



**Hepatocyte-specific activation of NF- $\kappa$ B does not aggravate chemical hepatocarcinogenesis in transgenic mice**

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4 **Hepatocyte-specific activation of NF- $\kappa$ B does not aggravate chemical**  
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6 **hepatocarcinogenesis in transgenic mice**  
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40 **Keywords:** Chemical hepatocarcinogenesis, diethylnitrosamine, I $\kappa$ B, IKK  
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46 **List of abbreviations:** DEN, diethylnitrosamine; I $\kappa$ B, inhibitor of nuclear factor kappa B;  
47 IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ ,  $\alpha$ ,  $\beta$  and  $\gamma$  subunits of I $\kappa$ B kinase complex; MEF, murine embryonic  
48 fibroblast; NF- $\kappa$ B, nuclear factor kappa B; TTR, transthyretin.  
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**Abstract**

The NF- $\kappa$ B signaling pathway plays important roles in liver organogenesis and carcinogenesis. Mouse embryos deficient in IKK $\beta$  die in mid-gestation due to excessive apoptosis of hepatoblasts. Although activation of the NF- $\kappa$ B signaling pathway has been demonstrated in human hepatocellular carcinoma, the direct effect of NF- $\kappa$ B in hepatocarcinogenesis is controversial. In this study, we have generated transgenic mice in which a constitutively active form of IKK $\beta$  was expressed in a hepatocyte-specific manner. Using electrophoretic mobility shift assay, we documented increased NF- $\kappa$ B activities and upregulated levels of NF- $\kappa$ B downstream target genes, Bcl-xL and STAT5, in the transgenic mouse livers. These results confirmed that the NF- $\kappa$ B pathway was activated in the livers of the transgenic mice. However, there was no significant difference in tumor formation between transgenic and wild-type mice up to an age of 50 weeks. When we treated the transgenic mice with the chemical carcinogen diethylnitrosamine (DEN), we observed no significant differences in the incidence and size of liver tumors formed in these mice with and without DEN treatment at 35 weeks of age, suggesting that activated NF- $\kappa$ B pathway in the livers of the transgenic mice did not enhance hepatocarcinogenesis. Interestingly, some of the transient transgenic embryos (E12.5) had abnormal excessive accumulation of nucleated red blood cells in their developing livers. In summary, NF- $\kappa$ B activation in hepatocytes did not significantly affect chemical hepatocarcinogenesis. In addition, the TTR/IKKCA transgenic mice may serve as a useful model for studying the role of NF- $\kappa$ B activation in hepatocarcinogenesis as well as inflammatory and metabolic diseases.

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The nuclear factor  $\kappa$ B (NF- $\kappa$ B) plays important roles in cell survival, inflammation, and innate immune response. In unstimulated cells, NF- $\kappa$ B is kept in the cytoplasm by the inhibitor of NF- $\kappa$ B (I $\kappa$ B), which shields the nuclear localization signal (NLS). Upon stimulation, the I $\kappa$ B kinase (IKK) complex, consisting of IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ /NEMO subunits, phosphorylates I $\kappa$ B and the phosphorylated I $\kappa$ B is then degraded through the ubiquitin-proteasome pathway. Once the inhibitor is degraded, the NLS is exposed. NF- $\kappa$ B is then translocated into the nucleus and activates expression of downstream target genes.

IKK $\alpha$  and IKK $\beta$  share a high degree of homology and are catalytic subunits, whereas IKK $\gamma$  are regulatory subunit. IKK $\alpha$  and IKK $\beta$ , however, do not respond to proinflammatory stimuli such as tumor necrosis factor (TNF- $\alpha$ ), interleukin 1 (IL-1), or lipopolysaccharide (LPS) in the same manner. It was demonstrated that IKK $\alpha$  was not required for the activation of NF- $\kappa$ B pathway by proinflammatory stimuli in IKK $\alpha$ -deficient murine embryonic fibroblast [1]. This finding implicates that the other catalytic subunit IKK $\beta$  may play a major role in inflammatory response. This is strongly supported by gene targeting experiments to disrupt the mouse IKK $\beta$  locus [2-4]. In these studies, loss of IKK $\beta$  led to embryonic lethality. Embryos deficient in IKK $\beta$  could not survive for more than 13.5 days. Excessive apoptosis of hepatoblasts was demonstrated in the embryonic livers. This phenotype was similar to that of mice deficient in RelA (p65), a subunit of NF- $\kappa$ B [5]. Also, marked reduction of NF- $\kappa$ B activity was observed in IKK $\beta$ -deficient MEF treated with proinflammatory stimuli [2-4]. However, IKK $\beta$ -deficient hepatocytes did not show reduced level of NF- $\kappa$ B activation or increased level of apoptosis upon treatment with TNF- $\alpha$  [6]. The different phenotypes observed in constitutive and hepatocyte-specific IKK $\beta$ -deficient mice implicate that IKK $\beta$  may play different roles in development and adult organs [2-4].

