

# Hepatocellular Carcinoma Repression by TNF $\alpha$ -Mediated Synergistic Lethal Effect of Mitosis Defect-Induced Senescence and Cell Death Sensitization

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Hepatocellular carcinoma (HCC) is a cancer lacking effective therapies. Several measures have been proposed to treat HCCs, such as senescence induction, mitotic inhibition, and cell death promotion. However, data from other cancers suggest that single use of these approaches may not be effective. Here, by genetic targeting of Survivin, an inhibitor of apoptosis protein (IAP) that plays dual roles in mitosis and cell survival, we identified a tumor necrosis factor alpha (TNF $\alpha$ )-mediated synergistic lethal effect between senescence and apoptosis sensitization in malignant HCCs. Survivin deficiency results in mitosis defect-associated senescence in HCC cells, which triggers local inflammation and increased TNF $\alpha$ . Survivin inactivation also sensitizes HCC cells to TNF $\alpha$ -triggered cell death, which leads to marked HCC regression. Based on these findings, we designed a combination treatment using mitosis inhibitor and proapoptosis compounds. This treatment recapitulates the therapeutic effect of Survivin deletion and effectively eliminates HCCs, thus representing a potential strategy for HCC therapy. *Conclusion:* Survivin ablation dramatically suppresses human and mouse HCCs by triggering senescence-associated TNF $\alpha$  and sensitizing HCC cells to TNF $\alpha$ -induced cell death. Combined use of mitotic inhibitor and second mitochondrial-derived activator of caspases mimetic can induce senescence-associated TNF $\alpha$  and enhance TNF $\alpha$ -induced cell death and synergistically eliminate HCC. (HEPATOLOGY 2016;64:1105-1120)

Hepatocellular carcinoma (HCC) is the fifth-most common cancer and the third cause of cancer death worldwide.<sup>(1)</sup> The only approved drug for HCC treatment is sorafenib, which prolongs overall survival by around 3 months.<sup>(2)</sup> There is an urgent need to develop more-effective therapies.

A notable feature of HCC is that it usually develops in inflammatory conditions, for example, viral infection and metabolic disease-associated steatohepatitis.<sup>(3,4)</sup> One of the key players in regulating cancer-associated immune responses is cellular senescence.<sup>(5,6)</sup>

*Abbreviations:* CPC, chromosomal passenger complex; DEN, diethylnitrosamine; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidine; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; IAP, inhibitor of apoptosis proteins; mRNA, messenger RNA; PCR, polymerase chain reaction; PDX, patient-derived xenograft; PLK1, polo-like kinase 1; qPCR, quantitative PCR; SA- $\beta$ -gal, senescence-associated  $\beta$ -galactosidase activity; Ptges, prostaglandin E synthase; SAHF, senescence-associated heterochromatin foci; siRNAs, small interfering RNAs; SMAC, second mitochondrial-derived activator of caspases; TNF $\alpha$ , tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Received February 13, 2016; accepted April 26, 2016.

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep.28637/supinfo](http://onlinelibrary.wiley.com/doi/10.1002/hep.28637/supinfo).

This study is funded by the Ministry of Science and Technology (MOST) of China (2014CB910601, 2012CB945001, and 2012ZX10002), the National Science Foundation of China (NSFC) (31225016, 31501111, 81221061, 81471948, and 81471948), Shanghai Science and Technology Committee (15JC1400200, 14XD1404200). Dr. Dan Li is supported by the Postdoctor Research Program of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (2014KIP302), and the China Postdoctoral Science Foundation (2015M580361).

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DOI 10.1002/hep.28637

Potential conflict of interest: Nothing to report.

Cellular senescence, a state of irreversible cell-cycle arrest, is considered a tumor-suppressive mechanism.<sup>(7,8)</sup> Cellular senescence has been documented as a pivotal barrier for initiation and progression of HCC.<sup>(6,9)</sup> Moreover, senescent HCC-associated stromal cells secrete extracellular-matrix-degrading enzymes, which are believed to enhance immune surveillance and facilitate HCC resolution.<sup>(10)</sup> In these scenarios, both innate immune responses and T-cell-mediated adaptive immunity are involved.<sup>(6,9)</sup>

Approaches to induce efficient senescence *in vivo* have been actively investigated.<sup>(8)</sup> Recent studies showed that inhibiting the chromatin passenger complex (CPC) function caused cellular senescence.<sup>(11)</sup> CPC complex controls chromatin alignment during mitosis, the inhibition of which leads to mitosis arrest and proliferation halt.<sup>(12)</sup> Polo-like kinase 1 (PLK1) and Aurora B kinase are two key kinases in regulating CPC functions.<sup>(13)</sup> There are several highly selective compounds targeting against PLK1 or Aurora B. Both types of inhibitors caused remarkable mitosis arrest and senescence. Surprisingly, the outcomes of several clinical trials for these compounds were disappointing, making some debates on the therapeutic value for these mitotic inhibitors.<sup>(14-16)</sup> To that end, it was proposed to exploit these compounds in combination with other drugs, mainly chemotherapeutic drugs<sup>(15,17)</sup>; however, the underlying rationale for such use was not fully clarified.

Both senescence-based or mitosis arrest-based anticancer treatment induce a cytostatic status, but do not directly eliminate cell-cycle-arrested cells.<sup>(18,19)</sup> A desirable therapy would not only induce cytostasis, but also eliminate these cancer cells efficiently. It is proposed to take advantage of HCC-associated inflammation. For example, senescence and infiltration of immune cells trigger locally increased concentrations of cytokines in HCC tissues. Some of these inflammatory factors, such as tumor necrosis factor alpha (TNF $\alpha$ ), are cell death inducers.<sup>(20)</sup> The difficulty of this approach is that HCC cells are well protected from cell death by antiapoptotic proteins, including members of the family of inhibitor of apoptosis proteins (IAPs). It is thus tempting to develop a combinational strategy to eliminate cytostatic cancer cells by unleashing the death-inducing effects of inflammatory cytokines.

Survivin is the smallest member of the family of IAPs and is highly expressed in precancerous liver lesions and in malignant HCC cells.<sup>(21)</sup> Besides its functions in cell death, Survivin is also a major component of the CPC complex and controls mitosis.<sup>(12)</sup> Previous studies have proposed that Survivin could be a therapeutic target for cancer treatment. However, development of Survivin inhibitors has so far been unsuccessful.<sup>(22,23)</sup> Further delineation of the oncogenic properties of Survivin may provide insights for the development of novel anticancer strategies that bypass direct targeting of Survivin. In that respect, we have previously shown that Survivin promotes

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survival of HCC initiating cells by regulation of activator protein 1 and sirtuin 6.<sup>(21)</sup> However, it remains unknown whether Survivin controls HCC malignancy at late stages and, if so, how the molecular mechanism could be translated into a potential therapeutic strategy.

In this study, we found a near-complete HCC regression using genetic deletion of Survivin. A careful analysis of Survivin deletion in HCC led to an unexpected finding of a synergistic effect between mitosis defect-induced senescence and apoptosis sensitization, mediated by TNF $\alpha$ , on eliminating HCC cells. Survivin deletion causes mitosis defect and senescence, which further induces inflammation and TNF $\alpha$  expression locally. Remarkably, because of the hypersensitivity of Survivin-deficient HCC cells to TNF $\alpha$ -triggered cell death, Survivin-deficient HCC cells undergo extensive cell death, thereby leading to drastic HCC regression. By taking advantage of these findings, we additionally designed and validated a new HCC therapeutic strategy by combination use of mitotic inhibitor and second mitochondrial-derived activator of caspases (SMAC) mimetic to induce mitosis arrest-associated senescence and enhance TNF $\alpha$ -induced cell death, respectively.

## Materials and Methods

### PRIMARY CULTURED HCC CELLS AND PATIENT-DERIVED XENOGRAFT

Human HCC samples used for primary HCC cell culture and patient-derived xenograft (PDX) transplantation were collected from Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China). All procedures of human sample collection were approved by the Ethical Committee of Eastern Hepatobiliary Surgery Hospital.

For primary HCC cell culture, HCC tissues were snipped followed by collagenase digestion. HCC cells were cultured in RPMI 1640 medium with 10% fetal calf serum on collagen-coated dishes. For PDX transplantation, HCC tissues were cut into pieces and subcutaneously transplanted into nod-scid mice and then transplanted into athymic nude mice where tumors were allowed to grow.

