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Analysis of the IKKβ/NF-κB Signaling Pathway during Embryonic Angiogenesis

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Abstract

The NF-κB signaling pathway regulates cellular growth, survival, differentiation and development. In this study, the functions of IκB kinase (IKK)β in angiogenesis during mouse development were examined. Conditional disruption of the *Ikkβ* locus in endothelial cells using the well-characterized *Tie2-Cre* transgene resulted in embryonic lethality between E13.5-15.5. Examination of the mutant embryos revealed that while deletion of *Ikkβ* occurred in endothelial cells throughout the embryo, only the vascular network in the fetal liver was affected. Disruption of the fetal liver vasculature was accompanied by decreased cell proliferation and increased apoptosis of hepatocytes, but hematopoiesis was not affected. Increased apoptosis was not observed outside of fetal liver in the mutant embryos. These results indicate that the IKKβ/NF-κB pathway plays a previously unappreciated role in development of the sinusoidal vasculature in the fetal liver and additionally that this pathway is critical in the crosstalk between endothelial cells and hepatocytes during mouse development.

Keywords

IKK (IκB kinase); Angiogenesis; Development

INTRODUCTION

Blood vessels develop through two consecutive processes, vasculogenesis and angiogenesis. Vasculogenesis occurs via differentiation of endothelial cells from hemangioblasts in the blood island of the extra embryonic mesoderm. Angiogenesis is a physiological process involving the growth of new blood vessels from the endothelial cells of existing blood vessels beginning at embryonic day 8.5 in the mouse (Risau and Flamme, 1995; Risau, 1997; Mojzis et al., 2008). Vasculogenesis and angiogenesis are regulated differently. Although the exact mechanisms are still not entirely defined, both vasculogenesis and angiogenesis are driven by growth factors like vascular endothelial growth factor (VEGF) and angiopoeitins, and additionally involve cell-cell and cell-matrix interactions (Tanjore *et al.*, 2008).

Nuclear factor-κB (NF-κB) regulates expression of a number of genes involved in immune and inflammatory responses, cellular growth, survival, differentiation and development (Chen and Castranova, 2007; Hayden and Ghosh, 2008). There are five NF-κB family

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members in mammals: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel and RelB. These factors share an N-terminal Rel homology domain (RHD) responsible for both DNA binding and homo- and heterodimerization (Hayden and Ghosh, 2008). In resting cells, NF-κB complexes are retained in the cytoplasm by interaction with IκB inhibitory proteins. Upon stimulation by inflammatory signals, such as TNF-α, IL-1 or LPS, IκB proteins become phosphorylated and ubiquitinated and are subsequently degraded by the proteasome. This results in the translocation of NF-κB proteins to the nucleus where they regulate expression of target genes (Hayden and Ghosh, 2008). Phosphorylation of IκB proteins is mediated by IκB kinase (IKK). The IKK complex contains two highly homologous kinase subunits, IKKα/IKK1 and IKKβ/IKK2, and a regulatory subunit NEMO/IKKγ (Hacker and Karin, 2006). IKKβ is involved in canonical NF-κB signaling in response to inflammatory signals, while the noncanonical pathway depends on the IKKα subunit that phosphorylates NF-κB2/p100 leading to its processing to the p52 protein (Bonizzi and Karin, 2004).

The NF-κB pathway has been previously implicated in angiogenesis. VEGF gene expression can be regulated by NF-κB (Chilov *et al.*, 1997; Ishdorj *et al.*, 2008). Other genes that are important for angiogenesis, such as MMPs, uPA, VCAM-1, ICAM-1, COX-2, GRO-1, iNOS, jagged-1, HMGB1 receptor, and HIF-1α are also regulated by NF-κB (Koch *et al.*, 1995; Taylor *et al.*, 1998; Stetler-Stevenson, 1999; Yasuda *et al.*, 2002; Zhou *et al.*, 2003; Rius *et al.*, 2008; Sainson *et al.*, 2008; van Beijnum *et al.*, 2008). *Ikkβ* deficient mice died between E12.5 and E14.5 because of severe liver defects (Li *et al.*, 1999a; Li *et al.*, 1999b; Tanaka *et al.*, 1999). Thus, it is not possibloe to study the role of the NF-κB pathway in endothelial cells using this model.

In order to directly address the potential roles of the NF-κB pathway in angiogenesis, we took an *in vivo* genetic approach using the Cre/*LoxP* system to specifically disrupt *Ikkβ* in Tie2-Cre positive endothelial cells. Conditional *Ikkβ* ablation in endothelial cells led to embryonic lethality between E13.5 and E15.5. The blood vessel network in the embryonic liver was specifically affected in the mice with endothelial cell deletion of *Ikkβ*. This led to apoptosis of hepatocytes and liver degeneration. Our results demonstrate that IKKβ is critical for fetal liver angiogenesis *in vivo*.

