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Connexin32 activates necroptosis through Src-mediated inhibition of caspase 8 in hepatocellular carcinoma

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Abstract

Necroptosis is an alternative form of programmed cell death that generally occurs under apoptosis-deficient conditions. Our previous work showed that connexin32 (Cx32) promotes the malignant progress of hepatocellular carcinoma (HCC) by enhancing the ability of resisting apoptosis in vivo and in vitro. Whether triggering necroptosis is a promising strategy to eliminate the apoptosis-resistant HCC cells with high Cx32 expression remains unknown. In this study, we found that Cx32 expression was positively correlated with the expression of necroptosis protein biomarkers in human HCC specimens, cell lines, and a xenograft model. Treatment with shikonin, a well-used necroptosis inducer, markedly caused necroptosis in HCC cells. Interestingly, overexpressed Cx32 exacerbated shikonin-induced necroptosis, but downregulation of Cx32 alleviated necroptosis in vitro and in vivo. Mechanistically, Cx32 was found to bind to Src and promote Src-mediated caspase 8 phosphorylation and inactivation, which ultimately reduced the activated caspase 8-mediated proteolysis of receptor-interacting serine-threonine protein kinase 1/3, the key molecule for necroptosis activation. In conclusion, we showed that Cx32 contributed to the activation of necroptosis in HCC cells through binding to Src and then mediating the inactivation of caspase 8. The present study suggested that necroptosis inducers could be more favorable than apoptosis inducers to eliminate HCC cells with high expression of Cx32.

KEYWORDS

caspase 8, connexin32, hepatocellular carcinoma, necroptosis, Src

Yu-ke Xiang, Fu-hua Peng, Yun-quan Guo, and Hui Ge contributed equally to this work.

1 | INTRODUCTION

Connexin (Cx) family proteins are the constituent elements of gap junction (GJ) channels, which are located on the cytomembrane between two adjacent cells and mediate direct cell-to-cell communication by transferring small molecules.[1] Although GJs are well recognized as tumor suppressors,[2] the specific nonjunctional roles of internalized Cxs in tumorigenesis remain controversial.[3] Recently, we reported that internalized and accumulated connexin32 (Cx32) is highly correlated with the malignant clinicopathological parameters of cervical cancer[4] and hepatocellular carcinoma[5] (HCC) patients, and protects tumor cells from chemotherapy-induced apoptosis in vitro by activating the epidermal growth factor receptor (EGFR) pathway. These results suggest that cytoplasmic Cx32 might be responsible for cancer cells evading apoptosis. Herein, we explore the strategy of eliminating tumor cells with high Cx32 expression that are insensitive to apoptotic inducers. Bypassing the apoptosis pathway to eliminate cancer cells is considered to be a promising approach to overcoming this problem.

Recently, necrosis has been found to not be absolutely unregulated, which was called "necroptosis."[6] Under apoptosis-deficient conditions, such as the inactivation of caspase 8 or the loss of its adaptor Fas-associated protein with death domain (FADD)[7] or treatment with pancaspase inhibitors, necroptosis could be activated to execute cell death as an alternative mode of cell death.[8] In contrast to apoptosis, which is mediated by the cascade activation of caspases, necroptosis critically requires the activation of receptor-interacting serine-threonine protein kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like (MLKL).[6] In brief, following the induction of necroptosis by various stimuli, activated RIP1 interacts with RIP3 to form a complex called the "necrosome."[9] Subsequently, autophosphorylated RIP3 recruits and phosphorylates MLKL, causing the oligomerization and activation of MLKL.[10] Active MLKL then translocates to the cytomembrane and disrupts the integrity of the plasma membrane, thus leading to necrosis-like cell death.[11] Based on the fact that apoptosis is generally negatively regulated in several cancers, activating necroptosis becomes an important way to kill cancer cells.[12,13]

Herein, we found that Cx32 expression levels were significantly positively associated with the levels of necroptosis biomarkers in 30 HCC human specimens, HCC cell lines, and a xenograft model. In vitro and in vivo, Cx32 contributed to amplifying shikonin (SHN)-induced necroptosis. Connexin32 interacted with Src and promoted Src-mediated caspase 8 phosphorylation, decreasing FADD and increasing the short splice variant of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-$FLIP_S$), which ultimately suppressed the activation of caspase 8 and thus protected RIP1 and RIP3 from activated caspase 8-mediated proteolysis. Our results revealed the mechanism by which Cx32 promotes necroptosis and suggested that inducing necroptosis might be a promising strategy for HCC patients with high Cx32 expression who are resistant to apoptosis inducers.