### MICE AND LIVER TUMORIGENESIS PROTOCOL

*Mx-cre*; *Survivin*<sup>f/f</sup> (*svv* <sup>$\Delta$ li\*</sup>) and *Alb-cre*; *Survivin*<sup>f/f</sup> mice were generated by crossing *svv*<sup>f/f</sup> mice to *Alb-cre*

mice and *Mx-cre* mice. Genotyping was performed by polymerase chain reaction (PCR) of tail genomic DNA. To induce HCCs in mice, a single dose of diethylnitrosamine (DEN; 25 mg per kilogram of body weight; Sigma-Aldrich, St. Louis, MO) was injected into 2-week-old male mice intraperitoneally and mice were euthanized at the age of 10 months. Genotyping primers are listed in the [Supporting Tables](#). Mice were kept under specific pathogen-free conditions at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Biochemistry and Cell Biology and performed in accord with this committee's guidelines.

### MICROARRAY ANALYSIS

Total RNAs were hybridized to whole-mouse gene expression microarray (Agilent Technologies, Inc., Lexington, MA) in accord with the manufacturer's instructions. Data were normalized using Gene-Spring (Agilent). Original data are available in the NCBI Gene Expression Omnibus (GSE64804).

### STATISTICAL ANALYSIS

For most statistical analyses, the unpaired Student *t* test was applied for calculating statistical probability. For survival analyses, the Kaplan-Meier log-rank test was applied. Statistical calculations were performed using the Statistical Program for Social Sciences (SPSS) software (IBM Corp., Armonk, NY). For all statistics, data from at least three independent samples or repeated experiments were used.

## Results

### SURVIVIN DELETION INDUCES REGRESSION OF MALIGNANT HCCs

We previously showed that Survivin promotes survival of HCC-initiating cells.<sup>(21)</sup> However, genetic evidence is still lacking on whether Survivin controls HCC progression and malignancy. We generated *Mx-cre*, *svv*<sup>f/f</sup> mice by crossing mice carrying floxed *Survivin* alleles (*svv*<sup>f/f</sup>) and mice carrying *Mx1* promoter-controlled *Cre* recombinase (*Mx-cre*). Poly(IC) treatment induces *Cre* expression in hepatocytes and hematopoietic cells in *Mx-cre*, *svv*<sup>f/f</sup> mice.<sup>(24)</sup> To specifically delete Survivin in

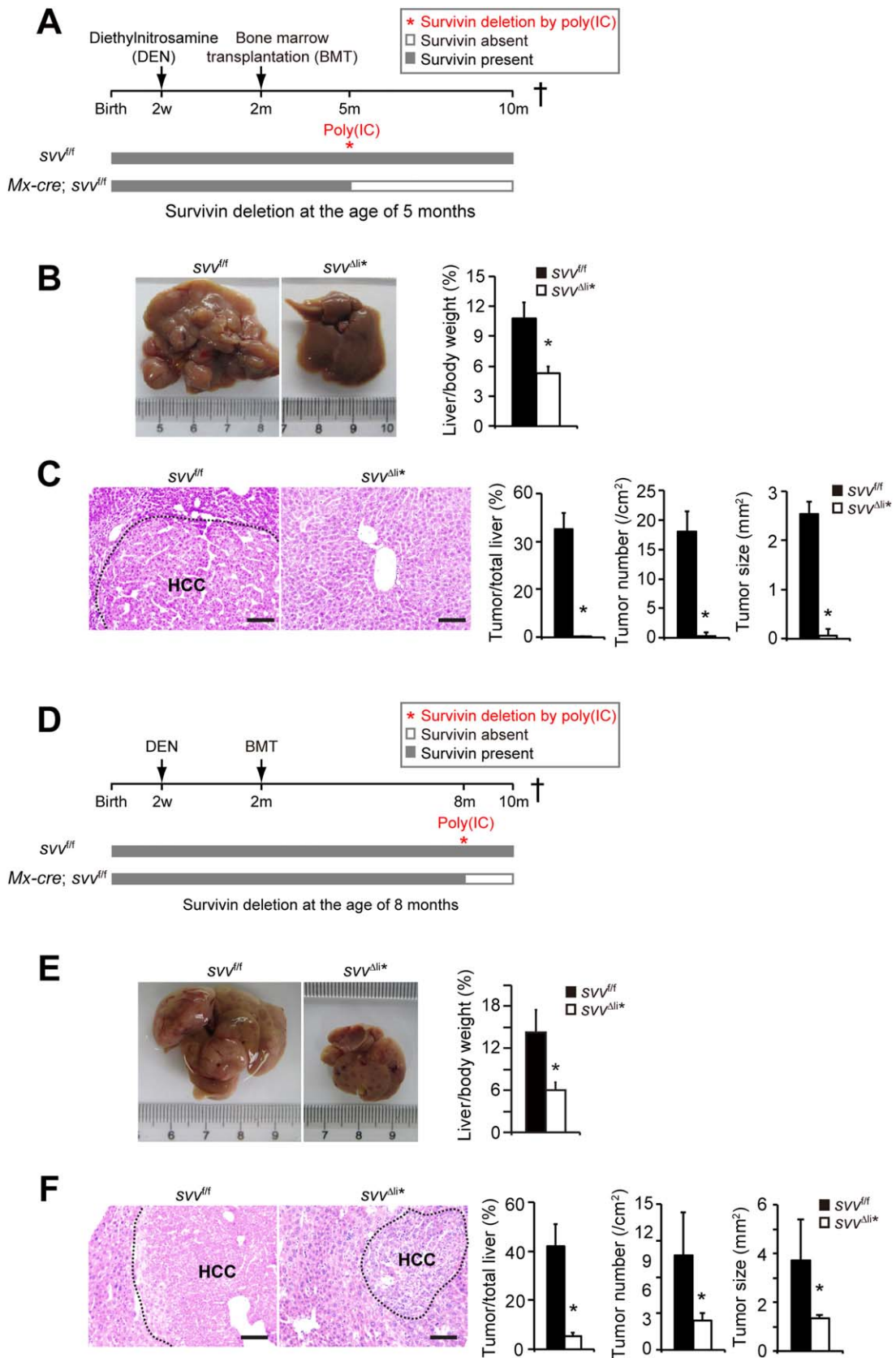


FIG. 1



hepatocytes, *Mx-cre*, *svv<sup>f/f</sup>* mice were irradiated and transplanted with bone marrows from *svv<sup>f/f</sup>* siblings before poly(IC) treatment (*svv<sup>Δli\*</sup>*). In line with our previous report, the morphology and function of hepatocytes remained normal in *svv<sup>Δli\*</sup>* mice.<sup>(24)</sup>

HCCs were induced with DEN in *svv<sup>f/f</sup>* and *Mx-cre*, *svv<sup>f/f</sup>* mice at the age of 2 weeks. Both *svv<sup>f/f</sup>* and *Mx-cre*, *svv<sup>f/f</sup>* mice then received bone marrow transplantation at the age of 2 months and injected with poly(IC) to delete Survivin either at the age of 5 (Fig. 1A) or 8 months (Fig. 1D). Poly(IC) treatment efficiently deleted Survivin in livers and HCCs in *Mx-cre*, *svv<sup>f/f</sup>* mice, but not in bone marrow cells or tumor-infiltrated Kupffer cells and T cells (Supporting Fig. S1A,B). In both experimental settings, the formation of HCCs was comparable between *svv<sup>f/f</sup>* and *Mx-cre*, *svv<sup>f/f</sup>* mice before Survivin deletion (Supporting Fig. S1C,D). At the age of 10 months, mice were sacrificed and HCCs were quantified. Control *svv<sup>f/f</sup>* mice developed a large number of HCCs (Fig. 1B,C). By contrast, HCCs were almost undetectable in *svv<sup>Δli\*</sup>* mice with Survivin deletion at the age of 5 months (Fig. 1B,C), and HCCs were markedly reduced in *svv<sup>Δli\*</sup>* mice with Survivin deletion at the age of 8 months (Fig. 1E,F). Serum alanine aminotransferase, aspartate aminotransferase, and total bilirubin levels were decreased in *svv<sup>Δli\*</sup>* mice (Supporting Fig. S1E,F), suggesting improved liver function. Moreover, cancer-bearing *svv<sup>Δli\*</sup>* mice survived much longer than *svv<sup>f/f</sup>* mice (Supporting Fig. S1G).