RESULTS

Ablation of *Ikkβ* in Tie2-positive endothelial cells leads to embryonic lethality between E13.5 and E15.5

Conditional deletion of *Ikkβ* using *Tie2-Cre* (Kisanuki *et al.*, 2001) was used to study the function of the canonical NF-κB pathway in endothelial cells, as previous work has demonstrated that targeting this kinase leads to ablation of the canonical NF-κB pathway (Li *et al.*, 1999a; Li *et al.*, 1999b; Tanaka *et al.*, 1999). Initially mice homozygous for the conditional *Ikkβ* floxed allele (*Ikkβ*$^{F/F}$*/Tie2-Cre*) were studied. The majority of *Ikkβ*$^{F/F}$*/Tie2-Cre* embryos died between E13.5 and E17.5, but infrequently mice with this genotype were born and grew to adulthood (e.g., 25 expected of genotype *Ikkβ*$^{F/F}$*/Tie2-Cre*, but only 2 survived; supplementary data, Table 1). Endothelial cells were isolated from the aorta of the atypical mice that survived to adulthood. PCR genotyping of the aortic endothelial cells demonstrated that the null *Ikkβ* allele was not present (Supplementary Figure 1A). In addition, analysis of IKKβ by Western blot analysis revealed that IKKβ was expressed at near normal levels in endothelial cells from the mice that survived to adulthood (Supplementary Figure 1B).

Because these results indicated that the *Tie2-Cre*-mediated deletion of the *Ikkβ* conditional allele was incomplete, we adopted the strategy of studying mice with one conventional knockout allele and one conditional allele, *Ikkβ*$^{-/F}$, so that only one allele needed to be

deleted in endothelial cells through the action of *Tie2-Cre*. The strategy used to generate mice of genotype of *Ikkβ*$^{-/F}$*/Tie2-Cre* is outlined in Figure 1A. Briefly, a conventional knockout allele was created by breeding *Ikkβ*$^{F/+}$ mice with *Sox2-Cre* mice (Hayashi *et al.*, 2002). Mice with the knockout allele were bred with *Tie2-Cre* mice and *Ikkβ*$^{-/+}$*/Tie2-Cre* male mice were identified. The *Ikkβ*$^{-/+}$*/Tie2-Cre* males were bred to *Ikkβ*$^{F/F}$ female mice to generate the experimental and control genotypes.

Mice of *Ikkβ*$^{-/F}$*/Tie2-Cre* genotype, expected at a Mendelian frequency of 25%, were not observed beginning at E15.5 (Table 1). Further analysis indicated that *Ikkβ*$^{-/F}$*/Tie2-Cre* mice were present at expected frequencies at E12.5. However, embryonic lethality was observed beginning at E13.5 (Table 1). All other possible genotypes from this genetic cross were observed at expected frequencies (not shown). These results indicated that embryonic lethality occurred in the period between E13.5 and E15.5.

To confirm that IKKβ was absent in endothelial cells, PECAM/CD31 positive cells were isolated from E12.5 embryos, before embryonic lethality occurred, by high speed fluorescence activated cell sorting (FACS). The PECAM-1 positive cells constituted approximately 5% of the total cells isolated from each individual embryo (Supplementary Figure 2). Total RNA was extracted from the enriched PECAM-1-positive cells and subjected to qRT-PCR analysis. The level of *Ikkβ* mRNA in *Ikkβ*$^{-/F}$*/Tie2-Cre* endothelial cells was reduced by 16-fold compared to controls, similar to levels observed in conventional knockout mice (Figure 1B). VE-cadherin, another endothelial cell marker, was expressed at the similar level in *Ikkβ*$^{-/F}$*/Tie2-Cre*, *Ikkβ*$^{-/F}$ and *Ikkβ*$^{-/-}$ embryos (Figure 1B). Additionally, the expression level of six NF-κB target genes in the PECAM-1 positive cells was also studied by qRT-PCR (Figure 1C). The mRNA levels of three target genes, IL-6, VEGF-C and COX-2, were reduced 2-fold in *Ikkβ*$^{-/F}$*/Tie2-Cre* endothelial cells compared to *Ikkβ*$^{-/F}$ control cells (Figure 1C). The relatively small effect on expression of these NF-κB target genes may reflect that basal levels and not cytokine induced levels of expression were determined. The data are consistent with a deletion of the *Ikkβ* gene and downregulation of the NF-κB pathway in endothelial cells isolated for *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos.

As a complementary approach to confirm *Ikkβ* deletion in endothelial cells, IKKβ expression was studied by immunohistochemistry in experimental and control mice (Figure 1D). Significantly reduced IKKβ expression was detected in the majority of endothelial cells of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos compared to controls (arrows in Figure 1D; quantified in Figure 1E). This commercial antibody did not work in our hands for indirect immunofluorescence using frozen sections (data not shown).The results support the conclusion that *Ikkβ* was deleted in Tie2-positive endothelial cells throughout the embryo.

Angiogenesis in livers of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos is impaired

Ikkβ$^{-/F}$*/Tie2-Cre* embryos at E13.5 had normal appearance at dissection (Figure 2A). Histological analysis of sagittal sections of viable E13.5 *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos revealed specific abnormalities in the livers of these mutant embryos compared to controls (Figure 2B). The architecture of the liver was disrupted and there were regions of apparent cell death. This phenotype was very similar to *Ikkβ*$^{-/-}$ embryos, but cell death occurred regionally in the livers of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos compared to through the entire livers in *Ikkβ*$^{-/-}$ embryos at the same stage. No obvious defects were detected in other organs or regions of the embryos (Figure 2C).