2 | MATERIALS AND METHODS

2.1 | Hepatocellular carcinoma tissues

Hepatocellular carcinoma tissues were collected at the Affiliated Cancer Hospital of Xinjiang Medical University, Xinjiang, China. Ethical approval (K-201354) of clinical data and tissues was obtained from the Research Committee of Ethics in the Affiliated Cancer Hospital of Xinjiang Medical University. Written informed consent for the experimental studies was obtained from the patients or their guardians.

2.2 | Cell culture

The PLC/PRF/5 and SMMC-7721 cells (ATCC) were cultured in MEM and RPMI-1640 mediums (Invitrogen), respectively, supplanted with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in an atmosphere containing 5% CO_2.

2.3 | Necroptosis induction and inhibitor treatment in vitro

Necroptosis was induced by SHN at 6 μM or 12 μM for 7 hours in PLC/PRF/5 or SMMC-7721 cells, respectively. Pretreatment with necrostatin-1 (Nec-1; 100 μM, 2 hours), z-VAD-FMK (zVAD; 50 μM, 2 hours), or 2-aminoethoxydiphenyl-borate (2-APB; 50 μM, 2 hours) was used to inhibit necroptosis, apoptosis, or GJ, respectively. Shikonin, Nec-1, and zVAD were from Selleck, and 2-APB was acquired from Sigma-Aldrich.

2.4 | Cell viability assay

Cells were pretreated with Nec-1, zVAD, 2-APB, or not, and subsequently treated with SHN for 7 hours. After removing the supernatant, the cells were incubated with fresh culture media containing 10% CCK-8 reagent (Dojindo Molecular Technologies) at 37°C for 2 hours. The optical density (OD_{450}) was measured by a microplate reader (BioTek Instruments).

2.5 | Stable cell clone establishment

Connexin32 shRNA vector and negative control vector (pLVX-shRNA-tdTomato-Puro) were constructed by Landbiology. Connexin32 overexpressed vector (Ex-A05140-Lv105) and negative control vector (pEZ-Lv105) were designed by Genecopoeia. The establishment of stable cells was carried out as described in a previous report.[5] The sequence for the shRNA targeting Cx32 (shCx32) was: 5′-GCTGCAACAGCGTTTGCTACTCGAGTAGCAAACGCTGTTGCAGCTTTTTTT-3′.

2.6 | Plasmid transfection and RNA interference

Plasmid transfection and RNA interference experiments were undertaken as previously described.[5] The Src overexpressed vector (Ex-B0107-M09) and negative control vector (EX-NEG-M09) were constructed by Genecopoeia. The sequences for the synthesized Src siRNA (RiboBio) were as follows, and siRNA-Src-1 was chosen for all experiments: siRNA-Src-1, 5′-CAAGAGCAAGCCCAAGGAT-3′; siRNA-Src-2, 5′-CAGGCTGAGGAGTGGTATT-3′; and siRNA-Src-3, 5′-GCAGTTGTATGCTGTGGTT-3′.

2.7 | Hoechst 33 342/propidium iodide staining

Hoechst/propidium iodide (PI) staining buffer was obtained from Beyotime Biotechnology. Cells were pretreated with Nec-1 or zVAD or not, and then treated with SHN. Cells were then washed twice with PBS and incubated with the Hoechst/PI buffer for 30 minutes at 4°C. Subsequently, cells were washed twice with PBS and observed with a fluorescence microscope (Olympus IX83).

2.8 | Annexin V-FITC/PI flow cytometry

The rate of survival ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$ and $FITC^+/PI^+$), and necrotic ($FITC^-/PI^+$) cells were detected by using annexin V-FITC/PI detection kits (BD Biosciences) as previously reported.[5] The data were measured by a FACScan (Beckman Instruments) and analyzed by FlowJo 7.6 software.

2.9 | Caspase 8 activity assay

The Caspase 8 Activity Assay Kit was from Beyotime. Cells were washed with 4°C PBS, added with lysis buffer, and incubated at 4°C for 15 minutes. After centrifuging at 16 000 *g* (4°C for 15 minutes), the supernatant was collected. The cleaved-caspase 8 in the supernatant reacts with the substrate peptides Ac-IETD-pNA and then produces p-nitroanilide (pNA). The OD_{450} was determined to qualify the activity of caspase 8.

2.10 | Western blot analysis

Western blotting was carried out as previously described.[5] Connexin32 Ab was purchased from Santa Cruz Biotechnology. The GAPDH Ab was obtained from Ray Antibody Biotech. β-Tubulin Ab and HRP-conjugated secondary Abs were acquired from Sigma-Aldrich. The RIP1, RIP3, MLKL, p-RIP1, p-MLKL, and Src Abs were obtained from Cell Signaling Technology. Phosphor-caspase 8 (Tyr380) primary Ab was from Abcam.