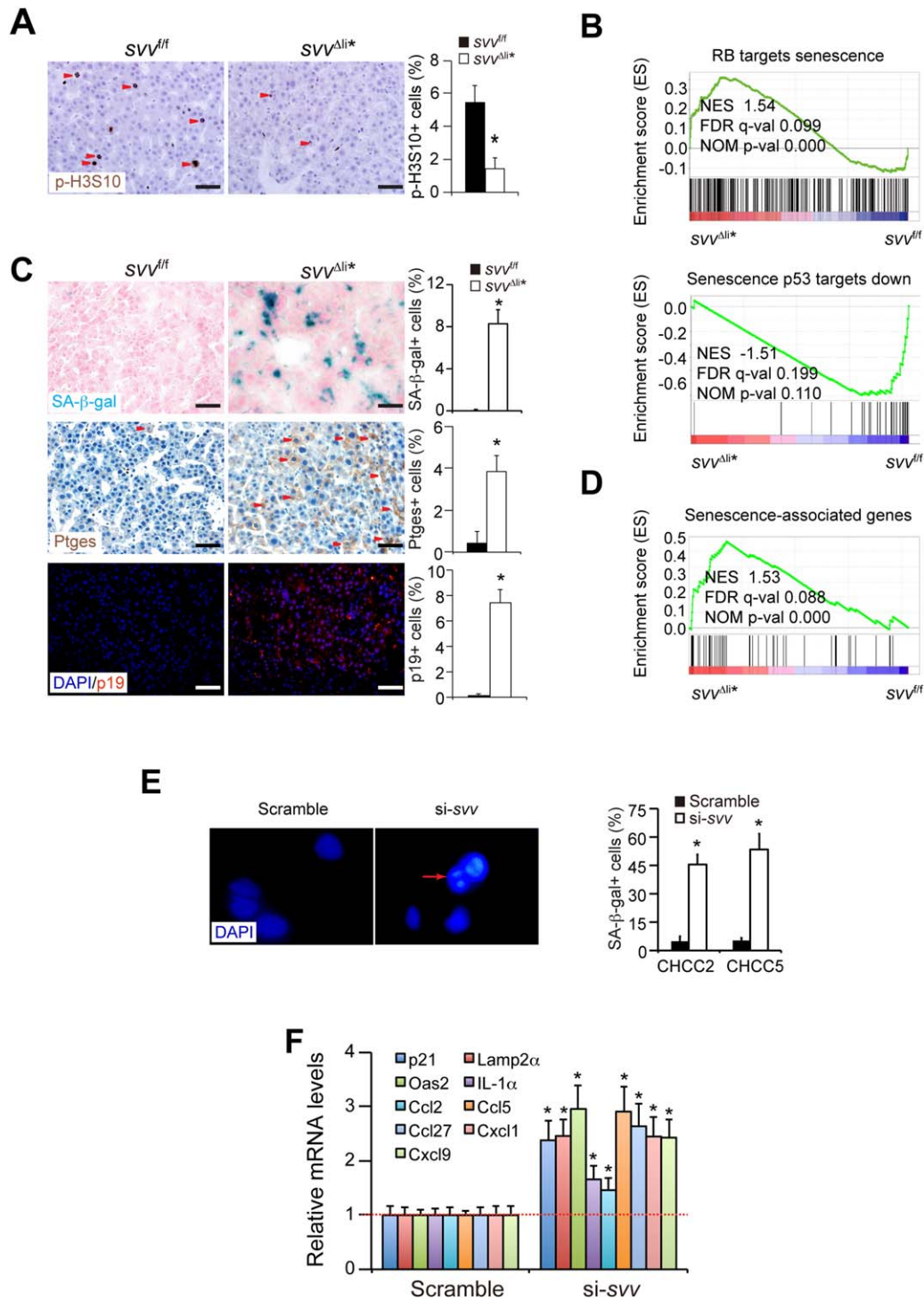
To validate this phenotype of HCC inhibition in Survivin-deficient livers, HCCs were induced in another liver-specific Survivin knockout mouse line, *Alb-cre*, *svv<sup>f/f</sup>*. We previously showed that Survivin was deleted in >90% of hepatocytes within 2 weeks after birth in these mice.<sup>(24)</sup> Notably, DEN induced very few HCCs in *Alb-cre*, *svv<sup>f/f</sup>* mice compared to control *svv<sup>f/f</sup>* mice (Supporting Fig. S2A,B), and *Alb-cre*, *svv<sup>f/f</sup>* mice showed improved liver function and survival (Supporting Fig. S2C,D). The robustness of our find-

ings was demonstrated with a c-Met and  $\Delta N90$ - $\beta$ -Catenin-induced HCC model (Supporting Fig. S10), in which we also observed reduced HCC formation in *svv<sup>Δli\*</sup>* mice (Supporting Fig. S2E). The results from different mouse models indicate that lack of Survivin results in marked inhibition of HCC and provide compelling genetic evidence that targeting Survivin might be an effective therapeutic strategy.

## SURVIVIN DEPLETION TRIGGERS MITOSIS DEFECT AND SENEESCENCE IN HCC CELLS

Although a potentially attractive therapeutic oncotarget, it is yet difficult to develop inhibitors against Survivin.<sup>(22,23)</sup> YM155 is the most clinically advanced Survivin inhibitor.<sup>(25)</sup> However, YM155 failed to show clear efficacy in phase II clinical trials for primary melanomas and non-small-cell lung cancers.<sup>(26,27)</sup> Moreover, remarkable toxicity of YM155 was reported in clinical trials. In line with these findings, we found that YM155, at the dose of 5 mg/kg body weight per day, showed significant toxicity on HCC-bearing mice, which lost over 30% of their body weight after 1 month of treatment (Supporting Fig. S3A) and imposed poorly repressive effects on primary HCCs (Supporting Fig. S3B). We therefore explored the mechanisms underlying Survivin deletion-induced inhibition of HCC growth, with a view to utilizing those mechanisms for the development of novel anti-HCC strategies. HCCs were analyzed in *svv<sup>Δli\*</sup>* mice with Survivin depletion at the age of 8 months, which was relevant to treatment of late-stage HCCs. We first asked whether Survivin controls the function of CPC complex and mitotic transition in HCCs. We found that the number of p-H3S10-positive HCC cells was decreased in *svv<sup>Δli\*</sup>* HCCs 1 week after Survivin depletion (Fig. 2A). Moreover, immunofluorescent staining showed that Aurora-B kinase, the enzymatic member

**FIG. 1.** Survivin is essential in HCC development. (A) Experimental design for the DEN-induced HCC model. *Mx-cre*, *svv<sup>f/f</sup>* and *svv<sup>f/f</sup>* control mice were treated with DEN at 2 weeks of age and  $\gamma$ -irradiated, followed by transplantation with bone marrows from *svv<sup>f/f</sup>* mice at the age of 2 months. No HCCs were detectable at the time of bone marrow transplantation. Five-month-old *Mx-cre*, *svv<sup>f/f</sup>* and control mice were injected with poly(IC) intraperitoneally to induce liver-specific Survivin gene depletion. *Mx-cre*, *svv<sup>f/f</sup>* mice were referred to as *svv<sup>Δli\*</sup>* after poly(IC)-induced depletion of Survivin. All mice were sacrificed for HCC analysis at the age of 10 months. (B,C) DEN-induced HCCs were analyzed in *svv<sup>Δli\*</sup>* and *svv<sup>f/f</sup>* mice 5 months after poly(IC) treatment. (B) Liver/body weight ratios were measured. n = 5 for *svv<sup>f/f</sup>* mice; n = 6 for *svv<sup>Δli\*</sup>* mice. (C) Hematoxylin and eosin (H&E) staining of liver sections. HCC was encircled with a dashed line. HCCs were quantified. n = 5 for *svv<sup>f/f</sup>* mice; n = 5 for *svv<sup>Δli\*</sup>* mice. (D) Similar treatment as described in (A) was applied to *Mx-cre*, *svv<sup>f/f</sup>* and *svv<sup>f/f</sup>* mice. However, Survivin was depleted with poly(IC) in *Mx-cre*, *svv<sup>f/f</sup>* mice at the age of 8 months. (E,F) DEN-induced HCC was analyzed in *svv<sup>Δli\*</sup>* mice at the age of 10 months. (E) Liver/body weight ratios were measured. n = 7 for *svv<sup>f/f</sup>* mice; n = 5 for *svv<sup>Δli\*</sup>* mice. (F) H&E staining of liver sections. HCCs were encircled with dashed lines, and HCCs were quantified. n = 5 for *svv<sup>f/f</sup>* mice; n = 5 for *svv<sup>Δli\*</sup>* mice. Scale bars, 100  $\mu$ m. \**P* < 0.05, *t* test.



