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Targeted deletion of hepatocyte *Ikkβ* confers growth advantages

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Abstract

Mice lacking hepatocyte IKK β (*Ikkβ^{Ahep}*) are defective in TNF α -activation of hepatocellular transcription factor NF- κ B, and highly susceptible to hepatotoxicity. Following diethylnitrosamine (DEN) exposure, *Ikkβ^{Ahep}* mice develop more hepatocellular carcinoma (HCC) than control mice due partly to enhanced DEN-induced hepatocyte death. Here we show that *Ikkβ^{Ahep}* hepatocytes display growth advantages over normal hepatocytes consisting of precocious PCNA and cyclin D1 expression during liver regeneration (shortened hepatocyte G₀ → G₁ transitions), and enhanced recovery efficiency, cyclin D1 expression and cell proliferation after plating. *Ex vivo* deletion of *Ikkβ* also accelerates hepatocyte growth. *Ikkβ^{Ahep}* hepatocyte proliferative responses show heightened sensitivity to TGF α and TNF α , and heightened expression of fibronectin, collagens I/III, nidogen, β -actin and integrin β 1 mRNAs. These findings suggest that altered mitogen signaling and expression of extracellular matrix and its associated components underlie growth advantages. Increased HCC development in *Ikkβ^{Ahep}* mice may also be caused by growth advantages of surviving *Ikkβ*-deleted hepatocytes.

Keywords

Growth-suppressor; Precocious S-phase onset; Mitogen sensitivity; Extracellular matrix

IKK kinase β (IKK β) is required for activation of NF- κ B [1,2], a transcription factor that regulates liver inflammation and protection from injury [2,3]. Although its liver growth-controlling role is controversial [4], current evidence suggests that NF- κ B activation is unnecessary for partial hepatectomy (PH)-induced liver regeneration [5–7]. Our results also suggested slightly accelerated regeneration in *Ikkβ^{Ahep}* mice which carry Cre-mediated deletions of hepatocyte *Ikkβ* exon 3 [7]. In correlation with defective NF- κ B activation and failed survival gene induction, *Ikkβ^{Ahep}* mice also show enhanced susceptibility to concanavalin-A (ConA)-induced liver failure [6] and to diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC [7]).

In trying to understand the basis for the enhanced HCC susceptibility, we found that *Ikkβ^{Ahep}* mice showed increased liver cell death and cytokine-driven compensatory hepatocyte

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2009.01.085.

proliferation [7]. Indeed, further work with *Ikk β ^{Δhep}* mice showed that HCC-development was partly due to IL-1 α , released from damaged hepatocytes, which induced Kupffer cell IL-6-expression and activated IL-1 receptors [8–10]. Here, we extend these investigations by comparing growth properties of hepatocytes cultured from *Ikk β ^{Δhep}* and *Ikk β ^{F/F}* mice. We find that *Ikk β* deletion confers direct growth advantages to hepatocytes, which consist of higher recovery efficiency (R.E.) and enhanced cell proliferation, possibly related to increased mitogen sensitivities and to augmented extracellular matrix (ECM), basement membrane, cytoskeletal, and integrin mRNA expression. Both advantages implicate a growth-suppressor role of IKK β , and should be considered along with the cell death hypothesis under conditions of induced hepatotoxicity and hepatocarcinogenesis.

Materials and methods

Animals

Ikk β ^{F/F} and *Ikk β ^{F/F}; Alb-Cre* (referred to as *Ikk β ^{Δhep}*) mice were maintained per NIH guidelines [6]. Homogeneous backgrounds were obtained by backcrossing *Ikk β ^{F/F}* with C57BL/6J mice for ≥ 8 generations. *Ikk β ^{F/F}; Alb-Cre* ♂ mice were bred with *Ikk β ^{F/F}* ♀ mice to generate *Ikk β ^{F/F}* and *Ikk β ^{Δhep}* ♂ littermates; these and C57BL/6J mice were used at 6–8 weeks.