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It has been shown that NF- $\kappa$ B is activated in hepatocellular carcinoma (HCC) [7, 8]. Pikarsky et al. [9] have demonstrated that NF- $\kappa$ B promoted hepatocarcinogenesis in the *Mdr2* knockout mice. The above studies strongly suggest that NF- $\kappa$ B is a tumor promoting factor. However, in a recent study from another group using diethylnitrosamine (DEN) to treat hepatocyte-specific IKK $\beta$ -knockout mice, the mice showed an increased incidence of hepatocarcinogenesis [10]. Also, it was shown in the same study that mice lacking IKK $\beta$  in both hepatocytes and hematopoiesis-derived Kupffer cells exhibited a decreased incidence of hepatocarcinogenesis when treated with DEN. The authors suggested that activation of NF- $\kappa$ B in Kupffer cells promoted hepatocarcinogenesis. Nevertheless, the role of hepatocyte-specific NF- $\kappa$ B activation in hepatocarcinogenesis remains unclear. In this study, we generated transgenic mice with liver-specific expression of constitutively active IKK $\beta$ , designated TTR/IKKCA, to examine if activated NF- $\kappa$ B pathway had influence on hepatocarcinogenesis. We also treated the TTR/IKKCA transgenic mice with diethylnitrosamine (DEN) to induce hepatocarcinogenesis. Here we show that NF- $\kappa$ B activation in hepatocytes did not significantly affect hepatocarcinogenesis and chemical hepatocarcinogenesis. We also generated transient transgenic embryos to examine liver organogenesis.

## Materials and Methods

**Transgene constructs.** We generated the transgenic mice, designated TTR/IKKCA, and transient transgenic embryos. It has been demonstrated that serine residues 177 and 181 of IKK $\beta$  are important phosphorylation sites of the subunit [11]. Phosphorylation of these sites activates the complex and leads to subsequent activation of the NF- $\kappa$ B pathway. Replacement of these serine residues with alanine (S177A/S181A) abolishes IKK activation

