SUPPLEMENTAL INFORMATION (Hep-08-1899.R1)

Supplemental Materials and Methods

Materials. Hamster monoclonal anti-mouse Fas antibody (Jo2) was purchased from PharMingen (San Diego, CA). The antibodies against PARP, Mcl-1, Bcl-x_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against EGFR, phospho-PTEN (Ser^{380}), phospho-Akt (Ser^{473}/Thr^{308}), Akt were purchased from Cell Signaling (Beverly, MA). The antibody against GAPDH was purchased from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The COX-2 inhibitor NS398 was purchased from Calbiochem (San Diego, CA). The EGFR inhibitor AG1478 and the caspase substrates (Ac-DEVD-AFC for caspase 3, Ac-IETD-AFC for caspase 8, and Ac-LEHD-AFC for caspase 9) were obtained from EMD Bioscience (Gibbstown, NJ).

Immunoblotting. Mouse liver tissues were homogenized on ice in a modified NP-40 lysis buffer (150 mM NaCl, 1.0 % NP-40, and 50 mM Tris, pH 8.0, 1 mM DTT) containing protease inhibitor cocktail tablets (Roche Diagnostics GmbH). The liver extracts was collected by centrifugation at the speed of 15,000g at 4ºC for 15 minutes to remove cell debris and stored in aliquots at -80ºC until use. The protein concentrations in the liver extracts were determined by the Bio-Rad protein assay (Bio-Rad, CA). 30 μg of liver protein was subjected to SDS-PAGE and the separated proteins were electrophoretically transferred onto the nitrocellulose membranes (BioRad, CA). Nonspecific binding was blocked with PBS-T (0.05% Tween 20 in PBS) containing 5% non-fat milk for 1 hr at room temperature. The membranes were then incubated overnight at 4°C with antibodies against PARP, EGFR, phospho-PTEN (Ser^{380}), phospho-Akt (Ser^{473}/Thr^{308}), Akt, Mcl-1, Bcl-x_L in PBS-T containing 1% non-fat milk at

the dilutions specified by the manufacturers. Following three washes with PBS-T, the membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies at 1:10,000 dilution in PBS-T containing 1% non-fat milk for 1 hour at room temperature. The membranes were then washed 3 times with PBS-T and the protein bands were visualized with the ECL Western blotting detection system according to the manufacturer's instructions. GAPDH was used as the loading control.

Supplemental Figure Legends

Supplemental Figure S1. The level of EGFR in mice with altered expression of COX-2. The COX-2 Tg, COX-2 KO and wild type mice were injected intraperitoneally with saline (A) or Jo2 (0.5 μg/g body weight) (B) and the livers were harvested 4 hours after the injection. The liver tissues were homogenized and the obtained cellular proteins were subjected to SDS-PAGE and Western blot analysis to determine the protein levels of EGFR and GAPDH. The blots in this figure were obtained from additional 2-3 mice for each group.

Supplemental Figure S2. The level of p65 NFκB in mice with altered expression of COX-2. Liver tissue samples from the COX-2 Tg, COX-2 KO and wild type mice were subjected to SDS-PAGE and Western blot analysis to determine the protein levels of p65 NFκB, phospho-NF-κB p65 (Ser^{276}), and phospho-NF-κB p65 ($Ser^{468/536}$). Western blot for GAPDH was shown as the loading control. The COX-2 transgenic livers have higher level of NFκB p65 as well as phosphorylated NFκB p65 than the wild type livers.

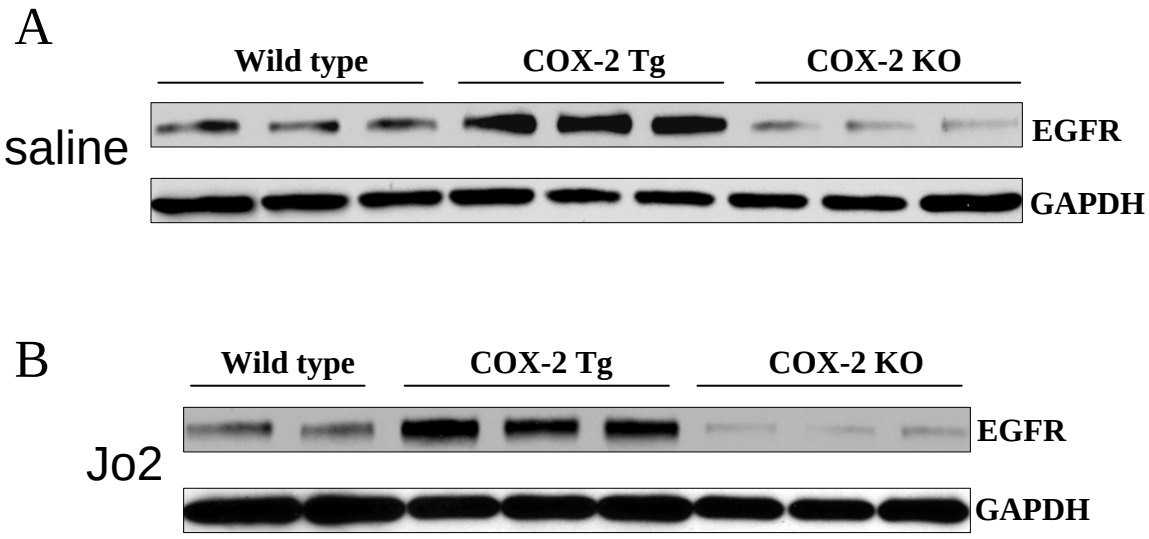
Supplemental Figure S3. Increased binding of NF-κB to its response element in EGFR promoter. Equal amounts of liver tissue nuclear extracts from three wild type mice and three COX-2 transgenic mice were incubated with biotinylated EGFR promoter NF-κB response element oligonucleotide followed by immunoblotting with NF-κB p65. The first lane represents cold competition (nuclear extract from COX-2 Tg liver incubated with biotinylated EGFR promoter NF-κB response element oligonucleotide plus 20-fold cold unlabeled oligonucleotide).