Liver regeneration and tissue processing

Seventy percent PH and sham hepatectomy, and liver analyses (histology; DNA, RNA, and proteins) were described elsewhere [7]. Apoptotic cells were quantified by TUNEL assays [7].

Genotyping, deletion analysis, PCR and real time Q-PCR

Cre and F/F alleles, and *Ikk β* deletions were assessed by PCR assays of tail, liver and cultured hepatocyte DNA [6,7]. Q-PCR was performed on liver, and freshly isolated and cultured hepatocyte RNA (Suppl. Table 1 lists primers); results were normalized to glyceral-dehyde phosphate dehydrogenase mRNA levels [11].

Primary culture

Hepatocytes were isolated by intraportal perfusion using a 23G-needle with 5 ml digestion buffer containing 0.5 mg Sigma Type I collagenase/ml [12;Appendix A]. Cells were plated (N_0 = initial cell concentrations) into uncoated plastic 35 mm-diameter tissue culture dishes or 10 mm-diameter 24-multiwell plates (NUNC) containing 2 ml or 1 ml plating medium [12], respectively. Human TGF α and mouse TNF α (R&D) were aliquoted into serum-free medium and stored at -20°C until use. Attached cells were trypsinized from cultures washed 3 $\times$ with PBS and Coulter-counted.

Adenoviruses

Ad-CMV-Cre adenovirus (Vector Biolabs) was amplified in HEK293 cells. Ad-CMV-GFP was constructed by inserting GFP cDNA into a pshuttle-CMV transfer vector (Stratagene); GFP-expressing adenovirus was obtained via homologous recombination between pshuttle-CMV-GFP and pAdEasy-1 (Stratagene). Titers were obtained using HEK293 cells [13]. Hepatocytes were infected at a multiplicity of infection = 10.

BrdU L.I., immunofluorescence and Western blots

L.I. measurements were made in cultures exposed 18 h to 10 μM BrdU, followed by washing, overnight fixation in 4% formalin and re-washing. Immunofluorescence staining (BrdU = red, albumin = green) was performed using 1 $^\circ$ -rabbit anti-BrdU and goat anti-albumin antibodies (Megabase), and 2 $^\circ$ -donkey Alexa 594-conjugated anti-rabbit- and chick Alexa 488-

conjugated anti-goat antibodies (Invitrogen), respectively; no staining occurred in the absence of 1°- or 2°-antibodies. To circumvent problems arising from heterogeneously distributed cells, ≥ 2000 hepatocytes, visualized microscopically using a dual red–green filter (Chroma Technology), were scored over similar evenly-spaced sites/dish. Photomicrographs (Ni-kon DXM1200C camera) were archived using Nikon software. Western blots were performed using anti-PCNA and cyclin D1 (PC10 and M-20, Santa Cruz sc-56 and 718), IKK β (10AG2, Imgenex IMG129A) and anti-actin (Sigma A4700) antibodies [7].

Statistical analysis

Data were expressed as means $\pm$ S.E. Significance ($P \leq 0.05$) was judged by 1-way ANOVA or Student's *t*-tests. Areas under curves (AUC) were calculated with SigmaPlot v.7 software.

Results

Targeted *Ikk β* deletion accelerates PCNA and cyclin D1 expression and shortens hepatocyte $G_0 \rightarrow G_1$ transitions during liver regeneration after PH

$G_0 \rightarrow G_1$ transitions were shortened after 70% PH in *Ikk β^{Ahep}* mice (Fig. 1A), as shown by elevated and sustained levels of PCNA at 24–60 h, and cyclin D1 at 24–48 h. Similar changes, absent after sham hepatectomy, did not occur until 36 h in control *Ikk $\beta^{F/F}$* mice, in which cyclin D1 levels oscillated and peaked between 36 and 60 h (Fig. 1A).