To determine if angiogenesis was affected in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers, PECAM-1 antibody was used to visualize endothelial cells by indirect immunofluorescence (Figure 3A). The density of blood vessels was reduced by 2.6-fold in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers (Figure 3B). This decrease in blood vessel density was seen throughout the entire

embryonic liver. In contrast, angiogenesis through the remainder of the embryonic body was not affected in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos, as determined by whole mount PECAM-1 immunohistochemistry staining (Figure 3C). Blood vessels are composed of endothelial cells and supporting cells, such as vascular smooth muscle cells and pericytes (Cleaver and Melton, 2003). To study the blood vessel integrity in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos, PECAM-1 and smooth muscle α-actin double immunofluorescence staining was performed on frozen sections (Figure 3D). The analysis indicated that there was no obvious blood vessel disorganization in the embryonic vasculature in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos.

Hematopoiesis occurs in the liver beginning at E10 (Godin and Cumano, 2005). Since liver degeneration was observed in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos, and because *Tie2-Cre* was also active in hematopoietic stem cells (Kisanuki *et al.*, 2001), the status of fetal liver hematopoiesis was examined at E12.5 by performing flow cytometry with different hematopoietic cell lineage markers. Populations of c-Kit^{+}, Ter119^{+}, F4/80^{+}, Mac-1^{+} and Gr-1^{+} cells were intact in *Ikkβ*$^{-/F}$*/Tie2-Cre* fetal livers (Figure 3E). These results indicated that hematopoiesis in mutant livers was not impaired. Immunostaining with F4/80 indicated no significant differences in the Kupffer cells populations between mutant and control embryos (data not shown).

Decreased proliferation and increased apoptosis in livers of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos

Cell proliferation was measured in sagittal sections of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos using a BrdU incorporation assay (Figure 4). BrdU positive cells were reduced by 5-fold at E13.5 in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers compared to *Ikkβ*$^{-/F}$ controls (Figure 4A top panels, quantification in 4B), indicating that the number of cells in S phase was significantly reduced at E13.5 in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers. In contrast, no obvious difference between mutants and controls was detected at E12.5 (Figure 4A bottom panels, 4C).

To further characterize the phenotype of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers, immunohistochemistry with cleaved Caspase-3 (Asp175) antibody was performed on both *Ikkβ*$^{-/F}$*/Tie2-Cre* and *Ikkβ*$^{-/F}$ sections at E13.5 (Figure 5A) and E12.5 (Supplementary Figure 4) to analyze potential changes in apoptosis. Cells that were undergoing apoptosis increased 10-fold in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers at E13.5 compared to controls. This level of apoptosis was approximately 3.5-fold lower than in the conventional *Ikkβ* knockout mice (Figure 5B). Apoptosis in the liver at E13.5 was regional and occurred in an apparently random fashion. This was in contrast to the changes in blood vessel density which occurred throughout the fetal liver (Figure 3A). At E12.5, before embryonic lethality was first detected, *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers showed only a 2-fold increase in apoptosis compared to controls (Supplementary Figure 4; Figure 5B). There was no increased apoptosis detected in the heart, lung and kidney (Figure 5C). Apoptosis was also verified by TUNEL staining in an independent set of embryos (data not shown).

Two-color immunofluorescence with the cleaved Caspase-3 antibody and either PECAM-1 antibody staining to detect endothelial cells or alpha-fetoprotein antibody staining to detect embryonic hepatocytes, was performed to identify the cell type undergoing apoptosis. The experiments revealed that apoptosis was significantly increased in alpha-fetoprotein positive hepatocytes in mutant versus control embryos (Figure 6A). However, there was no significant increase in apoptosis in cells that were PECAM-1 positive (Figure 6B). These results indicated that hepatocytes were the major cell type undergoing apoptosis in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos.

To rule out the possibility that Tie2-Cre may have non-specifically deleted *Ikkβ* from hepatocytes leading to cell death, immunohistochemistry with IKKβ antibody was performed on transverse sections of *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers. Robust IKKβ

staining was detected in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers (Figure 6C), consistent with the expression of IKKβ in hepatocytes. In a control experiment, tissue from *Ikkβ*$^{-/-}$embryos was also studied, demonstrating the absence of IKKβ staining in these embryos (Figure 6C). In conventional *Ikkβ* knockout mice, apoptosis in the liver occurs due to increased expression and sensitivity to TNF-α (Li *et al.*, 1999a; Li *et al.*, 1999b; Tanaka *et al.*, 1999). However, TNF-α RNA levels were no different in *Ikkβ*$^{-/F}$*/Tie2-Cre* compared to controls (Figure 6D).