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3 by proinflammatory stimuli. In contrast, replacement of these serine residues with glutamate  
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5 (S177E/S181E) renders the complex constitutively active. In both, the constitutively active  
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7 form (S177E/S181E) of IKK $\beta$  was expressed in a hepatocyte-specific manner using the  
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9 transthyretin (TTR) promoter [12]. The TTR promoter has been extensively used to mediate  
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11 hepatocyte-specific expression in transgenic mice [13-16]. Serine residues 177 and 181 of a  
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13 mouse IKK $\beta$  expression vector [17] were mutated to glutamate by PCR site-directed  
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15 mutagenesis. The construct was designated as IKK $\beta$ -CA since this mutation renders the  
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17 kinase subunit constitutively active [11]. Oligonucleotides encoding two FLAG epitopes (5'  
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19 to IKK $\beta$ -CA) and the fragment containing the IKK $\beta$ -CA fragment were then subcloned into  
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21 pCR2, an expression vector with CMV promoter, and TTR1 exV3 vector (kind gift of T. van  
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23 Dyke). TTR1 exV3 is a hepatocyte-specific expression vector derived from pTTR1 [12]. The  
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25 hepatocyte-specific expression is mediated by the transthyretin (TTR) promoter. The  
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27 resulting CMV/FLAG-IKK $\beta$ -CA and TTR/FLAG-IKK $\beta$ -CA, respectively, (Figure 1a) were  
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29 used for transfection and microinjection to generate transgenics.  
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39 **Generation of transgenic mice and mouse embryos, PCR genotyping, and RT-**  
40 **PCR for the detection of IKK $\beta$ -CA mRNA.** The animal experiments complied with the  
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42 guidelines prepared by the Committee on the Use of Live Animals in Teaching and Research  
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44 of the University of Hong Kong. Transgenic mice and mouse embryos were generated as  
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46 described previously [18]. Briefly, fertilized oocytes obtained from FVB/N mice were used  
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48 for DNA microinjection. Transgenic mice and mouse embryos were genotyped by PCR using  
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50 DNA extracted from tails and yolk sacs. Primers for genotyping were: forward primer, 5'-  
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52 GCCACCATGGATTACAAGGA; reverse primer, 5'-CACCAGCGGTTTCTGTTCTT. **For**  
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54 **the detection of IKK $\beta$ -CA mRNA with RT-PCR, the following primers were used; forward**  
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3 primer, 5'-GCTGGACTGGTATTTGTGTCTG; reverse primer, 5'-  
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6 GAATTCCTTATCGTCGTCATCCTTG (Figure 1a).  
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11 **Immunohistochemistry.** Embryos were fixed with 4% paraformaldehyde and then  
12 embedded in paraffin. Four-micron-thick sections were cut and detected with anti-FLAG  
13 antibody (M2 mouse monoclonal antibody, Sigma, USA; 1:1000 dilution) using DAKO  
14 EnVision™ + System (DAKO, California, USA). For the detection of p65 nuclear  
15 localization on cryostat sections, anti-p65 antibody (Cat. #RB-1683, NeoMarker, Fremont,  
16 USA; dilution 1:200) was used.  
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28 **Western blotting analysis.** Mouse livers were harvested and homogenized in RIPA  
29 buffer containing 1 x Complete™ protease inhibitors (Roche Diagnostics GmbH, Mannheim,  
30 Germany). The protein concentrations were determined by Bradford assay (Bio-Rad  
31 Laboratories, Hercules, CA, USA). Proteins (20 mg) were separated on a 10% SDS-PAGE  
32 gel and transferred onto membranes. The following antibodies were used: rabbit polyclonal  
33 anti-p65, (sc-372, Santa Cruz, CA, USA, 1:500 dilution), rabbit polyclonal anti-IkBa (sc-371,  
34 Santa Cruz, CA, USA, 1:500 dilution), rabbit polyclonal anti-STAT5 (sc-835, Santa Cruz,  
35 CA, USA, 1:500 dilution), and mouse monoclonal anti-Bcl-x (Transduction Lab., 1:500  
36 dilution). Relative protein expression levels were determined with densitometry using  
37 AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, USA).  
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54 **Preparation of nuclear extracts.** Nuclear extracts were prepared as described  
55 previously [19] with some modifications. Briefly, frozen tissue samples were homogenized in  
56 300 µl cold hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM  
57 dithiothreitol (DTT), 1 x Complete™]. Cells were placed and swollen on ice for 15 min, then  
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3 centrifuged at 1500 rpm for 5 min at 4°C. The cell pellets were resuspended with 200 µl  
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5 buffer A with 0.5% Nonidet NP-40 (Sigma, St Louis, MO) and incubated on ice for an hour  
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7 to lyse the cytoplasmic membranes. After incubation the suspensions were centrifuged at  
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9 11,000 rpm for 5 min at 4°C. The nuclear pellets were resuspended in 50 µl of ice cold buffer  
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11 B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1mM EDTA, 1 mM DTT, 1 x Complete™] and  
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13 incubated on ice for 30 min. Nuclear extracts were obtained by centrifugation at 11,000 rpm  
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15 for 5 min at 4°C.  
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23 **Electrophoretic mobility assay (EMSA).** The NF-κB DNA-binding activity was  
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25 analyzed by EMSA as described previously [7]. Each binding reaction (20 µl) containing 5  
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27 µg of nuclear extracts, 10 mM HEPES (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 5 mM MgCl<sub>2</sub>,  
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29 1 mM DTT, 10% glycerol, 1 µg of poly(deoxyinosinic-deoxycytidylic acid), and 1 pmol 32P-  
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31 5'-endlabeled probe (5 x 10<sup>4</sup> cpm) containing NF-κB-binding site sequence (Santa Cruz, CA,  
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33 USA) was incubated for 30 min at room temperature. For supershift EMSA, rabbit anti-  
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35 p65(sc-372, Santa Cruz, CA, USA, 1:50 dilution) or anti-p50 (sc-114, Santa Cruz, CA, USA,  
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37 1:50 dilution) were added to binding reactions, without labeled oligonucleotide, and  
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39 incubated for 30 min at 4°C. After incubation, labeled oligonucleotides were added and  
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41 incubated at room temperature. DNA-protein complexes were resolved in 5% polyacrylamide  
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43 gels. Gels were dried and autoradiographed at -80°C. **Relative NF-κB activities were**  
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45 **determined by densitometry using AlphaEaseFC software (Alpha Innotech Corporation, San**  
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47 **Leandro, USA).**  
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56 **Diethylnitrosamine (DEN) treatment of mice.** The hepatocarcinogenesis protocol  
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58 was as follows. The 15-day-old male TTR/IKKCA transgenic mice and non-transgenic litter  
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60 mates were injected intraperitoneally with 25 mg/kg DEN in sterile PBS. At 21 days of age,



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3 mice were separated and genotyped. At 35 weeks of age, mice were sacrificed and their livers  
4 were harvested and examined. The livers were then fixed in 4% formaldehyde in PBS,  
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6 were harvested and examined. The livers were then fixed in 4% formaldehyde in PBS,  
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8 embedded in paraffin and cut into 4- $\mu$ m sections for histological examination. All  
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10 experiments were approved by the Committee on the Use of Live Animals in Teaching and  
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12 Research of the University of Hong Kong, and performed according to the institutional  
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14 guidelines for animal care.  
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## 25 Results