**FIG. 2.** Survivin deficiency induces a mitosis defect and cellular senescence. (A) Mitotic HCC cells were analyzed by immunohistochemical staining for phosphorylated H3S10 (p-H3S10). HCCs were harvested 1 week after poly(IC)-induced Survivin depletion. (B) GSEA analysis showed that the expression profile of RB-related senescence and p53-related senescence were enriched in *svv*<sup>Δli\*</sup> liver cancers. (C) SA-β-gal staining (blue) on cryosections of HCCs from *svv*<sup>Δli\*</sup> mice 1 week after polyIC treatment. SA-β-gal-positive HCC cells were quantified. Cancer cells expressing senescence marker genes Ptges and p19Arf were determined by immune staining on paraffin sections. Arrowheads indicate Ptges-positive cancer cells. Quantification of Ptges- and p19Arf-positive cancer cells are shown. n = 4 for each group for (A) and (C). (D) GSEA of expression profiles of senescence-associated genes in DEN-induced HCCs from *svv*<sup>fl/fl</sup> and *svv*<sup>Δli\*</sup> mice 1 week after poly(IC) treatment. Senescence-associated genes were taken from the study of Pribluda et al.<sup>(28)</sup> (E) Two primary human HCC cells were transfected with Survivin-specific siRNAs to silence Survivin. SAHFs were characterized by DAPI-DNA staining. SAHF are indicated by arrows. SA-β-gal-positive cells were quantified 72 hours after siRNA transfection. All results represent three independent experiments. \*P < 0.05, t test. (F) qPCR analysis of senescence-associated genes in Survivin-silenced human HCC cells. Results represent three independent experiments. Scale bars, 50 μm. \*P < 0.05, t test. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; FDR, false discovery rate; NES, normalized enrichment score; NOM, nominal.

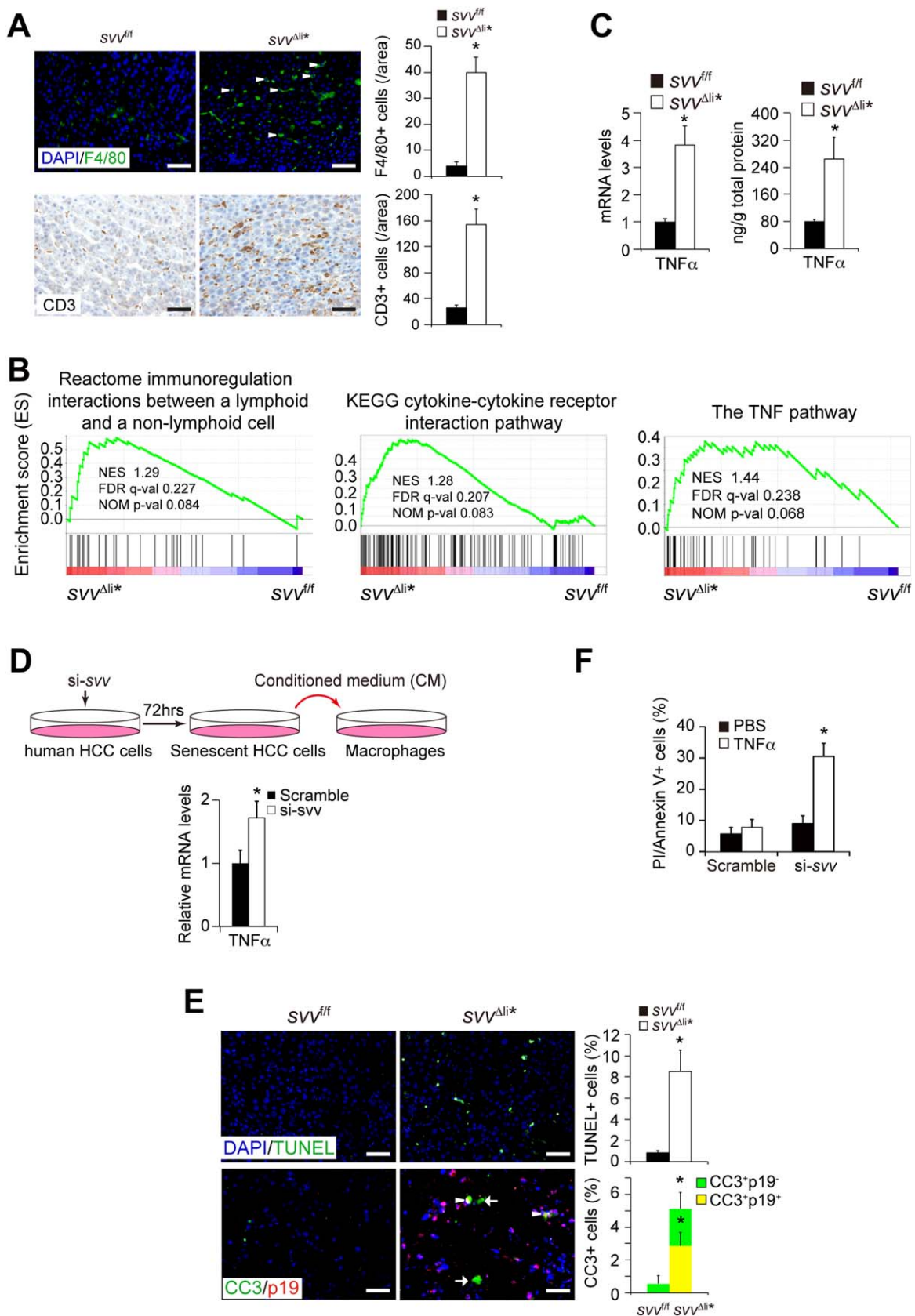


FIG. 3



of the CPC complex, diffused in chromosome arms and failed to locate to kinetochores in *svv*<sup>Δli\*</sup> HCC cells (Supporting Fig. S4A). These data suggest that Survivin deficiency causes impaired mitosis in HCC cells.

Whole-genome expression profiling of *svv*<sup>Δli\*</sup> HCCs 1 week after Survivin depletion was performed by microarray assay. Gene set enrichment analysis (GSEA) showed that genes involved in mitosis and cell-cycle transition were significantly down-regulated in *svv*<sup>Δli\*</sup> HCCs (Supporting Fig. S4B). Moreover, expression of genes involved in RB-related senescence and p53-related senescence were enriched in *svv*<sup>Δli\*</sup> HCCs (Fig. 2B). Because mitotic arrest has been previously shown as a cause of cellular senescence,<sup>(11)</sup> we characterized senescence in Survivin-deficient HCCs. Senescence-associated β-galactosidase activity (SA-β-gal) was markedly increased in *svv*<sup>Δli\*</sup> HCCs (Fig. 2C). Senescence marker genes, including prostaglandin E synthase (Ptges) and p19Arf, were similarly up-regulated in *svv*<sup>Δli\*</sup> HCCs<sup>(28)</sup> (Fig. 2C). Furthermore, a panel of senescence-associated genes<sup>(28)</sup> were up-regulated in *svv*<sup>Δli\*</sup> HCCs, as shown by GSEA analysis (Fig. 2D) and confirmed by quantitative PCR (qPCR; Supporting Fig. S4C). Overall, these data indicate that Survivin depletion induces senescence in HCC cells.

To assess the clinical relevance of our findings, primary human HCC cells were transfected with small interfering RNAs (siRNAs) targeting Survivin. Efficient knockdown of Survivin was confirmed by qPCR (Supporting Fig. S4D). Survivin knockdown resulted in impaired mitosis in primary human HCC cells at 24 to 48 hours (Supporting Fig. S4E). After impaired mitosis, prominent senescence was validated with senescence-associated heterochromatin foci (SAHF) staining and SA-β-gal staining (Fig. 2E) and increased

expression of senescence-associated genes in Survivin-deficient human HCC cells by qPCR (Fig. 2F). Together, our data indicate that Survivin deficiency causes mitosis defect-associated senescence in HCC cells.

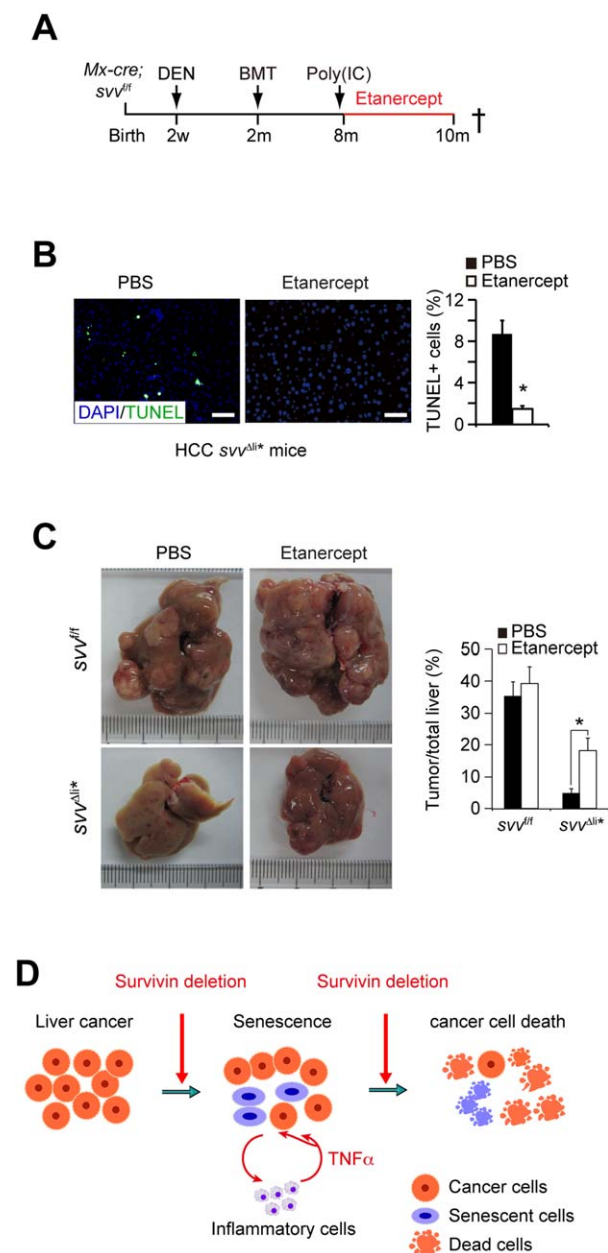
## SURVIVIN PROTECTS HCC CELLS FROM TNF $\alpha$ -INDUCED CELL DEATH