DISCUSSION

Conditional deletion of *Ikkβ* using *Tie2-Cre* resulted in embryonic lethality with obvious defects in fetal liver angiogenesis and liver degeneration. The restricted phenotype was not due to liver-specific ablation of *Ikkβ* as deletion of the gene was pan-endothelial. An inductive role for endothelial cells in liver development beginning at E8.5 has been previously established (Matsumoto *et al.*, 2001). However, based on the later onset of the phenotype in the *Ikkβ*$^{-/F}$*/Tie2-Cre* mice, a role for the IKKβ/NF-κB pathway in early endothelial inductive events appears unlikely. The results presented here indicate that the IKKβ/NF-κB pathway plays a novel role in liver homeostasis during embryonic development. The sinusoidal endothelium of the liver is structurally and functionally distinct from the general endothelium of the embryo (Couvelard *et al.*, 1996; Braet and Wisse, 2002) and these differences may explain the apparent lack of phenotype in other tissues and organs.

The phenotype of the *Ikkβ*$^{-/F}$*/Tie2-Cre e*mbryos was unexpectedly similar to the phenotype observed in the conventional knockout, albeit not as severe (Li *et al.*, 1999a; Li *et al.*, 1999b; Tanaka *et al.*, 1999). Hepatocyte apoptosis occurs later in the *Ikkβ*$^{-/F}$*/Tie2-Cre* mice and hepatocyte apoptosis was initially more regional, limited to a few lobes at E13.5, compared to the *Ikkβ* deficient mice at the same or earlier stages. In contrast, hepatocyte-specific knockout of *Ikkβ* using the *Alfp-Cre* transgene did not result in significant embryonic lethality, despite the activity of this Cre-driver beginning around E10.5 (Kellendonk *et al.*, 2000; Luedde *et al.*, 2005). Taken together, this information suggests that ablation of *Ikkβ* in endothelial cells could contribute to the conventional knockout phenotype. One caveat concerning this possible interpretation is that a different targeted allele of *Ikkβ*, a construct that targeted exons 6–7, was utilized in the *Alfp-Cre* experiments (Luedde *et al.*, 2005). It is possible this allele is hypomorphic, or that *Alfp-Cre* is not expressed early enough in embryonic development to duplicate the results with the conventional knockout allele. A second caveat is that in the results reported here, the one copy of *Ikkβ* deleted in hepatocytes may be necessary for the phenotype seen with *Tie2-Cre* ablation of *Ikkβ* in endothelial cells. However, hepatocyte apoptosis was also observed in *Ikkβ*$^{F/F}$*/Tie2-Cre* studied in embryos at E15.5 (data not shown), indicating that the phenotype observed does not depend on deletion of one allele of *Ikkβ* in hepatocytes.

The decreased cell proliferation and increased cell death observed in hepatocytes in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos is non-cell autonomous, dependent apparently on the deletion of the *Ikkβ* gene in endothelial cells. It is well appreciated that communication between the distinct cell types present in liver is required to affect organ structure and functions. For example, the endothelium has been demonstrated to protect hepatocytes independent of its evident role in supplying nutrients and oxygen (LeCouter et al., 2003). Similarly, co-cultivation of hepatocytes with endothelial cells *in vitro* can potentiate expression of hepatocyte-specific genes, such as albumin and ApoA-I (Harimoto *et al.*, 2002; Kurosawa *et al.*, 2005). Additionally, crosstalk between hepatocytes and closely juxtaposed endothelial cells mediated via vascular endothelial growth factor is essential for sinusoidal endothelial growth and differentiation (Edwards *et al.*, 2005). Our results define the IKKβ/NF-κB pathway as a key element in this intercellular communication during embryonic development. The exact

molecular nature of the intercellular dialogue, and how its disruption results in the phenotype observed in these studies, remains to be determined. One obvious candidate, TNF-α does not seem to be involved, since hepatocytes still expressed IKKβ and TNF-α RNA Levels were not altered. Which factors or cytokines are responsible for this crosstalk deserves further investigation.

EXPERIMENTAL PROCEDURES

Mice

Floxed mice (*Ikkβ*$^{F/F}$) were described previously (Li *et al.*, 2003). *Ikkβ* conventional knockout mice were generated by crossing *Ikkβ*$^{F/F}$ mice with *Sox2-Cre* mice (Hayashi *et al.*, 2002). *Tie2-Cre* mice were described previously (Kisanuki *et al.*, 2001). All animals were maintained on a FVB/N background (>10 generations). Animals were housed in the animal facility at The Ohio StateUniversity Biomedical Research Tower under sterile conditionsmaintaining constant temperature and humidity and were fed astandard diet. Mice were genotyped by PCR analysis from preparedtail DNA. All protocols for the use of mice were approved by the OSU Institutional Animal Use and Care Committee.

Quantitative real-time PCR

Total RNA was extracted from CD31 sorted endothelial cells by Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA was purified using the TURBO DNA-*free* kit (Ambion, Austin, TX). Total RNA was reverse transcribed by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamer primers. qPCR was performed as previously described (Wei *et al.*, 2004). All PCR reactions were performed in duplicate. Primer sequences are available upon request.