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27 **Activation of NF- $\kappa$ B in cultured cells and in transgenic mouse livers.** To test the  
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29 utility of plasmid CMV/FLAG-IKK $\beta$ -CA for expression of FLAG-IKK $\beta$ -CA and activation  
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31 of the NF- $\kappa$ B pathway, the construct was transfected into HepG2 hepatoblastoma cells. As  
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33 shown by immunofluorescence staining, we observed nuclear localization of p65 in HepG2  
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35 cells expressing FLAG-IKK $\beta$ -CA (Figure 1b). As expected, transgenic mice generated with  
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37 the TTR-FLAG/IKK $\beta$ -CA construct (Figure 1a) showed liver-specific expression of IKK $\beta$ -  
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39 CA mRNA as detected by RT-PCR (Figure 1c). Also, p65 was found to be upregulated in the  
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41 livers of these transgenic mice (Figure 1e), and enhanced p65 nuclear localization was  
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43 detected with immunohistochemistry in the hepatocytes of transgenic mouse livers (Figure  
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45 1d). In addition, the level of I $\kappa$ B $\alpha$  was found to be lower than that in the non-transgenic  
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47 mouse livers (Figure 1e), suggesting that the exogenous FLAG-IKK $\beta$ -CA phosphorylated  
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49 and triggered the degradation of I $\kappa$ B $\alpha$ . With electrophoretic mobility shift assay (EMSA), we  
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51 showed that NF- $\kappa$ B activities were upregulated, at about 3-fold, in the transgenic mouse  
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53 livers (Figure 2). Furthermore, the downstream target genes of NF- $\kappa$ B, Bcl-xL [20] and  
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55 STAT5 [21], were upregulated in the livers of the transgenic mice (Figure 1e). Taken  
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3 together, these results confirmed that the NF- $\kappa$ B pathway was activated in the transgenic  
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5 mouse livers [at least within 2 to 50 weeks of age being studied](#).  
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10 **Hepatocarcinogenesis in transgenic mice.** To address the controversy as to whether  
11 liver-specific NF- $\kappa$ B activation or inactivation promotes hepatocarcinogenesis, we compared  
12 male TTR/IKKCA mice (n = 7) and male non-transgenic littermates (n = 8) up to an age of  
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22 **Chemical hepatocarcinogenesis in transgenic mice.** To address the controversy as  
23 to whether liver-specific NF- $\kappa$ B activation or inactivation promotes chemical  
24 hepatocarcinogenesis, we treated the 15-day old male TTR/IKKCA mice and non-transgenic  
25 littermates with DEN. When the mice reached 35 weeks of age, they were dissected and their  
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51 **Liver organogenesis in embryos with activation of NF- $\kappa$ B pathway.** The NF- $\kappa$ B  
52 pathway has been documented to be essential for the development of embryonic livers and  
53 prevent uncontrolled apoptosis in livers [5, 10]. We therefore investigated whether activation  
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3 harvested embryos at different embryonic stages from 11.5 to 13.5 dpc. These transient  
4 transgenic embryos from 11.5 dpc to 13.5 dpc during embryogenesis were examined. Most of  
5 the transient transgenic embryos did not have any observable phenotypes (Table 2). However,  
6 the livers of four transient transgenic embryos at 12.5 dpc were intensely red as compared  
7 with those of the others (Figures 4a and 4b). These four embryos had remarkably numerous  
8 nucleated red blood cells accumulated in their livers (Figures 4c-4f). Also, there appeared to  
9 be altered vasculature with enlarged sinusoids. With immunohistochemical staining with anti-  
10 FLAG antibody, we were able to demonstrate that the FLAG-IKK $\beta$ -CA was expressed in the  
11 hepatocytes of these embryos (Figures 4g and 4h). However, FLAG-IKK $\beta$ -CA expression  
12 was much lower or undetectable in other transgenic embryos without the above phenotype  
13 (data not shown). This may explain why only a small fraction (4 out of 25) of transgenic  
14 embryos had the above phenotype.  
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## Discussion