Previous studies showed that senescent cancer cells have a role in increase local inflammatory responses in tumors.<sup>(7,8)</sup> Accordingly, increased numbers of macrophages and CD3<sup>+</sup> T lymphocytes were found in *svv*<sup>Δli\*</sup> HCCs (Fig. 3A). Moreover, several inflammation response-related pathways were remarkably enriched in *svv*<sup>Δli\*</sup> HCCs by GSEA analysis (Fig. 3B). Importantly, we found that genes downstream of the TNF $\alpha$  pathway were strikingly increased (Fig. 3B). Given that TNF $\alpha$  is a prominent cell death inducer, we focused our study on this cytokine. Increased messenger RNA (mRNA) and protein levels of TNF $\alpha$  were validated in *svv*<sup>Δli\*</sup> HCCs (Fig. 3C). To characterize whether senescent cells have a role in inducing TNF $\alpha$  in inflammatory cells, we generated senescent cells by silencing Survivin and applied supernatant from the culture of senescent cells to macrophages. It was notable that macrophages produced higher TNF $\alpha$  upon addition of the supernatant. These data suggest a causative role of senescence in TNF $\alpha$  induction (Fig. 3D).

Because Survivin protects cells from death ligand-triggered cell death, we considered whether senescence-associated TNF $\alpha$  might cause enhanced cell death in Survivin-deficient HCCs. Both terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and immunostaining for cleaved caspase 3

**FIG. 3.** Survivin deficiency induces senescence-associated inflammation and TNF $\alpha$  expression. (A) Immunofluorescent staining of F4/80 and CD3 showed increased infiltration of macrophages and T cells, respectively, in *svv*<sup>Δli\*</sup> liver cancers. F4/80-positive and CD3-positive cells were quantified. n = 4 for each group. (B) GSEA analysis showed enrichment of inflammation pathway and the TNF $\alpha$  pathway genes in *svv*<sup>Δli\*</sup> liver cancers. (C) TNF $\alpha$  mRNA and protein levels in *svv*<sup>Δli\*</sup> HCCs were determined by qPCR and enzyme-linked immunosorbent assay. n = 4 for each group. (D) Conditioned medium of senescent human HCC cells stimulates THP1-derived macrophages to secrete TNF $\alpha$ . Human HCC cells were induced to be senescent by siRNA against Survivin. Conditioned medium from senescent culture were collected at 72 hours. THP-1-derived macrophages were cultured with conditioned medium of senescent HCC cells for another 24 hours. mRNA levels of TNF $\alpha$  were analyzed by qPCR. Results represent two independent experiments. \*P < 0.05, t test. (E) Cell death was detected by TUNEL staining in HCCs 1 week after poly(IC) treatment. TUNEL-positive cancer cells were quantified. n = 4 for each group. Costaining of cleaved caspase 3 (CC3; for dead cells) and p19Arf (p19; for senescent cells) on HCCs 1 week after poly(IC) treatment. CC3-positive p19Arf-negative cells are indicated by arrows. CC3-positive p19Arf-positive cancer cells are indicated by arrowheads. Quantification of CC3-positive and p19Arf-positive cells showed both senescent and neighboring nonsenescent cells undergoing cell death. n = 3 for each group. (F) Two primary human HCC cells were transfected with siRNAs against Survivin and then treated with 10ng/mL of human TNF $\alpha$  (hTNF $\alpha$ ) to induce cell death. Dead cells were stained by propidium iodide and Annexin V and then analyzed by fluorescence-activated cell sorting. Results represent three independent experiments. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; NOM, nominal; PBS, phosphate-buffered saline.





**FIG. 4.** Survivin protects HCC cells from inflammatory TNF $\alpha$ -mediated cell death. (A) Experimental scheme of etanercept treatment. Survivin was deleted in HCC-bearing *Mx-cre; svv<sup>fl/fl</sup>* at the age of 8 months. These mice were then treated with etanercept at 10 mg/kg body weight intraperitoneally twice a week. (B) Cell death in *svv<sup>Δli\*Δli\*</sup>* HCCs was dramatically reduced after etanercept treatment for 1 week as determined by TUNEL staining. (C) HCC development was analyzed in *svv<sup>Δli\*Δli\*</sup>* mice after etanercept treatment. HCCs were quantified.  $n = 3$  for each group.  $*P < 0.05$ ,  $t$  test. (D) Schematic of TNF $\alpha$ -mediated synergistic lethal effect of senescence and apoptosis sensitization; Survivin depletion induced impaired mitosis and senescence in cancer cells, whereas infiltration of inflammatory cells and senescence-associated TNF $\alpha$  triggered cell death in senescent and neighboring nonsenescent cancer cells lacking Survivin. Abbreviations: BMT, bone marrow transplantation; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate-buffered saline.

revealed extensive cell death in *svv<sup>Δli\*Δli\*</sup>* HCCs (Fig. 3E and Supporting Fig. S5A). In addition, gene expression profiling showed that a panel of cell death and apoptosis genes were increased in *svv<sup>Δli\*Δli\*</sup>* HCCs (Supporting Fig. S5B). It is also noteworthy that both senescent and neighboring nonsenescent HCC cells underwent cell death in *svv<sup>Δli\*Δli\*</sup>* tumors (Fig. 3E), suggesting that a paracrine effect to induce cell death in Survivin-deficient HCC cells.

We additionally analyzed these findings using cultured primary human HCC cells. TNF $\alpha$  treatment alone induced a low level of cell death in primary HCC cells, which were consistent with previous findings that cancer cells are resistant to TNF $\alpha$ -induced cell death.<sup>(29)</sup> In contrast, siRNA-mediated Survivin silencing notably augmented TNF $\alpha$ -induced cell death (Fig. 3F). These results indicated that Survivin-deficient cells are hypersensitive to TNF $\alpha$ -induced cell death, which may explain the dramatic HCC regression in *svv<sup>Δli\*Δli\*</sup>* mice.

These findings together suggested a role of TNF $\alpha$  in mediating mitosis defect-induced senescence and sensitized cell death in Survivin-deficient HCC cells. To validate this hypothesis, we analyzed the anticancer effect of TNF $\alpha$  *in vivo* by blocking TNF $\alpha$  signaling with the TNF $\alpha$  antagonist, etanercept.<sup>(30)</sup> (Fig. 4A). We found that expression of TNF $\alpha$  was already reduced in *svv<sup>Δli\*Δli\*</sup>* HCCs after 2 weeks of etanercept treatment (Supporting Fig. S5C), confirming an inhibitory effect of etanercept on TNF $\alpha$  *in vivo*.<sup>(31)</sup> Moreover, decreased number of SA- $\beta$ -gal-positive cells further validated the blockage of TNF $\alpha$  (Supporting Fig. S5D), because TNF $\alpha$  has a positive-feedback effect on senescence.<sup>(32)</sup> Importantly, cell death in *svv<sup>Δli\*Δli\*</sup>* HCCs was significantly attenuated after 2 weeks of etanercept treatment (Fig. 4B). As a consequence of reduced cell death, HCC regression in *svv<sup>Δli\*Δli\*</sup>* mice was largely reverted after etanercept treatment for 2 months (Fig. 4F). In contrast, etanercept had no effect on liver tumorigenesis in *svv<sup>fl/fl</sup>* mice (Fig. 4C), supporting a key role for Survivin in antagonizing cell death induced by senescence-related TNF $\alpha$ . Based on these findings, we proposed that Survivin targeting causes mitosis arrest-induced senescence and increased TNF $\alpha$  levels in HCCs. Moreover, Survivin depletion additionally sensitizes cancer cells to TNF $\alpha$ -induced cell death (Fig. 4D).