Intergenotypic differences (Fig. 1A) agreed with published hepatocyte BrdU L.I. values: S-phase onset times, S_t [14], were shortened in *Ikk β^{Ahep}* mice, as shown by a 200-fold higher BrdU L.I. (10%) compared to controls (0.05%) at 24 h ($P < 0.0001$); BrdU L.I. levels peaked normally in both groups [15] by 36 h, and BrdU-staining occurred only in hepatocytes [7]. *Ikk β^{Ahep}* mitotic indexes (M.I.) were higher than *Ikk $\beta^{F/F}$* controls by ~40% at peak mitotic times (48 h; Fig. 1B); however, apoptotic hepatocyte fractions were equivalent in both strains at 0–36 h (Fig. 1C). α_1 -Fetoprotein positive oval-like cells (not shown), inflammatory cells or necrotic hepatocytes were not observed (Fig. 1B). Thus, *Ikk β* deletion enhanced hepatocyte proliferation during liver regeneration without affecting apoptosis.

Differences between plating and growth properties of *Ikk $\beta^{F/F}$* and *Ikk β^{hep}* hepatocytes are revealed in primary culture

To determine whether *Ikk β* deletion directly affects growth, primary cultures were prepared in the absence of non-parenchymal cells. Hepatocytes spread and flattened between d1 and d4, as expected [12] (Suppl. Fig. 1A); consistent with growth (Fig. 2A), more hepatocytes were seen between d4 and d24 and, after ~3 population doublings, large differentiated hepatocyte-aggregates appeared (Suppl. Fig. 1A).

Growth curves (Fig. 2A) showed intergenotypic differences when cells were plated at varying concentrations. At low N_0 (1.5×10^5 cells/dish), d1 *Ikk β^{Ahep}* hepatocyte R.E. [15] exceeded controls (*Ikk β^{Ahep}* ~ 58–68% > *Ikk $\beta^{F/F}$* ~ 36–38%, Suppl. Fig. 1A). Because R.E. curves were linear over a broad N_0 range (Suppl. Fig. 2), such differences were unlikely results of differential media conditioning [16]. When plateau phase was attained (d12), *Ikk β^{Ahep}* hepatocyte numbers exceeded controls by ~1.5-fold ($P < 0.01$). At progressively higher N_0 (3×10^5 or 6×10^5), although d2–d8 growth rates and d12–d21 plateau densities were similar, intergenotypic R.E. differences were sustained (*Ikk β^{Ahep}* > *Ikk $\beta^{F/F}$* , Suppl. Fig. 2), *Ikk β^{Ahep}* hepatocytes/dish exceeded controls until d8 ($P < 0.0001$), and *Ikk β^{Ahep}* lag phases relative to controls were attenuated ($P < 0.003$, Fig. 2A).

Additional findings support cell-autonomous growth differences between *Ikk $\beta^{F/F}$* and *Ikk β^{Ahep}* hepatocytes. First, because both kinds of hepatocytes came from age- and weight-

matched littermates, and were isolated with similar yields and viabilities (Suppl. Table 2), the results could not be explained by differences in these parameters. Second, non-parenchymal cells could not have contributed measurably to growth because cell isolation and plating conditions select against non-hepatocyte survival [10,12,16]; this was visually evident from uniform d1 hepatocyte morphologies (Suppl. Fig. 1) and findings that >90% of cells expressed hepatocyte-specific albumin in d1 cultures (Fig. 2D [top curves]; Suppl. Fig. 1B). Genotyping analysis failed to reveal significant levels of non-parenchymal *Ikkβ* DNA (an ~2-kb product generated from intact *Ikkβ* genomes) in freshly isolated (0-time) or cultured *Ikkβ^{Ahep}* hepatocytes on d3–d4 (Fig. 2B) or d8 (not shown). The origins of trace 2-kb bands in d1–d2 cultures is unclear; such bands represented <0.1% of observed PCR products and were absent in 0-time cells (they may have come from rare non-hepatocyte contaminants, undetected at 0-time and lost from cultures as selection-pressure prevailed). Non-hepatocyte-derived IKKβ proteins were also undetectable in d1 *Ikkβ^{Ahep}* cultures [6], consistent with ~6-fold intergenotypic differences in *Ikkβ* mRNA levels in d1–d6 cultures (*Ikkβ^{F/F}* > *Ikkβ^{Ahep}*, $P < 0.00004$; Fig. 2C). Thus targeted *Ikkβ* deletion conferred growth advantages to *Ikkβ^{Ahep}* hepatocytes that are independent of non-parenchymal cells.