Histology and Immunohistochemistry

Whole-mount embryo immunohistochemistry was performed as described (Suri *et al.*, 1996). Tissue was fixed in 10% buffered formalin (Fisher, Middletown, VA). After routine processing, paraffin-embedded sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for histological analysis. Immunohistochemistry was performed on paraffin-embeddedsections (5 μm thick), and immunofluorescence studies were performed on cryo-preserved sections (7 μm thick). Paraffin sections were dewaxed and rehydrated through a graded series of ethanol washes, and maintained in phosphate buffered saline (pH 7.4; PBS). Frozen sections were air dried for 30 minutes and then placed in PBS. Sections were treated with 3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity and placed in Dako target retrieval solution at sub-boiling temperature for 60 minutes to unmask the antigens. Protein Block (Dako, Carpinteria, CA) was applied for 30 minutes to reduce non-specific binding of the antibodies. Sections were treated with primary antibody, followed by biotin conjugated secondary antibody (Rockland, Gilbertsville, PA). Alexa Fluro dye 488 or 594 streptavidin (Molecular Probes, Eugene, OR) were used for immunofluorescence. Vectorstain ABC kit (Vector Laboratories, Burlingame, CA) was used for immunohistochemistry, followed by DAB to visualize immunocomplexes. Primary antibodies were: anti-PECAM antibody (MEC13.3, rat anti-mouse monoclonal, Pharmingen, San Diego, CA), anti-smooth muscle α-actin antibody (Spring Bioscience, Fremont, CA), anti-cleaved Caspase-3 antibody (Asp175) (9661, cell signaling, Danvers, MA), anti-Brdu antibody (M0744, DAKO, Carpinteria, CA), anti-AFP antibody (MAB1368, R&D Sytems, Minneapolis, MN) and anti-IKKβ antibody (ab55404, abcam, Cambridge, MA).

Flow cytometry

Embryos and embryonic livers were isolated at E12.5. DNA prepared from yolk sacs was used to genotype the embryos. A single cell suspension of embryonic livers or whole embryos was prepared by established method (Motoike *et al.*, 2000) and treated with red blood cell lysing buffer (Sigma, St. Louis, MO). 1×10^6 cells for flow or 1×10^7 cellsfor sorting were incubated with antibodies (0.5 mg/1×10^6 cells) in DMEM +2% FCS on ice for 30 minutes in the dark. Cells were washed once with 1X PBS + 1% FCS. Cells fixed in 1% formalin were analyzed with the FACS Calibur (Beckton-Dickinson, San Jose, CA), or directly sorted without fixation on the FACS Aria (Beckton-Dickinson, San Jose, CA). Monoclonal antibodies against the following antigens were used: Gr-1-FITC, F4/80-PE, Mac-1-FITC, Ter119-PE, c-Kit- FITC and CD31-PE (eBioscience, San Diego, CA and Caltag, Carlsbad, CA). Appropriate isotype controls were also analyzed.

BrdU incorporation assay

100 μg BrdU (5-Bromo-2'-deoxyuridine, Sigma, St. Louis, MO) per gram of body weight in 1X PBS was injected ventrally into the abdomen of pregnant mice avoiding the embryos. Mice were sacrificed two hours after injection. Embryos were fixed in 10% buffered formalin and embedded in paraffin. BrdU incorporation was measured by anti-BrdU antibody immunohistochemistry. Three individual embryos were analyzed in each genotype group at each time point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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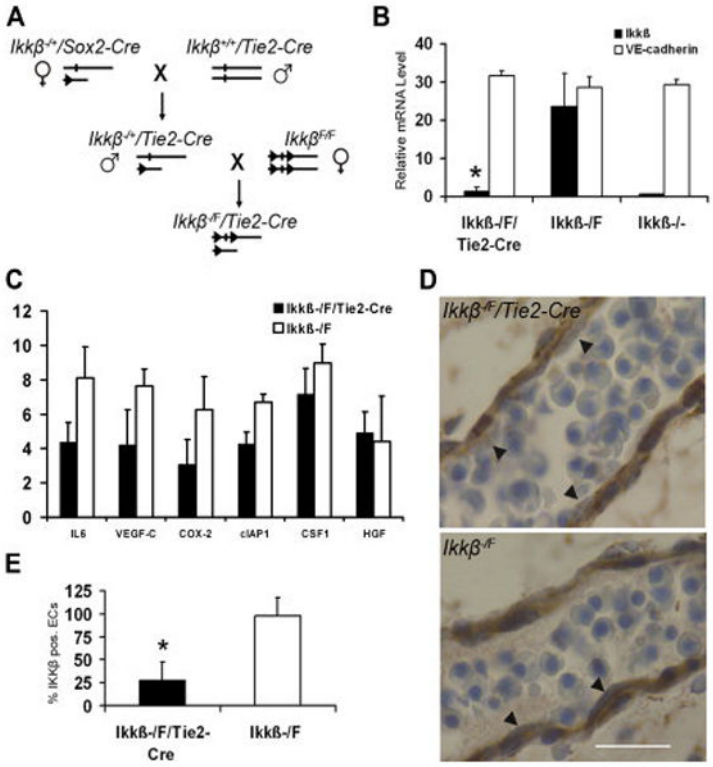