## COMBINATION OF MITOSIS INHIBITOR AND SMAC MIMETIC ELIMINATES MURINE HCCs

We asked whether we could take advantage of the anticancer mechanisms by mimicking mitosis arrest-

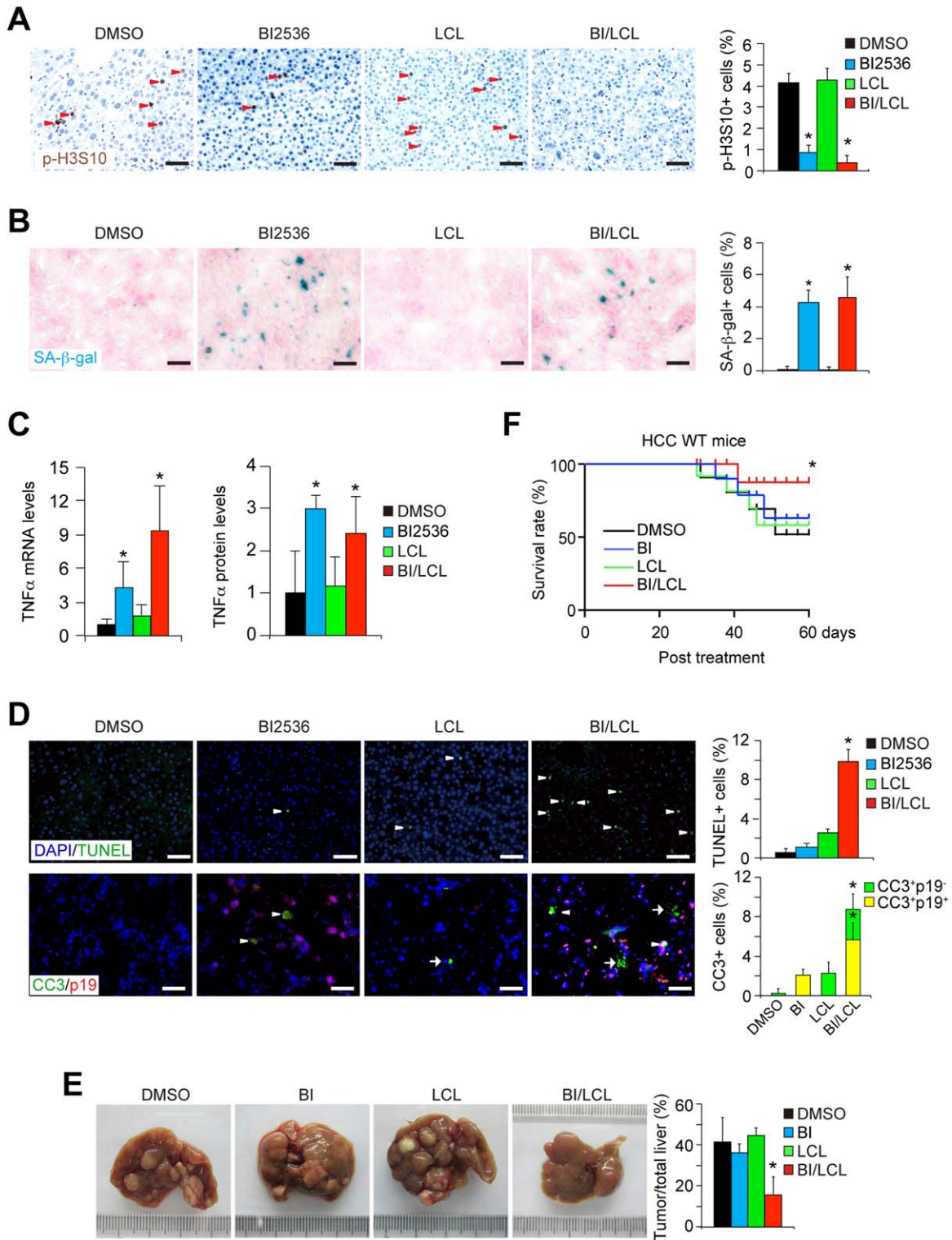


FIG. 5

caused senescence and sensitization to TNF $\alpha$ -induced cell death. We hypothesized that combination of a drug triggering mitosis arrest and senescence with a drug enhancing TNF $\alpha$ -induced cell death may phenocopy the Survivin deficiency effect on HCC. To that end, we used the PLK inhibitor, BI2536, to induce mitosis arrest and senescence and the SMAC mimetic, LCL161, to sensitize HCC cells to TNF $\alpha$ -induced cell death.

BI2536 is a highly selective PLK1 inhibitor,<sup>(33)</sup> which targets PLK1, a critical activator of the CPC complex and causes mitosis defect.<sup>(34)</sup> Exposure of primary human HCC cells to BI2536 caused mitosis defect (Supporting Fig. S6A) and senescence, as shown by SA- $\beta$ -gal staining (Supporting Fig. S6B), SAHF (Supporting Fig. S6C) and augmented expression of senescence-associated genes (Supporting Fig. S6D). We analyzed the *in vivo* effect of BI2536 on wild-type mice bearing DEN-induced HCCs. A decrease in p-H3S10-positive cell numbers was found in HCCs after BI2536 treatment for 2 weeks at a dose of 25 mg per kilogram body weight (mg/kg) every other day (Fig. 5A). BI2536-treated HCCs showed senescence, evidenced by SA- $\beta$ -gal staining and increased expression of senescence-associated genes (Fig. 5B and Supporting Fig. S6E). Notably, HCCs from BI2536-treated mice were infiltrated with inflammatory cells (Supporting Fig. S6F), and TNF $\alpha$  levels were elevated in BI2536-treated HCCs (Fig. 5C). However, BI2536 treatment induced a much lower level of cell death than in *svv*<sup>Al<sup>i</sup>\*</sup> HCCs (Fig. 5D), suggesting that the HCC cells may be protected by anticell death mechanisms. These data were in line with recent reports that BI2536 used alone did not appear to be an effective agent for cancer therapy.<sup>(14,35)</sup>

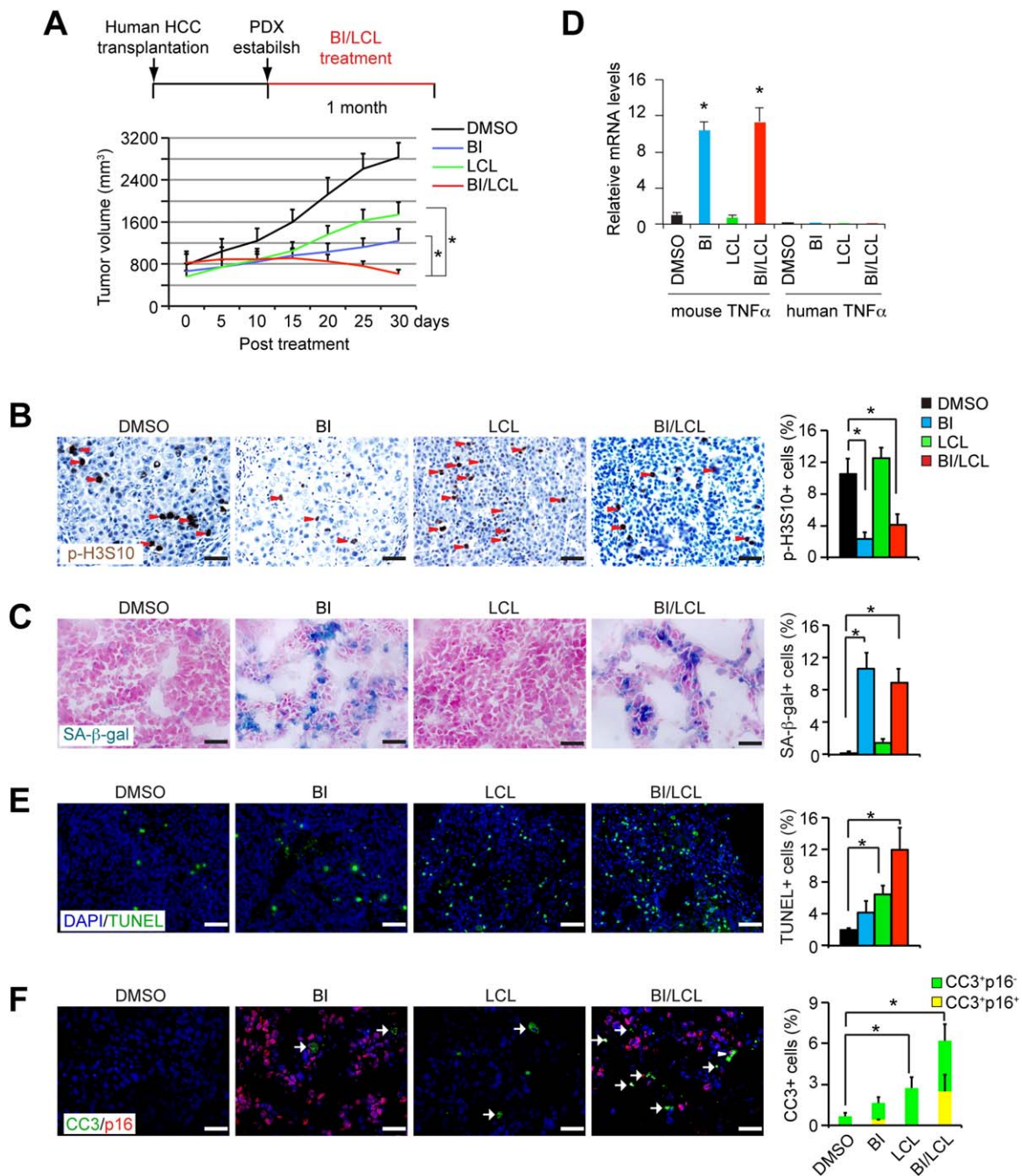
To boost the sensitivity to TNF $\alpha$ -induced cell death, we chose a clinically advanced SMAC mimetic, LCL161.<sup>(36,37)</sup> We first confirmed that LCL161 increased sensitivity to TNF $\alpha$ -induced cell death in primary human HCC cells (Supporting Fig. S6G). However, when LCL161 alone was applied to mice bearing DEN-induced HCC, it had a negligible effect on mitosis and senescence in HCC cells (Fig. 5A,B), and LCL161 only induced a mildly increased cell death in HCCs (Fig. 5D). As a consequence, LCL161 alone did not repress the progress of DEN-induced primary HCCs at a dose of 25 mg/kg every other day for 2 months (Fig. 5E).