Targeted *Ikkβ* deletion causes precocious expression of cyclin D1 and enhanced S-phase entry in primary hepatocytes

To investigate growth-associated cell-cycle parameters, S-phase entry and cyclin D1 expression were measured d1–d8 post-plating. If precocious $G_{0,1} \rightarrow S$ transitions were properties of growth-stimulated *Ikkβ^{Ahep}* hepatocytes, BrdU L.I. and cyclin D1 expression levels should be augmented compared to controls. Indeed, S-phase entry (visualized by scoring BrdU + albumin + cells, Suppl. Fig. 1B) was specifically 2-fold higher in *Ikkβ^{Ahep}* hepatocytes between d1 and d4 ($P < 0.009$; Fig. 2D, bottom curves). Using 18h labeling intervals to exclude scoring log-phase cells transiting S-phase more than once (given ~72 h population doubling times, Fig. 2A), BrdU labeled mitotic hepatocytes were also detected (Fig. 2E). Cyclin D1 mRNA expression peaked bimodally d3 and d6, and declined towards baseline on d8 in both groups; however, *Ikkβ^{Ahep}* hepatocyte rates and peak amplitudes of cyclin D1 mRNA expression exceeded controls ($P < 1.8 \times 10^{-5}$, Fig. 2F). Albumin expression did not parallel intergenotypic $G_1 \rightarrow S$ transition differences; instead, it changed inversely with growth-state (12): highest initially on d1, with its L.I. falling to 80–82% (Fig. 2D, top curves) as log-growth ensued (Fig. 2A and D [bottom curves]). Thus, constitutive *Ikkβ* deletion directly enhanced hepatocyte proliferative transitions.

Ex vivo deletion of *Ikkβ* stimulates hepatocyte proliferation

To circumvent strain-dependent R.E., the effects of abrupt *Ikkβ* deletion were investigated in equal numbers of *Ikkβ^{F/F}* hepatocytes ($N_0 = 3 \times 10^5$) infected with Cre recombinase-expressing (Ad-Cre) or control GFP-expressing adenovirus (Ad-GFP). Ad-Cre infection effectively deleted *Ikkβ* (Suppl. Fig. 3A) and stimulated proliferation compared to controls ($P < 0.0009$; Fig. 3A, top panel). Ad-Cre infection also augmented rates and levels of hepatocyte BrdU labeling ($P < 0.05$; Fig. 3A, bottom panel). Net increases in cells/dish and L.I. were similar to uninfected *Ikkβ^{Ahep}* hepatocytes (Figs. 2A and D [bottom curves]). Ad-Cre infection also augmented cyclin D1 ($P < 0.05$; Fig. 3B, top panel) and reduced IKKβ mRNA expression ($P < 0.0001$; Fig. 3B, bottom panel) compared to controls. Neither Cre expression artifacts [17] nor cytotoxicity could have caused Ad-Cre-augmented responses because equivalent growth (Suppl. Fig. 3B) and albumin expression curves (not shown) of Ad-Cre, Ad-GFP- or vehicle-treated C57BL/6 hepatocytes were observed.