38 We successfully generated a transgenic mouse model with hepatocyte-specific  
39 expression of constitutively active IKK $\beta$ . We also confirmed that NF- $\kappa$ B was significantly  
40 activated in the livers of this transgenic mouse model. In the transgenic mouse livers, NF- $\kappa$ B  
41 activities and the levels of NF- $\kappa$ B downstream targets, Bcl-xL and STAT5, were upregulated.  
42 Bcl-xL, an anti-apoptotic member of the Bcl-2 family, has been shown to be elevated in  
43 human HCCs [22, 23] and demonstrated to be anti-apoptotic in HepG2 cells [23]. STAT5 is  
44 implicated in cancer cell survival [24] and tumor progression [25] in prostate cancer, and in  
45 tumor invasiveness in human hepatocellular carcinomas [26]. STAT5 is also a direct activator  
46 of Bcl-xL [27].  
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3 However, our results showed that activation of the NF- $\kappa$ B pathway in hepatocytes did not  
4 have significant effects on the incidence of formation of HCC, up to an age of 50 weeks.  
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6 Furthermore, such specific activation of the NF- $\kappa$ B pathway in hepatocytes did not affect the  
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8 incidence of formation of HCC after DEN treatment at 35 weeks of age.  
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15 In recent study, Pikarsky et al. [9] investigated the role of the NF- $\kappa$ B pathway in the  
16 development of HCC with a hepatocyte-specific controllable I $\kappa$ B-super-repressor system in  
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18 Mdr2-knockout mice. Mdr2-knockout mice develop HCC in a similar manner as the  
19 development of human HCC, which develops progressively through inflammation, dysplasia,  
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21 dysplastic nodules, carcinoma and metastasis. The authors found that the super-repressor  
22 could suppress tumor progression through inactivation of the NF- $\kappa$ B pathway and switching  
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24 off the super-repressor enhanced tumor progression. They therefore suggested that NF- $\kappa$ B  
25 promoted hepatocarcinogenesis in the Mdr2-knockout mice. Our results were different from  
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27 those observed by Pikarsky et al. We showed that activation of NF- $\kappa$ B did not promote  
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29 chemical-induced hepatocarcinogenesis. These observations suggest that the underlying  
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31 mechanisms of hepatocarcinogenesis in these mouse models may be different, and hence the  
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33 NF- $\kappa$ B pathway may not affect hepatocarcinogenesis in these models in a similar manner.  
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46 Another recent report from Maeda et al. [10] showed that hepatocyte-specific deletion of  
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48 IKK $\beta$  promoted DEN-induced hepatocarcinogenesis in the IKK $\beta^{\Delta\text{hep}}$  mice. This finding was  
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50 also supported by a recent report that hepatocyte-specific IKK $\gamma$ /NEMO deficient mice, in  
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52 which the NF- $\kappa$ B pathway was inhibited in hepatocytes, were prone to develop steatohepatitis  
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54 and HCC [28]. Maeda et al. [10] also suggested that Kupffer cells, rather than hepatocytes  
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56 themselves, played a more important role in DEN-induced hepatocarcinogenesis. They  
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58 proposed a model that DEN-treated hepatocytes caused Kupffer cells to produce growth  
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3 factors, such as IL-6, TNF $\alpha$  and HGF, which in turn promoted the survival of hepatocytes  
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5 accumulating DEN-induced mutations by stimulating compensatory proliferation. Sakuri et al.  
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7 [29] further supported this compensatory proliferation model of hepatocarcinogenesis in the  
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9 IKK $\beta$ <sup>Ahep</sup> mice by showing that loss of JNK1 reversed the increased susceptibility to  
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11 hepatocarcinogenesis in these mice. Our findings that activation of the NF- $\kappa$ B pathway in  
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13 hepatocytes had no significant effects on chemical hepatocarcinogenesis did not contradict  
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15 and might even be in accordance with this proposed model.  
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23 Some of these transient transgenic embryos at 12.5 dpc had abnormal accumulation of  
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25 nucleated red blood cells (RBCs) in their developing livers. Normally, nucleated RBCs are  
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27 generated in primitive erythropoiesis in embryonal and fetal stages and will be replaced by  
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29 anucleated RBCs in erythropoiesis after birth. The accumulation of nucleated red blood cells  
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31 in the livers of our transgenic mice might be due to activation of NF- $\kappa$ B mediated by the  
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33 constitutively active form of IKK $\beta$  in the hepatoblasts of these embryos. We hypothesize that  
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35 during embryonic liver development, NF- $\kappa$ B in hepatoblasts plays an important role in  
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37 controlling erythropoiesis. This is supported by the following lines of evidence. Koibuchi et  
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39 al. (2004) has shown that hepatocyte growth factor (HGF) regulates primitive hematopoiesis.  
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41 Although the authors did not investigate NF- $\kappa$ B signaling in their study, HGF has been  
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43 shown to cause rapid nuclear translocation of the p65 (RelA) subunit of NF- $\kappa$ B in epithelial  
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45 cells by another group [30]. This suggests that NF- $\kappa$ B activation in hepatoblasts may be  
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47 essential for erythropoiesis in developing embryos.  
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57 Cai et al. [31] have generated a mouse line, designated as LIKK, in which a low-level  
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59 activation of NF- $\kappa$ B was observed in the livers of these mice. The low-level activation of NF-  
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61  $\kappa$ B resulted in subacute inflammation, as demonstrated by increased level of transcription of

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3 proinflammatory cytokine genes such as IL-1 $\beta$ , IL-6, and TNF $\alpha$  in the livers of LIKK mice. It  
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5 was suggested that cytokines secreted by hepatocytes could induce local and systemic insulin  
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7 resistance. The design of our TTR/IKKCA mice was similar to that of the LIKK mice, except  
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9 that the hepatocyte-specific expression in our mice was mediated by the mouse TTR  
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11 promoter, whereas that in the LIKK mice was mediated by the mouse albumin promoter.  
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13 Also, the murine form of IKK $\beta$  was used in our mice and the human counterpart was used in  
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15 the LIKK mice. Thus, our TTR/IKKCA mice will also be a good model for studying the link  
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17 among inflammation, obesity, insulin resistance and type 2 diabetes.  
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## Legend to figures

