ B2 (Lombardi et al., 1995), while NF κ B1/p50 expression is regulated by a combination of p50 and RelA (Ten et al., 1992). Similarly, c-Rel is also capable of autoregulation (Hannink and Temin, 1990). Because COUP-TFII suppresses genes that are targets of the NF κ B pathway, this would also be expected to lead to reduced expression of the NF κ B subunits as observed. Consistent with our data showing higher NF κ B subunit and target gene expression in LCC9 endocrine-resistant cells, NF κ B subunits *NFKB1*, *NFKB2*, *RELA*, and *REL* as well as the NF κ B gene targets *ICAM1* and *TNFAIP3* are also upregulated in inflammatory breast cancer patient samples (Lerebours et al., 2008). These data also suggest that COUP-TFII inhibits both the classical and alternative (canonical and noncanonical) NF κ B pathways. A previous study showed that overexpression of COUP-TFII inhibited 15-PGJ2-induced IL-8 mRNA expression and promoter activity in U937 human myeloid cells, a result which the authors attributed to COUP-TFII competing with PPAR γ to form a heterodimer with RXR or by forming a COUP-TFII/RXR heterodimer and competing with PPAR γ /RXR for DNA binding (Zhang et al., 2001).

COUP-TFII was recently identified as one of four NRs that are significant predictors of metastasis-free survival in tamoxifen-treated breast cancers, independent of ER α expression (Muscat et al., 2013). We previously observed that COUP-TFII expression was reduced with

histological grade (Litchfield et al., 2012) and this was confirmed by other investigators in (Muscat et al., 2013). Here we report for the first time that COUP-TFII, *i.e.*, *NR2F2* gene expression was inversely correlated with NFκB subunit genes *NFKB2*, *REL*, *RELA*, and *RELB* and NFκB target genes *ICAM1*, *IL6*, and *TNFAIP3* in microarray data from 298 ERα+ breast tumors from patients treated with TAM for 5 years (Table 1).

Mechanistically, we have demonstrated that COUP-TFII interacts with NFκB subunit RelB and NFκB1 p50 and inhibits NFκB-DNA-binding. COUP-TFII also inhibited the ability of coactivators SRC-1, SRC-3, and CBP to enhance NFκB transactivation and TNFα-induced phosphorylation of RelA on Ser 529, required for p300 coactivator recruitment and RelA transactivation function (Chew et al., 2009). The effects of COUP-TFII on NFκB lead to inhibition of cell proliferation, with endocrine-resistant LCC9 cell proliferation effectively reduced when treated with an NFκB inhibitor. Overall, these data suggest that the general downregulation of NFκB target gene expression by COUP-TFII may play a role in endocrine sensitivity. As NFκB is activated in endocrine-resistant cells, blockade of downstream NFκB target gene expression by COUP-TFII offers a possible mechanism by which COUP-TFII enables breast cancer cells to maintain endocrine sensitivity. Natural product inhibitors of NFκB activity such as parthenolide (Riggins et al., 2005), curcumin (Jobin et al., 1999; Singh and Aggarwal, 1995), and ellagic acid (Edderkaoui et al., 2008), as well as synthetic inhibitors (Burke et al., 2003; Zhou et al., 2005a,b), provide compounds for future experimental and possible translational purposes. The surprising observation that the combination of 4-OHT with lower concentrations of the NFκB inhibitor BMS-345541 stimulated the proliferation of LCC9 cells treated with TNFα (but not MCF-7 cells, data not shown) offers a cautionary note that requires further *in vivo* study. Overall, these data point to the potential for evaluating COUP-TFII as a marker for breast tumors that may be sensitive to the combination of NFκB inhibitors with antiestrogen therapies, *e.g.* tamoxifen, to promote endocrine sensitivity and prevent disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Komen for the Cure grant KG080365 to C.M.K. L.M.L. was supported in part by a predoctoral fellowship from NIEHS T32 ES011564.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mce.2013.10.010>.

Abbreviations

COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
NFκB/NFkappa B	nuclear factor κB

SERM	selective estrogen receptor modulator
TAM	tamoxifen
ERα	estrogen receptor
SERD	selective estrogen receptor downregulator
4-OHT	4-hydroxytamoxifen
TNFα	tumor necrosis factor
RE	response element
qPCR	quantitative real-time PCR
IL6	interleukin 6 (IL6)
ICAM1	intercellular adhesion molecule 1
A20/TNFAIP3	TNF α -induced protein 3

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Fig. 1.

COUP-TFII suppresses NF κ B activity in LCC9 cells. (A) MCF-7 and LCC9 cells were transfected with a NF κ B luciferase reporter and increasing concentrations of pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before performing dual luciferase assay (Promega). Firefly luciferase values are shown relative to protein concentration for a representative experiment of quadruplicate values $\pm$ SEM. *Significantly different $P < 0.05$ from pcDNA + TNF α in LCC9. The numerical values over the data bars for LCC9 cells are fold relative to pcDNA-transfected LCC9. (B) Comparison of NF κ B subunit mRNA expression in MCF-7 vs. LCC9 cells. Values are the mean of 4–8 separate experiments $\pm$ SEM. * $P < 0.05$ vs. MCF-7.

Fig. 2.

COUP-TFII inhibits expression of NFκB target genes. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNFα for 6 h before preparing RNA and cDNA. mRNA expression of NFκB target genes identified in the NFκB pathway array (A) *IL6*, (B) *ICAM1*, (C) *TNFAIP3* was analyzed by qPCR. Note that relative expression of each gene was normalized to pcDNA-transfected, control treated cells within each cell line. Values are the mean of 2–7 separate experiments ± SEM. Bars indicate significant differences ($p < 0.05$) between the indicated samples, demonstrating the effect of COUP-TFII on basal or TNFα-induced gene expression in each cell line.