Ikk β deletion affects hepatocyte growth-signaling systems

Signaling system components, including mitogens and mitogen receptor/ECM/basement membrane/cytoskeleton/integrin complexes concertedly regulate hepatocyte growth [14,18]. Therefore, selected components were investigated for growth advantage-correlations in *Ikk β ^{Ahep}* cells. Firstly, increased receptor sensitivities to mitogens were monitored by quantifying growth-responses to TGF α or TNF α . Mitogens were added after attachment (d1) and replicate cell counts measured d1–d9 (Suppl. Fig. 4 and Table 3). Again, *Ikk β ^{Ahep}* hepatocyte R.E. exceeded controls by ~2-fold. Without added mitogens, following a long lag-phase >3–5d (perhaps due to differences between multiwells and dishes), *Ikk β ^{Ahep}* hepatocyte growth surged between d5 and d7, ahead of controls d7–d9. With added mitogens, lag phases shortened; by d7, *Ikk β ^{Ahep}* hepatocytes responded better than controls to TNF α ($P < 0.0005$) and TGF α ($P < 0.03$): Suppl. Fig. 4 shows nearly doubled peak numbers of cells. These results suggested that *Ikk β ^{Ahep}* hepatocytes were more sensitive to mitogens than controls, a conclusion more evident when data were plotted as mitogen dose-response curves (Fig. 4A). Left-shifts occurred in both groups. *Ikk β ^{Ahep}* responses to TGF α plateaued before controls (attenuated responses were not observed.). In contrast, both groups showed biphasic TNF α responses, heightened in *Ikk β ^{Ahep}* cultures in which TNF α -dependent growth was considerably higher at low or blunted at high ligand concentrations (Fig. 4A).

Secondly, correlations with ECM, basement membrane, cytoskeletal and mitogen receptor-associated integrin mRNA expression were investigated [19]. Specifically elevated changes (*Ikk β ^{Ahep}* > *Ikk β ^{F/F}*) were observed in 6 of 12 candidate mRNAs surveyed between d1 and d6 (Fig. 4B): fibronectin, collagens I and III, nidogen, β -actin, and EGF receptor-associated integrin B1. No differences were seen in laminin, elastin, FAK, SPARC, or mitogen receptor-associated integrin B3 (c-MET) or B4 (insulin) mRNAs, or in freshly isolated hepatocyte, or *Ikk β ^{Ahep}* and control liver mRNAs.

Discussion

We have shown that constitutively *Ikk β* deleted (*Ikk β ^{Ahep}*) or *ex vivo* *Ikk β* deleted *Ikk β ^{F/F}* hepatocytes have growth advantages over *Ikk β* -expressing hepatocytes. These advantages are associated with attenuated levels of IKK β mRNA and augmented levels of cy-clin D1 mRNA; cell-cycle expression patterns of IKK β and cyclin D1 proteins and activities will require further clarification. In contrast, when intrahepatic mitogen mRNA expression is reduced throughout liver by the deletion of *Ikk β* in hepatocytes and hematopoietic-derived cells (*Ikk β ^{ALIV}* mice), *in vivo* hepatocyte growth advantages expected of *Ikk β* deleted hepatocytes following 70% PH are absent [7]. Thus, IKK β is a bifunctional regulator of hepatocyte proliferation and HCC, with different roles played by hepatocytes and liver non-parenchymal cells; enhanced HCC in DEN-treated *Ikk β ^{Ahep}* mice requires the effects of IKK β -dependant Kupffer cell-derived cytokines and the loss of growth-inhibitory hepatocyte IKK β . Because primary culture studies, and dual investigations with *Ikk β ^{Ahep}* and *Ikk β ^{ALIV}* knockout mice [7], were needed to reach both conclusions (otherwise missed by unicellular targeting), the potential regulation of HCC-suppressors including pRb, p53 and p21 [20] by non-parenchymal cells should not be overlooked.