Figure 1. Activation of NF- $\kappa$ B pathway in HepG2 cells and livers of transgenic mice expressing constitutively active IKK $\beta$  (IKK $\beta$ CA). (a) Schematic diagrams of constitutively active IKK $\beta$  constructs. Arrows represent primers used for RT-PCR to detect IKK-CA mRNA. Black boxes represent the exons of transthyretin vector; grey boxes represent FLAG epitopes; white boxes represent IKK $\beta$ CA coding sequence. (b) Confocal images showing nuclear localization of p65 in HepG2 cells expressing FLAG-tagged IKK $\beta$ CA. (c) Liver-specific expression of IKK-CA mRNA in transgenic mice (6 weeks old) by RT-PCR. Sk. Muscle, skeletal muscle. (d) Enhanced nuclear localization of p65 in livers of transgenic mice (6 weeks old) detected by immunohistochemistry on cryostat sections. Arrows indicate p65 nuclear localization in hepatocytes. (e) Western Blotting showing increased levels of p65 subunit of NF- $\kappa$ B and NF- $\kappa$ B downstream targets, Bcl-xL and STAT5, and decreased level of I $\kappa$ B in livers of transgenic mice (from 2 to 50 weeks old). NTg, non-transgenic; Tg, Transgenic; NS, non-specific. Numbers indicate the relative levels determined by densitometry (normalized with actin level).

Figure 2. Electrophoretic mobility shift assays (EMSA) showing increased NF- $\kappa$ B activities in nuclear extracts from livers of transgenic mice. (a) Increased level of nuclear factors, which bound to radioactive-labeled probes containing NF- $\kappa$ B-binding site sequence, in nuclear extracts from livers of transgenic mice (6 weeks old). Lane 1, negative control, no nuclear extract added; Lanes 2 - 3, nuclear extracts from non-transgenic mouse livers; Lanes 4 - 6, nuclear extracts from transgenic mouse livers. Numbers indicate the relative NF- $\kappa$ B activities determined by densitometry (normalized with lamin B level). Arrow indicates mobility shift. Western blotting with anti-lamin B as loading control. (b) EMSA with nuclear extracts from transgenic mouse livers showing the nuclear factors specifically bound to the

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3 labeled probes containing NF- $\kappa$ B-binding site sequence. Lane 1, no competitor added; Lane 2,  
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5 50X unlabeled specific competitor (SC) added; 50X unlabeled non-specific competitor (NSC)  
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7 added. (c) Super-shift assay showing the nuclear factors which bound to the labeled probed  
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9 were p50/p50 homodimer and p50/65 heterdimer of NF- $\kappa$ B. Lane 1, no antibody added; Lane  
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11 2, anti-p65 added; Lane 3, p-50 added; Lane 4, Control IgG added. Arrowheads indicate  
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13 “super-shift”.  
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20 Figure 3. NF- $\kappa$ B activation in hepatocytes did not affect chemical hepatocarcinogenesis. (a)  
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22 The numbers of liver tumor and (b) maximum tumor sizes (diameters in mm) of DEN-treated  
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24 mice at the age of 35 weeks. Mean  $\pm$  standard deviations are shown at the top of the graphs  
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26 and the statistical analysis was done using the student *t*-test. Livers of DEN-treated (c)  
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28 TTR/IKKCA mouse and (d) wild type mouse at the age of 35 weeks. (e – h), Haematoxylin  
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30 and Eosin (H&E) stained liver sections of mice without DEN treatment. (i – l), H&E stained  
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32 liver sections of mice with DEN treatment.  
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39 Figure 4. Nucleated red blood cells accumulated in livers of transgenic embryos expressing  
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41 constitutively active IKK $\beta$ . (a) and (b), Representative non-transgenic and transgenic  
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43 embryos at 12.5 dpc. (c) and (d), Embryonic liver sections stained with H & E. (e) and (f),  
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45 High power magnification of E and F respectively. (g) and (h), Embryonic liver sections  
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47 stained with anti-FLAG antibody.  
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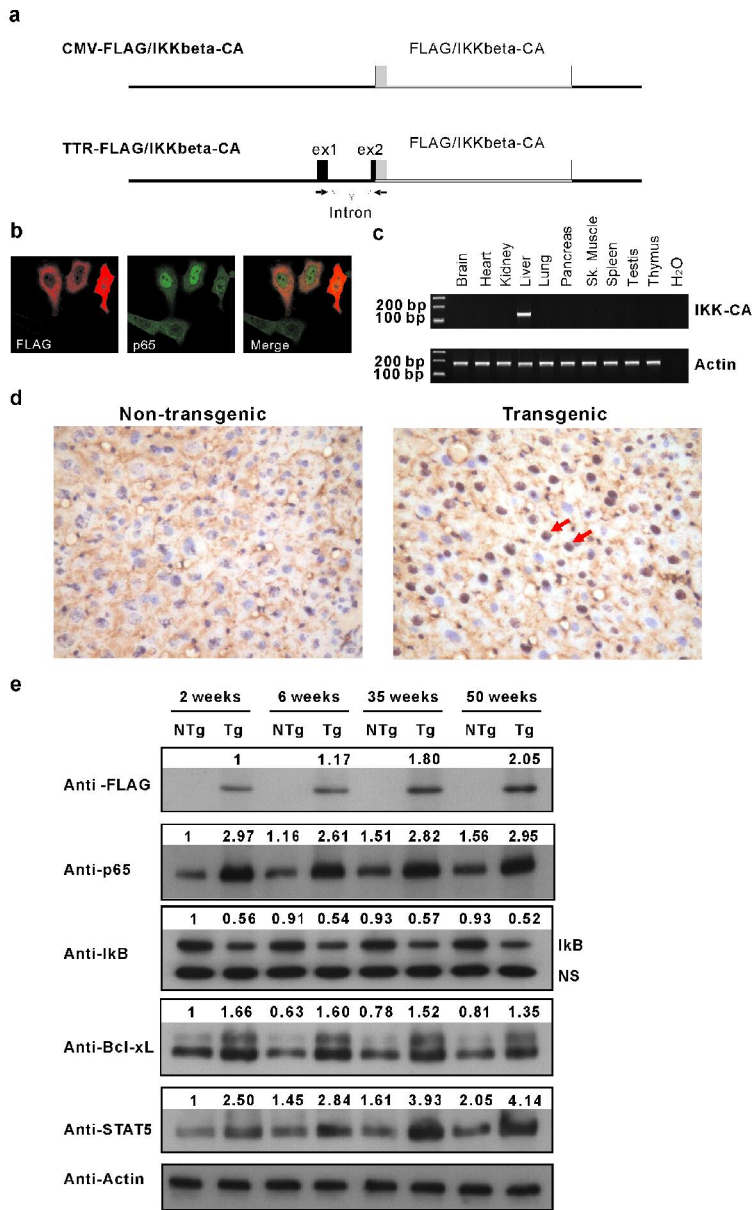
**Table 1. Summary of tumor formation in wild-type and transgenic mice in chemical hepatocarcinogenesis using DEN.**