Figure 1. Conditional deletion of *Ikkβ* in endothelial cells

A. Generation *Ikkβ*$^{-/F}$*/Tie2-Cre* mice. A conventional *Ikkβ* knockout allele was generated with *Sox2-Cre* and *Ikkβ*$^{-/+}$*/Tie2-Cre* male mice were crossed with *Ikkβ*$^{F/F}$ female mice. **B.** IKKβ and VE-cadherin mRNA expression levels in PECAM-1 positive embryonic endothelial cells isolated by FACS from *Ikkβ*$^{-/F}$*/Tie2-Cre*, *Ikkβ*$^{-/F}$ and *Ikkβ*$^{-/-}$ embryos. Cells from four individual embryos for each genotype were analyzed in duplicate experiments. Error bars indicate standard deviation. * indicates that the difference between *Ikkβ*$^{-/F}$*/Tie2-Cre* and *Ikkβ*$^{-/F}$ embryos is significant as determined by student t-test ($p<0.01$). **C.** NF-κB target gene mRNA expression levels in PECAM-1 positive endothelial cells from *Ikkβ*$^{-/F}$*/Tie2-Cre* and *Ikkβ*$^{-/F}$ embryos. Three individual embryos for each genotype were analyzed in duplicate experiments. Error bars indicate standard deviation. **D.** IKKβ expression in endothelial cells was examined with immunohistochemistry staining. Endothelial cells in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos showed less IKKβ staining compared to *Ikkβ*$^{-/F}$ embryos (arrows). 100X, scale bar 50 μm. **E.** Quantification of IKKβ positive cells in mutant versus control embryonic blood vessels studied. * indicates significance ($P<0.01$). Total 50 ECs were counted.

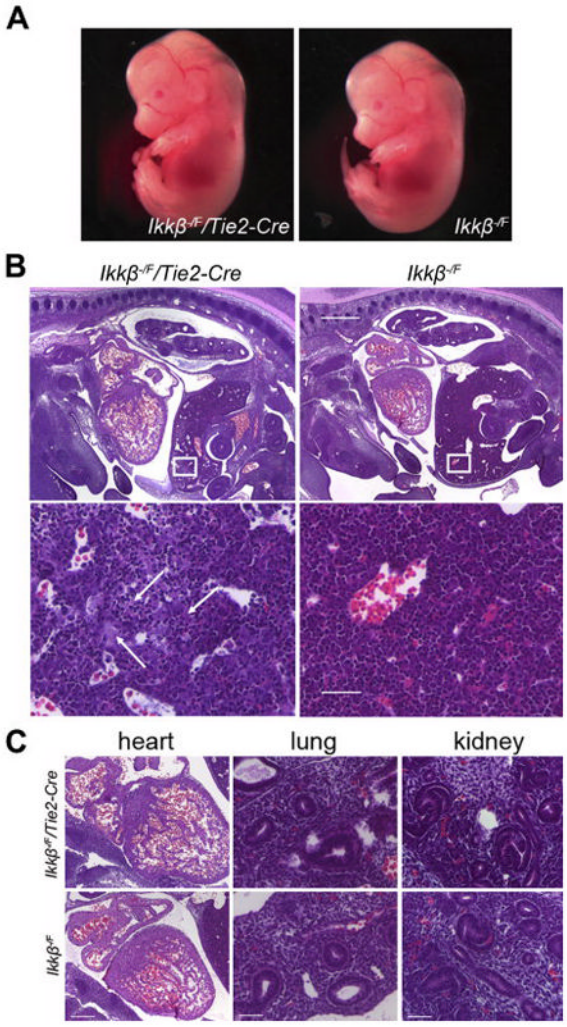

Figure 2. *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos have obvious liver defects

A. *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos are normal by their gross appearance (E13.5). **B.** H&E staining of sagittal sections. *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos show obvious liver defects. 4X, scale bar 500 μm. Boxed areas are showed in higher magnification at the bottom panel. 40X, scale bar 50 μm. Liver structure is disorganized and arrows highlight areas of cell death. **C.** H&E staining of other organs. Heart 10X, scale bar 200 μm. Lung and kidney 40X, scale bar 50 μm.