Bifunctional IKK β implicates cell-specificity. This is supported by studies with embryonic *Ikk β ^{-/-}* fibroblasts which grow faster but plate with identical efficiency compared to controls [21], and *Ikk β ^{-/-}* Hela cells which undergo mitotic arrest [22]. Investigations with *Ikk2 Δ hepa* mice [23,24] also suggest that hepatocyte IKK β survival functions depend on IKK α and IKK γ [25]; however, different *Ikk β* -targeting strategies may have differentially affected *Ikk2 Δ hepa* mice because findings of PH-induced inflammation indirectly shortening hepatocyte S_t and abnormally delayed post-PH BrdU L.I. peaks [15,24] disagree with findings in *Ikk β ^{Ahep}* mice [6,23].

G₀ and proliferating *Ikkβ^{Ahep}* populations consist only of hepatocytes. Therefore, exceedingly rare hepatocyte progenitors like oval cells, or putative progenitors like stellate cells that differentiate into albumin + cells [26], are unlikely to have undergone *Ikkβ* deletion in regenerating liver or primary culture. Defined G₀-culture conditions, which best simulate complex growth control processes free from confounding *in vivo* variables [27], should help clarify mechanisms by which *Ikkβ* deletion confers growth advantages. Although *in vitro* measurements were consistent with *in vivo* results [7], which suggested that 70% PH shortened *Ikkβ^{Ahep}* hepatocyte S_t without affecting DNA-synthesis entry rates (*S_Δ*), they were made in cells exposed to pre-plating stresses that induce early gene expression [28]. However, pre-plating artifacts like these or trace mitogens in the serum supplement are unlikely to have caused post-plating *Ikkβ^{Ahep}* hepatocyte growth advantages because *ex vivo* *Ikkβ* deletion also stimulates *Ikkβ^{F/F}* hepatocyte proliferation. Future G₀-culture investigations should also clarify whether *Ikkβ^{Ahep}* growth advantages result from signal-1 or -2 accelerated G_{0,1} → G₁ transitions with or without increased rates of *S_Δ* [14,29]. These investigations might be feasible with the culture system described here, which may have undergone G₀-arrest as evident from stable plateau phases d8–d24, reduced cyclin D1 mRNA expression and near-baseline BrdU L.I. levels d8–d10.

Our results show that increased sensitivities to mitogens, including increased expression of ECM, basement membrane, cytoskeletal and integrin mRNAs accompany increased *Ikkβ^{Ahep}* hepatocyte growth. TNFα-attenuated growth in *Ikkβ^{F/F}* and *Ikkβ^{Ahep}* cultures is consistent with TNFα-stimulated apoptosis at high (0.58 nM) but not low (0.058 nM) TNFα doses [6], suggesting that ligand interactions with TNFR2 may account for attenuated growth in high-dose TNFα-treated cells. In further agreement, biphasic dose-response curves of TNFα-stimulated AP-1 activation were also seen in transfected hepatocytes: AP-1 expression peaked at 0.01 nM and returned to basal levels at 1 nM [30]. Significant changes in the expression of *Ikkβ^{Ahep}* fibronectin, collagens I and III, nidogen, β-actin and TGFα/EGF receptor-associated integrin B1 mRNAs were not seen in tissues or cells from which the cells were plated; instead 1–2d were required before *Ikkβ^{Ahep}*-associated elevations were observed. This suggests that regulatory effects of *Ikkβ* deletion occurred post-plating after exposure to serum factors, to factors released by necrotic cells [10], and possibly to culture conditions like uncoated plastic used to preclude cell attachment bias. Further investigations are needed to determine how and if these signaling systems underlie the growth advantages of *Ikkβ^{Ahep}* hepatocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ikkβ	IκB kinase β
IKK	IκB kinase complex
PH	partial hepatectomy

DEN	diethylnitrosamine
HCC	hepatocellular carcinoma
ECM	extracellular matrix
TGF	transforming growth factor
PBS	phosphate-buffered saline
GFP	green fluorescent protein
FAK	focal adhesion kinase
SPARC	secreted protein acidic and rich in cysteine

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Fig. 1. *Ikk $\beta^{\Delta hep}$* liver regeneration is accelerated after 70% PH. (A) Precocious PCNA and cyclin D1 expression. Liver lysates were gel separated and immunoblotted. (B) Elevated hepatocyte M.I. Hemotoxylin-and-eosin stained liver sections 48 h post-PH (200 $\times$): mitotic hepatocytes ($\rightarrow$). (C) Apoptotic *Ikk $\beta^{F/F}$* and *Ikk $\beta^{\Delta hep}$* hepatocytes quantified by TUNEL assays ($N = 4$).