	<u>Without DEN treatment</u>		<u>With DEN treatment</u>	
	TTR/IKKCA	Non-transgenic	TTR/IKKCA	Non-transgenic
Tumor incidence	0	0	7	6
Number of mice	7	8	7	6

**Table 2. Summary of the transient embryos carrying the FLAG-IKK $\beta$ -CA transgene.**

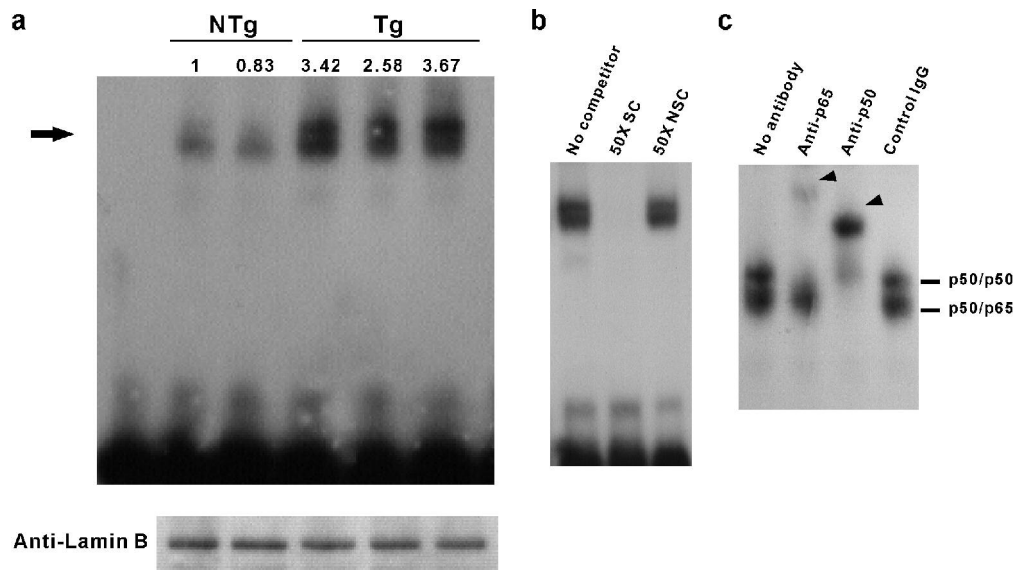
Embryonic stage	Number of transgenic embryos	Number of embryos
11.5	36	106
12.0	9	14
12.25	11	15
12.5	24 (4*)	49
13.5	8	15

\* Number of transgenic embryos showing accumulation of nucleated RBCs in their livers.