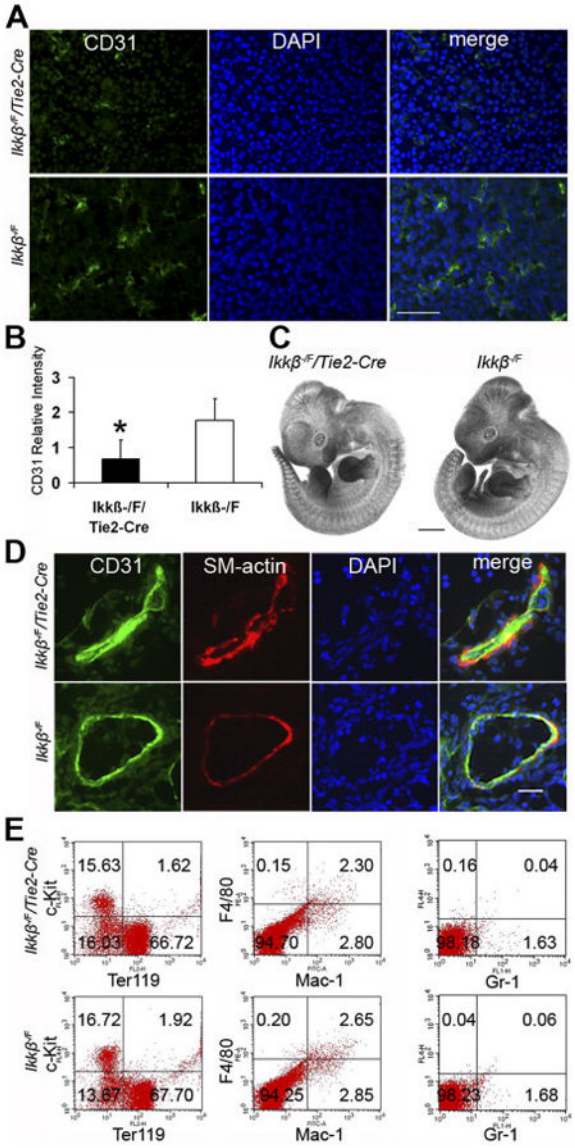

Figure 3. Vascular structure is impaired in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers

A. PECAM-1 (green) immunofluorescence staining of frozen transverse section of embryonic livers at E13.5. 40X, scale bar 50 μm. **B.** Relative PECAM-1 intensity was quantified with Image J software. Four individual embryonic liver sections with three fields for each sample were studied. * indicates that the difference is significant ($p<0.01$). **C.** Angiogenesis is intact in whole embryos. PECAM-1 IHC staining in whole mount. Scale bar 1 mm. Six individual embryos were studied. **D.** Cryosections of embryos were stained with anti-PECAM-1 antibody and anti-smooth muscle α-actin antibody. 40X, scale bar 20 μm. Data are representative examples for five individual embryos. **E.** FACS analysis of hematopoietic differentiation in livers for cell type markers c-Kit, Ter119, F4/80, Mac-1 and Gr-1. The percentage of cells in each quadrant is indicated. Data are representative examples for three individual embryonic livers studied.

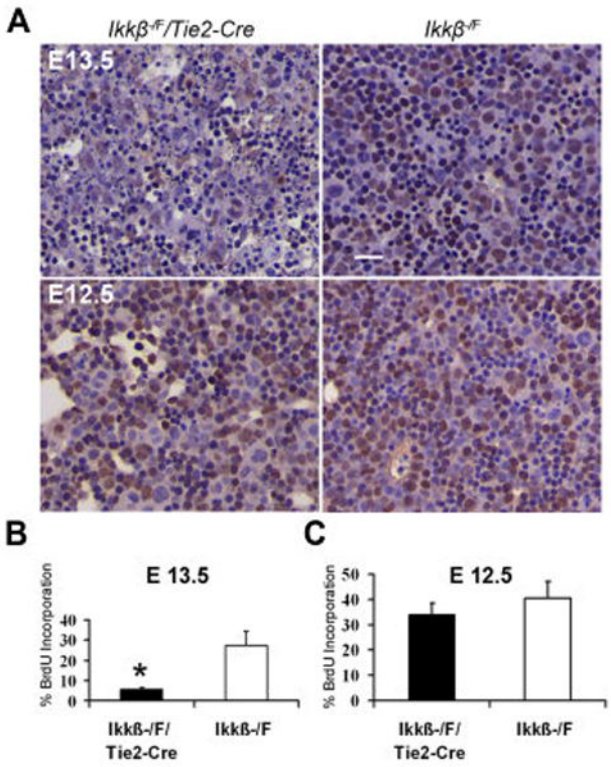

Figure 4. Cell proliferation is impaired in *Ikkβ*$^{-/F}$*/Tie2-Cre* embryonic livers

A. Proliferation was measured by BrdU incorporation. BrdU immunohistochemistry staining of embryonic livers at E13.5 and E12.5. 40X, scale bar 20 μm. **B.** and **C.** Percentage of BrdU positive cells were measured at E13.5 (B) and E12.5 (C). Three individuals were analyzed. * indicates that difference is significant measured by student t-test ($p<0.01$).