Fig. 2.

(A–C) Cultured $Ikk\beta^{\Delta hep}$ hepatocyte growth advantages. (A) Growth dependence upon N_0 . $N_0 = 1.5 \times 10^5$ ($N = 2$ dishes/point, 1 plating); $N_0 = 3 \times 10^5$ ($IKK\beta^{F/F}$ [$N = 137$] or $IKK\beta^{\Delta hep}$ [$N = 176$ dishes/curve]; 6–10 platings); $N_0 = 6 \times 10^5$ ($N = 2$ dishes/point, 1 plating). (B) Selection against non-hepatocytes. $Ikk\beta$ deletion analyses were performed at indicated times ($N_0 = 3 \times 10^5$). (C) Reduced $IKK\beta$ mRNA levels. $N_0 = 3 \times 10^5$; $N = 2$ dishes/point, 2 platings. (D–F) Augmented cultured $Ikk\beta^{\Delta hep}$ hepatocyte L.I. and cyclin D1 mRNA expression ($N_0 = 3 \times 10^5$). (D) L.I. through mid-log phase: albumin + (top curves) or BrdU + albumin + cells (bottom curves); $N = 6$ dishes/point, 3 platings. (E) $Ikk\beta^{\Delta hep}$ S- and M-phase hepatocytes (200 $\times$): mitotic cells ($\leftarrow$). (F) Augmented cyclin D1 expression. $N = 8$ dishes/point, 4 platings.

Fig. 3. *Ikkβ* deletion *ex vivo* stimulates cultured *Ikkβ^{F/F}* hepatocyte proliferation. (A) Growth and BrdU L.I. curves (*top* and *bottom* panels, respectively; additions, ↓). (B) Ad-Cre infection augments cyclin D1 and reduces IKK β mRNA expression (*top* and *bottom* panels, respectively). Panels (A and B): *N* = 6 dishes/point, 3 platings.

Fig. 4.

Effects of *Ikkβ* deletion on growth-signaling systems in cultured hepatocytes. (A) Heightened *Ikkβ^{Δhep}* sensitivity to mitogens. Duplicate wells of cells plated ($N_0 = 1.5 \times 10^5$) into 24-multiwell plates received serum-free plating medium (0-dose), TGFα (top panel) or TNFα (bottom panel). Curves are differences between the average AUC $\pm$ S.E._[each treatment] minus the average AUC $\pm$ S.E._[0-dose] integrated over d1–d9 cell counts. Average AUC values_[0-dose] subtracted from each AUC_[ligand dose] were 42026 (*Ikkβ^{F/F}*) and 61023 (*Ikkβ^{Δhep}*; see Suppl. Table 3). Intergenotypic *P* values between curves: TGFα, $P < 0.0005$; TNFα, $P < 0.03$. (B) Augmented *Ikkβ^{Δhep}* cytoskeletal, ECM, basement membrane and integrin mRNA expression. Curves are averages, 3 platings ($N_0 = 3 \times 10^5$); $N = 6$ dishes/primer pair/point (sister culture growth was similar to Fig. 2A). Primer pair results were combined (Suppl. Table 1), except for nidogen ($N = 6$ dishes/primer pair 1/point; $N = 4$ dishes, primer pair 2/point). Intergenotypic *P* values d0–d6: fibronectin ($P < 0.07$); collagen I ($P < 0.003$), collagen III ($P < 0.0003$); nidogen ($P < 0.02$); β-actin ($P < 0.03$); integrin B1 ($P < 0.0001$).