For preparation of nuclear extract, the liver tissues from either wild type mice or COX-2 transgenic mice were ground in liquid nitrogen and then homogenized with hypotonic buffer (including 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$ and 10 mM KCl). The Nuclei were recovered by centrifugation of the homogenate at 10,000g for 20 min. The nuclear proteins were extracted by gentle sonication of the nuclei in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5% of NP-40). The nuclear protein suspension was cleared by centrifugation at 20,000g for 5 min. The supernatants were collected and frozen at -80°C. All buffers contained DTT, protease inhibitor cocktail and phosphatase inhibitor. All the steps were carried out on ice or at 4°C. The protein concentrations in the nuclear extracts were measured by the Bio-Rad protein assay (Bio-Rad) using BSA as a standard.

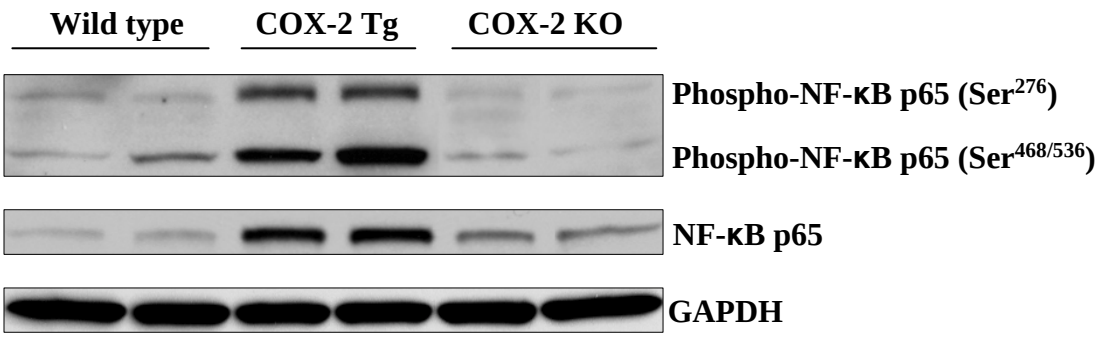
Biotinylated oligonucleotide precipitation assay was performed as described previously with minor modification (Hata et al. Cell 2000;100:229-240). The sequences of biotinylated oligonucleotides corresponding to putative binding site of NFκB in the EGFR promoter region, -504 to -514, are forward: 5'-GGAACGCCCC-3' and reverse: 5'-GGGGCGTTCC-3' [Tao et al, Cell Signal. 2004;16:781-790]. The 5'-biotinylated oligonucleotides were synthesized by Sigma-Genosys (Woodland, TX). Nuclear extracts from liver tissues were prepared according to our previously described above. The binding reaction in the equal amount of nuclear extract was carried out at 4°C for 16 h with 1 μg biotinylated double-strand oligonucleotides and 10 μg poly(dl-dC)·poly(dl-dC).

The DNA-bound proteins were precipitated using ImmunoPure streptavidin-agarose beads (Pierce, Rockford, IL) for 1 h at 4°C and subjected to detect NF-κB p65 by Western blotting.

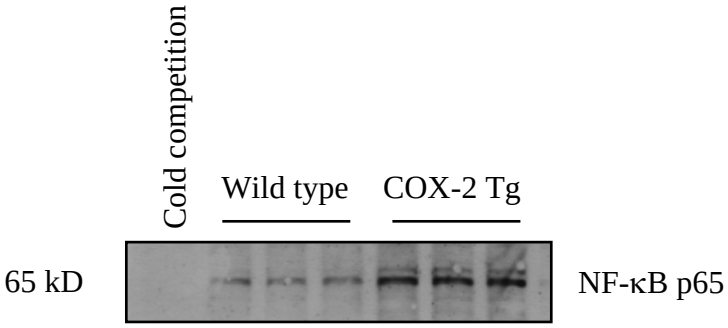
Supplemental Fig. S1



Supplemental Fig. S2



Supplemental Fig. S3



IP: Biotinylated NF-κB response element

IB: NF-κB p65