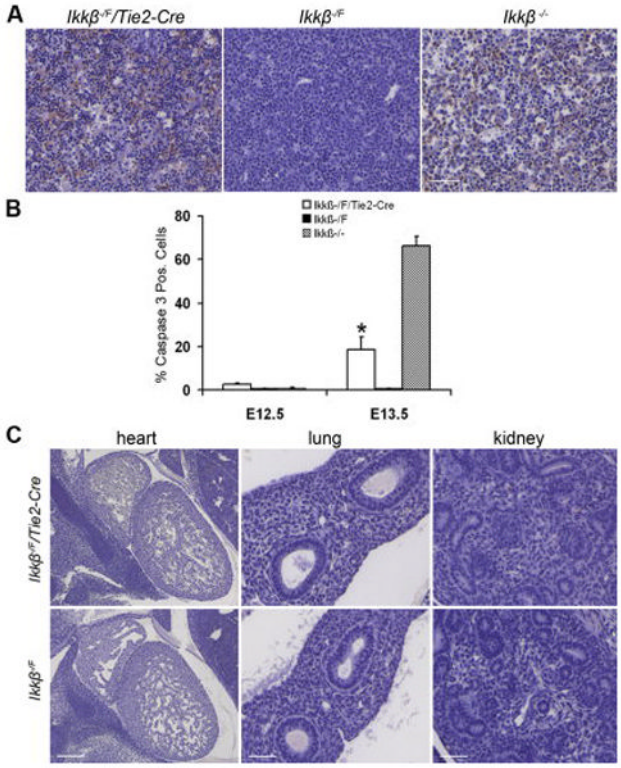

Figure 5. Increased cell apoptosis in *Ikkβ$^{-/F}$/Tie2-Cre* embryonic livers

A. Regional apoptosis was detected in *Ikkβ$^{-/F}$/Tie2-Cre* embryonic livers. Cleaved Caspase-3 (Asp175) antibody IHC staining of paraffin-embedded sections at E13.5. 40X, scale bar 50 μm. **B.** Ratio of apoptotic cells to total liver cells at E12.5 and E13.5. Five individual embryos for each genotype were analyzed. * indicates that the difference between *Ikkβ$^{-/F}$/Tie2-Cre* and *Ikkβ$^{-/F}$* embryonic livers at E13.5 is significant as determined by student t-test ($p<0.01$). **C.** Apoptosis was not increased in heart, lung and kidney at E13.5. Heart 10X, scale bar 200 μm. Lung and kidney 40X, scale bar 50 μm.

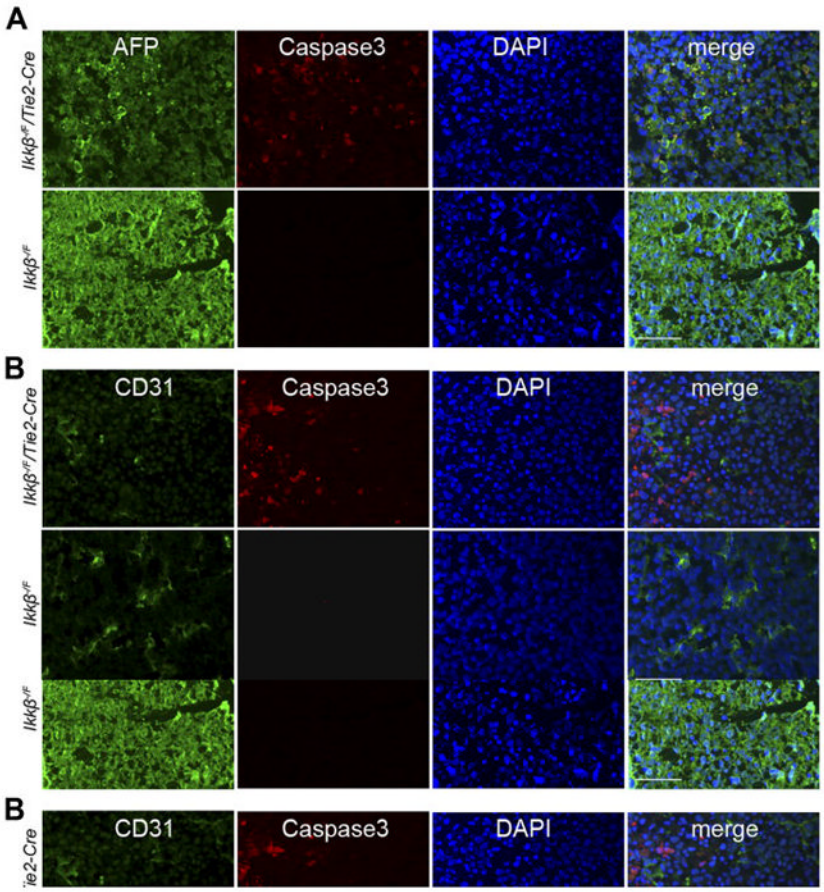

Figure 6. Apoptosis occurs in hepatocytes in *Ikkβ*$^{-/F}$*/Tie2-Cre embryos*

A. and **B.** AFP (green, A) or PECAM-1 (green, B) and cleaved Caspase-3 (red) double antibody staining of frozen sections from E13.5 embryonic livers. 40X, scale bar 50 μm. **C.** IKKβ immunohistochemistry staining in *Ikkβ*$^{-/F}$*/Tie2-Cre* and *Ikkβ*$^{-/-}$ embryonic livers. 40X, scale bar 50 μm. **D.** TNF-α mRNA expression levels in *Ikkβ*$^{-/F}$*/Tie2-Cre* and *Ikkβ*$^{-/F}$ whole embryonic livers.

Table 1

Lethality of *Ikkβ*$^{-/F}$*/Tie2-Cre* mice happened between E13.5 and E15.5 in FVB/N background.

Embryonic Day	Total embryos	*Ikkβ*$^{-/F}$*/Tie2-Cre* embryos	Expected *Ikkβ*$^{-/F}$*/Tie2-Cre* embryos
E11.5	57	14	14.25
E12.5	54	15	13.5
E13.5	63	5	15.75
E14.5	40	3	10
E15.5	76	0	19

Expected Menderian ratio is 25%.

Viable: heartbeat can be detected at dissection.