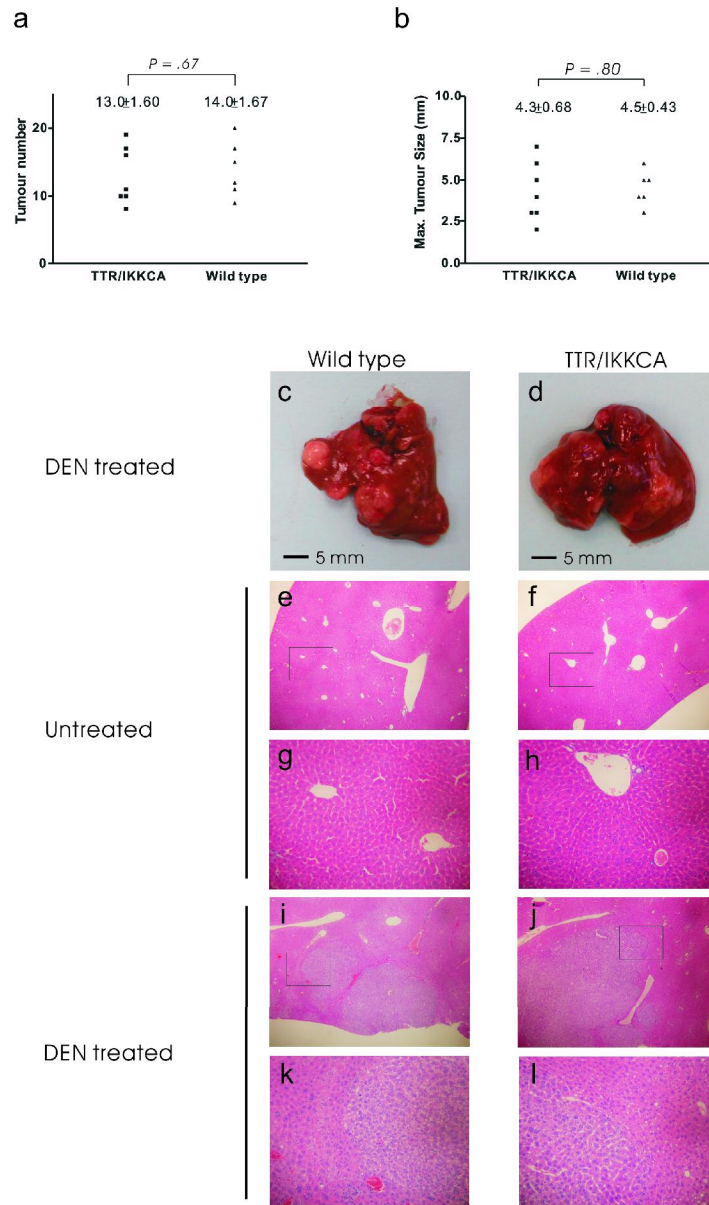
**Activation of NF-kappaB pathway in HepG2 cells and livers of transgenic mice expressing constitutively active IKKbeta.**

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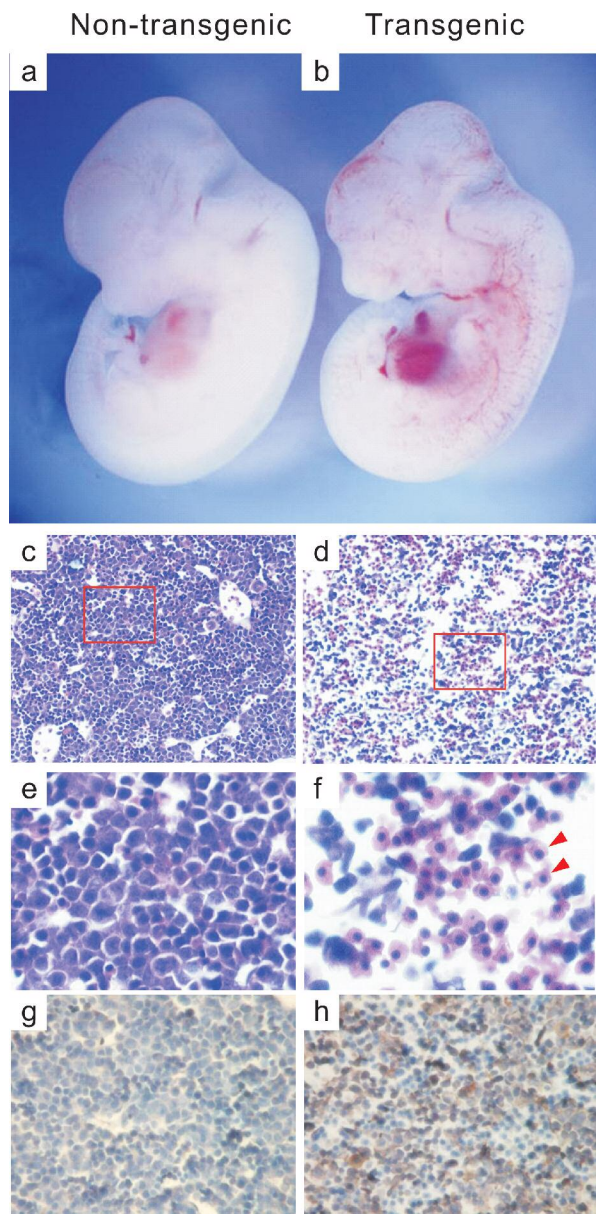
**Electrophoretic mobility shift assays (EMSA) showing increased NF-kappaB activities in nuclear extracts from livers of transgenic mice.**

Peer Review



**NF-kappaB activation in hepatocytes did not affect chemical hepatocarcinogenesis.**

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**Nucleated red blood cells accumulated in livers of transgenic embryos expressing constitutively active IKKbeta**