We then analyzed the therapeutic effect of combined use of BI2536 and LCL161 *in vivo*. mRNA levels of Survivin were not significantly changed in HCCs by BI2536 treatment or combined treatment of BI2536 and LCL161 (Supporting Fig. S6H). Combination treatment with BI2536 and LCL161, each administered at a dose of 25 mg/kg every other day for 2 weeks, triggered mitotic arrest (Fig. 5A). Moreover, cellular senescence was enhanced in HCC cells, detected by SA- $\beta$ -gal staining (Fig. 5B) and senescence-associated gene expression (Supporting Fig. S6I). Increased levels of TNF $\alpha$  were also found in combined treatment with BI2536 and LCL161 (Fig. 5C). Importantly, the combination treatment induced substantially more cell death in both senescent and neighboring nonsenescent HCC cells, when compared to that observed with either BI2536 or LCL161 alone (Fig. 5D).

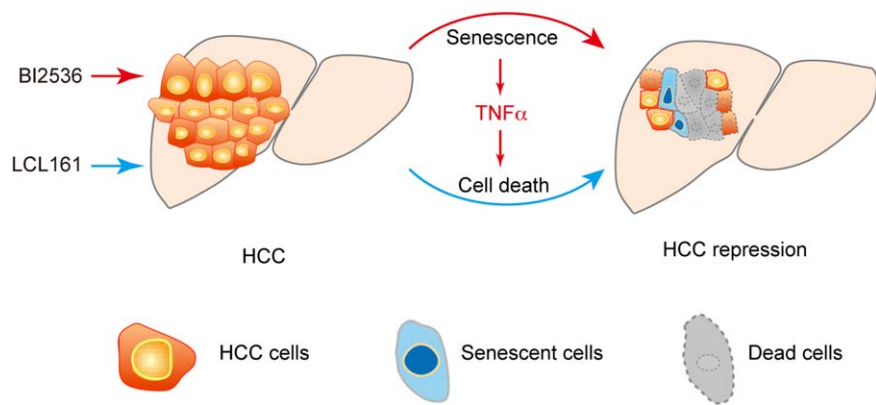
HCC-bearing mice were sacrificed after continuous treatment of both BI2536 and LCL161 for 2 months. Although HCCs were detectable in these mice, HCC progression was markedly arrested after combined treatment with BI2536 and LCL161, a finding that

**FIG. 5.** Regression of HCCs by combined treatment with mitotic inhibitor and SMAC mimetic. (A,B) HCCs were induced in wild-type mice at the age of 2 weeks. 25 mg/kg body weight BI2536 (BI), LCL161 (LCL) alone, or combination with BI2536 and LCL161 (BI/LCL) were injected into cancer-bearing mice every other day at the age of 8 months. Mitosis and senescence in HCCs were characterized 2 weeks after BI2536 treatment. Mitosis of HCC cells was analyzed by p-H3S10 immunohistochemical staining. p-H3S10-positive cancer cells were quantified (A). *n* = 4 for each group. SA- $\beta$ -gal staining showed increased senescence in BI2536-treated or BI/LCL combined-treated HCCs. SA- $\beta$ -gal-positive HCC cells were quantified (B). (C) TNF $\alpha$  mRNA and protein levels in HCCs from mice treated by BI2536, LCL161 alone, or BI/LCL combination for 2 weeks were determined by qPCR and enzyme-linked immunosorbent assay. *n* = 4 for each group. (D) Cell death was determined by TUNEL staining of HCCs 2 weeks after treatment. Positively stained cancer cells were quantified. Cleaved caspase 3 (CC3) and p19Arf were costained on HCC sections. CC3-positive p19Arf-negative cells are indicated by arrows. CC3-positive p19Arf-positive cancer cells are indicated by arrowheads. CC3-positive cells and p19Arf-positive cells were quantified. *n* = 3 for each group. (E) Therapeutic effects were monitored and HCCs were quantified after 2 months after BI2536, LCL161 alone, or BI/LCL combination treatment. *n* = 5 for DMSO group; *n* = 4 for BI group; *n* = 4 for LCL group; *n* = 5 for BI/LCL group. (F) Kaplan-Meier survival analysis showed that combined treatment of BI2536 and LCL161 significantly improved the survival of HCC-bearing mice (log-rank test, *P* < 0.01). *n* = 11 for DMSO group; *n* = 10 for BI2536 group; *n* = 7 for LCL group; *n* = 12 for BI/LCL group. \**P* < 0.05, *t* test. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.





**FIG. 6.** Mitotic inhibitor and SMAC mimetic synergistically eliminate human PDX HCCs. (A) PDX HCCs developed in athymic nude mice and treated with BI2536 and LCL161 alone or combined for 1 month. For each experimental group, we transplanted 5 human HCCs with 2 PDX tumors for each HCC. Growth of the PDX HCC was monitored during combination treatment with BI2536 (25 mg/kg) and LCL161 (25 mg/kg) for 1 month. *n* = 10 for each group. (B,C) Cell mitosis and senescence in PDX cancers were determined by p-H3S10 (B) and SA-β-gal (C) staining, respectively. Positively stained cancer cells were quantified. *n* = 3 for each group. (D) PDX liver cancers were treated with BI2536, LCL161, or combination of BI2536 and LCL161. Species-specific qPCR was applied to analyzed mouse and human TNFα mRNA levels in PDX cancers. *n* = 3 for each group. (E) Cell death was determined by TUNEL staining. Positively stained HCC cells were quantified. *n* = 3 for each group. (F) Cleaved caspase 3 (CC3) and p16 were costained on HCC tissues. CC3-positive p16-negative cells are indicated by arrows. CC3-positive p16-positive cancer cells are indicated by arrowheads. CC3-positive cells and p16-positive cells were quantified. *n* = 3 for each group. Scale bars, 50 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.



**FIG. 7.** Proposed therapeutic strategy. Schematic summarization of the proposed HCC therapeutic strategy, which shows that PLK1 inhibitor BI2536 induced mitosis defect and senescence, and the SMAC mimetic, LCL161, sensitized HCC cells to TNF $\alpha$ -induced cell death. Combined treatment with these two drugs markedly repressed HCC.

largely phenocopied Survivin-deficiency in HCCs (Fig. 5E). Moreover, combination therapy extended the life span of cancer-bearing mice (Fig. 5F). Overall, the combination of BI2536-induced cellular senescence and LCL161-sensitized cell death mimics the therapeutic effects of Survivin depletion in HCCs.