Fig. 3.

COUP-TFII inhibits mRNA expression of NFκB subunits in LCC9 cells. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNFα for 6 h before preparing RNA and cDNA. mRNA expression of NFκB subunits (A) *NFKB1*, (B) *NFKB2*, (C) *REL*, (D) *RELB*, (E) *RELA* was analyzed by qPCR. Bars indicate significant differences ($p < 0.05$) between the indicated samples, demonstrating the effect of COUP-TFII on basal or TNFα-induced gene expression in each cell line.

Fig. 4.

COUP-TFII inhibits protein expression of NFκB subunits. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNFα for 6 h before preparing whole cell extracts. Expression of NFκB subunits (NFκB1 p105/p50, NFκB2 p100, RelA p65, RelB, c-Rel) was analyzed by western blot with specific antibodies and normalized to the β-actin control in each lane. The NFκB subunit/β-actin value of the pcDNA control was set to 1 for comparison between separate experiments. The expression of each protein was normalized to pcDNA-transfected untreated MCF-7 cells which was set to 1. Note: NFκB2 p52 was not detectable.

Fig. 5.

COUP-TFII decreases NFκB1 p50 and RelA-DNA binding. TransAM NFκB family assays were performed to measure NFκB subunit activation by quantification of NFκB-DNA response element binding in an ELISA assay using 15 μg NE from MCF-7 and LCC9 cells transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with TNFα for 6 h. Values are the average of two determinations ± SEM. *Significantly different $p < 0.05$ from pcDNA-transfected cells.

Fig. 6. COUP-TFII interacts with NFκB subunits and inhibits coactivator function in NFκB activation. (A) 500 μg NE from MCF-7 cells treated with TNFα for 6 h was immunoprecipitated (IP) using a COUP-TFII antibody and separated on a 10% SDS PAGE gel prior to transfer to a PVDF membrane. Interaction with NFκB subunits NFκB1 p105/p50 and RelB was identified using specific antibodies on western blot. (B) LCC9 cells were transfected with a NFκB luciferase reporter and pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h +/- expression plasmids for the coactivators SRC-1, SRC-2, SRC-3, and CBP. Cells were treated with 10 ng/ml TNFα for 6 h before performing dual luciferase assay. Firefly luciferase values are shown relative to protein concentration for a representative experiment of quadruplicate values ± SEM. Values were normalized to TNFα-treated pcDNA vector (negative control) transfected cells. *Significantly different $P < 0.05$ from pcDNA-transfected cells. Bars connect values which are significantly inhibited by COUP-TFII-transfection vs. that particular coactivator alone ($p < 0.05$).

Fig. 7.

COUP-TFII inhibits phosphorylation of p65/RelA. MCF-7 and LCC9 cells were transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 48 h and treated with 10 ng/ml TNF α for 6 h before preparing whole cell extracts. Expression of total p65/RelA and p-RelA Ser 529 were analyzed by western blot with specific antibodies compared to β -actin control. Values represent p-RelA relative to total RelA.

Fig. 8.

LCC9 cells are growth inhibited by NF κ B inhibitor BMS-345541. MCF-7 and LCC9 cells were serum-starved for 24 h before being treated with the indicated concentrations of BMS-345541 for 5 days for an MTT assay. Relative proliferation for untreated cells within each cell line was set to 1. Values are the average of 4 separate treatments $\pm$ SEM. ** $p < 0.05$ vs. no added (0) BMS.

Fig. 9.

Overexpression of COUP-TFII increases endocrine sensitivity in LCC9 endocrine-resistant breast cancer cells. LCC9 cells were serum starved for 24 h before being transfected with pcDNA3.1 or pcDNA3.1-COUP-TFII for 24 h. Cells were treated with the concentrations of BMS-345541 indicated $\pm$ 1 μ M 4-OHT and 10 ng/ml TNF α (B) for 5 days before performing MTT assay to assess changes in cell viability. Values are the average of 4 separate treatments $\pm$ SEM. * p < 0.05 vs. vehicle control within each set of 4 treatment/transfection groups, *i.e.*, within each set of BMS concentrations (0–5 μ M) $\pm$ COUP-TFII transfection $\pm$ 4-OHT. ** p < 0.05 vs. 0 BMS for BMS comparisons only, *i.e.*, 0 BMS vs. 0.01–5 μ M BMS with no added 4-OHT or COUP-TFII transfection.

Table 1

Correlation of COUP-TFII expression to NFκB target and subunit gene expression in ERα+ breast cancer patients treated with tamoxifen. Spearman correlations of gene expression between COUP-TFII (*NR2F2*) and select NFκB-related genes obtained by analysis of microarray data (GSE17705) from 298 ERα+ breast cancer patients treated with tamoxifen for 5 years. Values in bold are statistically significant, as indicated.

Classification	Gene	Spearman Rho	p-Value	Correlation to COUP-TFII
NFκB target	ICAM1	−0.35	0.0000	Inverse
	IL6	−0.16	0.0063	Inverse
	TNFAIP3	−0.23	0.0000	Inverse
NFκB subunit	NFKB1	0.08	0.1638	
	NFKB2	−0.20	0.0004	Inverse
	REL	−0.28	0.0000	Inverse
	RELA	−0.34	0.0000	Inverse
	RELB	−0.12	0.0428	Inverse