### BI2536 AND LCL161 SYNERGISTICALLY INHIBIT HUMAN HCCs

To further assess the clinical utility of our findings, we tested whether the combination of BI2536 and LCL161 could restrain progression of human HCCs *in vivo* in the human PDX mouse model, in which primary human HCC tissues are transplanted into athymic nude mice (Fig. 6A). When HCC volume reached  $\sim 800 \text{ mm}^3$ , HCC-bearing mice were treated with BI2536 and LCL161 each at 25 mg/kg every other day for 1 month. Tumor size was monitored every 5 days (Fig. 6A). Intriguingly, single treatment with either BI2536 or LCL161 slowed the growth of PDX HCC, suggesting that these inhibitors alone have better therapeutic effects on xenografted HCCs than *de novo* HCCs. We speculated that this might be because the PDX HCC is a selective model for cancer cells with a higher mitotic index compared to *de novo* HCCs (compare Fig. 6C with Figs. 2A and 5A). Mice bearing the PDX HCCs were sacrificed after continuous treatment of combined BI2536 and LCL161 for 1 month. Importantly, combination treatment with BI2536 and LCL161 caused PDX HCCs to stop growing within 2 weeks and regress to  $\sim 75\%$  of their original size by the end of the experiment (Fig. 6A). The findings suggest that these two agents act synerg-

istically to achieve a beneficial response when combined.

The mechanisms of the responses were further evaluated. BI2536 treatment alone or in combination with LCL161 resulted in a profound cell cycle arrest and caused prominent cellular senescence in PDX cancers (Fig. 6B,C) without affecting expression of Survivin (Supporting Fig. S7A). High expression of human senescence-associated genes (Supporting Fig. S7B) and increased infiltration of inflammatory macrophages (Supporting Fig. S7C) were detected in PDX HCCs receiving BI2536 alone or the combination treatment. The expression level of mouse TNF $\alpha$ , rather than human TNF $\alpha$ , increased in the BI2536 treatment alone and in the combination treatment (Fig. 6D), indicating that mouse inflammatory cells, but not the human HCC cells, represent the sources of TNF $\alpha$ . In PDX HCCs treated with LCL161 alone, impairments in mitosis and senescence-associated features were not detectable (Fig. 6B,C). However, LCL161 treatment alone did increase the level of cell death in human PDX HCCs (Fig. 6E,F). Notably, combination treatment with BI2536 and LCL161 induced the highest level of cell death in both senescent and neighboring nonsenescent HCC cells (Fig. 6E,F), reinforcing the concept that synergistically boosting senescence and TNF $\alpha$ -triggered cell death is of value in treating HCC.

## Discussion

In this study, genetic deletion of Survivin induced mitotic defect and senescence in HCCs, which then caused inflammation and TNF $\alpha$  induction. Moreover, Survivin inactivation sensitized TNF $\alpha$ -induced cell

death in both senescent and nonsenescent HCC cells, which led to a marked regression of HCCs (Fig. 4D). Based on these findings, we proposed a new therapy to mimic the multiple anti-HCC mechanisms of Survivin deletion by triggering mitosis defect and senescence and enhancing TNF $\alpha$ -induced cell death. To exploit these findings, we used the PLK1 inhibitor, BI2536, to induce mitosis defect and senescence and the SMAC mimetic, LCL161, to sensitize HCC cells to TNF $\alpha$ -induced cell death (Fig. 7). Importantly, combined treatment with the mitotic inhibitor and the SMAC mimetic phenocopied Survivin deletion by inducing senescence and sensitizing TNF $\alpha$ -dependent cell death therefore supporting the proposed mechanistic model. Also, it provided a strategy bypassing direct targeting Survivin. Several important points are worth noting. First, senescence is an essential trigger of HCC regression by inducing inflammatory cytokine TNF $\alpha$ . Second, TNF $\alpha$  is critical for cell death in both cytostatic and proliferating HCC cells. Finally, depletion of Survivin or blocking the antiapoptotic pathway caused a dramatic increase in cell death in response to TNF $\alpha$ . Our results therefore demonstrate a paradigm to harness the synergistic power of mitosis arrest-induced senescence and boosting TNF $\alpha$ -induced cell death.

Both Survivin depletion and PLK1 inhibition caused mitotic defects and senescence in HCC cells, consistent with an earlier report showing that mitosis defect is efficient in induction of senescence.<sup>(38)</sup> Moreover, genetic depletion or chemical inhibition of Aurora kinase caused aberrant mitosis, which mediated senescence in cancer cells.<sup>(11,39)</sup> Notably, both p53- and Rb-associated senescence pathways were activated in Survivin-deficient HCCs. The underlying mechanisms of mitosis defect-associated senescence could be even more complicated, because senescence induced by Aurora kinase inhibition were independent of the function of p53 and Rb.<sup>(11,39)</sup> Although it remains unclear by which mechanisms mitosis arrest induces senescence, these findings indicate that mitotic inhibition is a plausible approach to induce senescence *in vivo*.

Our findings showed that cytostatic and proliferating HCC cells could be eliminated by sensitizing to TNF $\alpha$ -induced cell death. TNF $\alpha$  expression was strongly associated with senescence and infiltration of inflammatory cells. It was notable that Survivin-deficient HCC cells had heightened sensitivity to TNF $\alpha$ -induced cell death. TNF $\alpha$  has been reported to play multiple roles in liver tumorigenesis.<sup>(40)</sup> In the ini-

tiation of HCCs, and in the setting of nuclear factor kappa B inhibition, inflammation and TNF $\alpha$  cause cell death and compensatory proliferation; the latter contributes to tumorigenesis.<sup>(21,41)</sup> Although secondary tumorigenesis may be a concern post-TNF $\alpha$  induction, its major effect in our study appears to be to eliminate malignant HCC cells. After etanercept treatment, HCC cell death was markedly blocked, which led to partially restored liver tumorigenesis. However, etanercept treatment did not fully restore the inhibited HCC phenotype in *svv*<sup>Al<sup>i</sup>\*</sup> mice, likely attributed to the fact that the Survivin-deficient HCC cells did not proliferate normally.

Roles for Survivin in mitosis and cell death have been recognized in tumorigenesis for many years; however, it is unclear whether and how these two seemingly separate functions are intertwined.<sup>(42)</sup> Using a genetic model, our results showed, for the first time, that Survivin promotes tumorigenesis in both a cell autonomous and noncell autonomous manner. This has not previously been fully appreciated using xenograft models,<sup>(43)</sup> but is important because development of Survivin-specific inhibitors has so far been unsuccessful.<sup>(22,23)</sup> By uncovering the unique mechanisms regulated by Survivin, it is possible to bypass direct targeting of Survivin. Indeed, we proposed and tested a new therapeutic approach that mimics the anticancer mechanisms of Survivin-deletion by triggering mitosis defect and senescence and enhancing TNF $\alpha$ -induced cell death.

In this study, we tested the combination of the PLK1 inhibitor, BI2536, and the SMAC mimetic, LCL161, for HCC therapy. It is worth noting that we only attempted to use these compounds to mimic mitosis defect and senescence and cell death sensitization. It is not necessarily relevant whether PLK1 and IAPs are direct targets of Survivin. Interestingly, both compounds have shown limited therapeutic responses in clinical trials when used as a monotherapy.<sup>(14,35,36)</sup> Others have proposed that BI2536 or LCL161 might be used in combination with other cytotoxic compounds.<sup>(15,36,44)</sup> Our data strongly support the use of these two newly developed compounds in combination for HCC treatment. Furthermore, no adverse effects on liver functions or body weight were found with longer-term combined treatment in HCC-bearing mice (Supporting Fig. S8A,B) and in mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury (Supporting Fig. S8C,D). Pharmacokinetic studies reveal that clearance of BI2536, when combined with LCL161, was slightly increased,



and thus total exposure dose was decreased compared to single use (Supporting Fig. S9A). On the other hand, the clearance and total exposure dose of LCL161 were unchanged (Supporting Fig. S9A). Moreover, there were no drug-drug interactions between BI2536 and LCL161 in mice with DDC-induced liver injury (Supporting Fig. S9B). The pharmacological effects of BI2536 and LCL161 remained in combination treatment (Supporting Fig. S9C,D). Therefore, the synergistic anti-HCC effect of combination treatment is not likely attributed to drug-drug interactions between BI2536 and LCL161.

Based on our findings, we expect that additional experiments will reveal whether combinations of other mitotic inhibitors or proapoptotic compounds would exhibit similar therapeutic benefits (Fig. 7). We recognize that such an approach requires careful monitoring and dosing, so as to avoid adversely affecting healthy cells by inducing unwanted senescence and cell death. Overall, we speculate that the combination of a mitotic inhibitor and a SMAC mimetic will not only provide new ammunition against HCC, but also prove useful for the treatment of a range of other inflammation-related cancers.

*Acknowledgments:* We thank Zhengye Yang (USTC, China) for comments on mitosis and senescence, Liming Sun (SIBCB, China) and Zheng-Gang Liu (NIH, USA) for comments on cell death, and Dangsheng Li (Cell Research) for thorough discussion of the manuscript.

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## Supporting Information

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep.28637/supinfo](http://onlinelibrary.wiley.com/doi/10.1002/hep.28637/supinfo).