2.11 | Coimmunoprecipitation

The coimmunoprecipitation (Co-IP) analysis was carried out as previously described.[5] Nonspecific mouse or rabbit IgG was purchased from Beyotime. Protein G Plus/Protein A Agarose Suspension was from Merck Millipore.

2.12 | Immunohistochemistry

Immunohistochemistry (IHC) staining was carried out as previously described.[5] The PV-9000 DAB detection kit was obtained from ZSGB-Bio.

2.13 | Xenograft tumor model

BALB/c-nu mice (male, 4 weeks of age, 18-20 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd and Sun Yat-Sen University. All procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. The nude mice were subcutaneously inoculated with PLC-Vector cells and PLC-shCx32 cells (5×10^6 in 200 μL PBS) near the right scapula. In the experiments shown in Figure 6, the nude mice were killed on day 20 after implantation. In the experiments shown in Figure 7, after tumors had appropriately grown to 200 mm^3, the mice were randomly assigned into four groups with eight mice each: PLC-Vector+Vehicle, PLC-shCx32+Vehicle, PLC-Vector+SHN, and PLC-shCx32+SHN. The mice were intraperitoneally injected with SHN (3 mg/kg/d in 200 μL vehicle) or 200 μL vehicle (2% DMSO) every 2 days for a total of nine injections. The mice were killed 2 days after the last injection. The calculation of tumor volume was described in a previous report.[5]

2.14 | Statistical analysis

All experimental results were repeated at least in triplicate. Parametric data were analyzed by one-way ANOVA or Student's *t* test. A two-tailed value of $P < .05$ was considered statistically significant. The results are presented as the mean ± SD if the data were quantitative in nature. The data were statistically analyzed using the SPSS software package (version 16.0). The graph was developed by using GraphPad Software 5.

3 | RESULTS

3.1 | Connexin32 is positively correlated with necroptosis biomarkers in HCC specimens and cell lines

The expression of Cx32 and necroptosis biomarkers, including RIP1, p-RIP1, and p-MLKL, were detected by western blot analysis in 30 human specimens (Figure 1A). The correlation analysis indicated

that Cx32 expression was significantly positively associated with the expression of p-RIP1, RIP1, and p-MLKL (Figure 1B-D, respectively). In HCC cell lines, the expression of RIP1, RIP3, and MLKL in PLC/PRF/5 cells with naturally high expression of Cx32 was higher than that in SMMC-7721 cells with naturally low expression of Cx32 (Figure 1E). We then established stable Cx32-knockdown cell lines (PLC-shCx32) and Cx32-overexpression cell lines (SMMC-Cx32). As shown in Figure 1F, altering the expression of Cx32 significantly positively changed the levels of RIP1, RIP3, and MLKL in stable HCC cell lines. These results strongly suggested that the necroptosis pathway was more activated in HCC tissues and cell lines with high Cx32 expression.

3.2 | Knockdown of Cx32 alleviates SHN-induced necroptosis in PLC/PRF/5 cells

We examined whether Cx32 is responsible for necroptosis in HCC cells when exposed to SHN, a necroptosis inducer.[14] As shown in Figure 2A, treatment with 4-10 μM SHN for 7 hours induced significant cell death in PLC/PRF/5 cells, which was reversed by pretreatment with RIP1 inhibitor Nec-1 but not pancaspase inhibitor zVAD, indicating that SHN-induced cell death was necroptosis rather than apoptosis. Pretreatment with GJ inhibitor 2-APB had no influence on SHN-induced necroptosis, but the knockdown of Cx32 remarkably alleviated necroptosis (Figure 2A). Treatment with 6 μM SHN for 7 hours was chosen for subsequent experiments in PLC/PRF/5 cells. Shikonin treatment resulted in massive numbers of necrotic cells (annexin V-FITC$^-$/PI$^+$ cells in Figure 2B and PI-positive cells with red fluorescence in Figure 2C), which was evidently ameliorated by pretreatment with Nec-1 and knockdown of Cx32.

Moreover, SHN-induced significant upregulation of RIP1, RIP3, and MLKL confirmed the activation of necroptosis in PLC/PRF/5 cells, which was restored by pretreatment with Nec-1 (Figure 2D) and knockdown of Cx32 (Figure 2E). As shown in Figure 2F,G, knockdown of Cx32 markedly reduced the binding of RIP1 and RIP3 and the expression of membrane MLKL, all biomarkers for activation of necroptosis. The above results showed that SHN-induced necroptosis was suppressed by knockdown of Cx32 in PLC/PRF/5 cells.

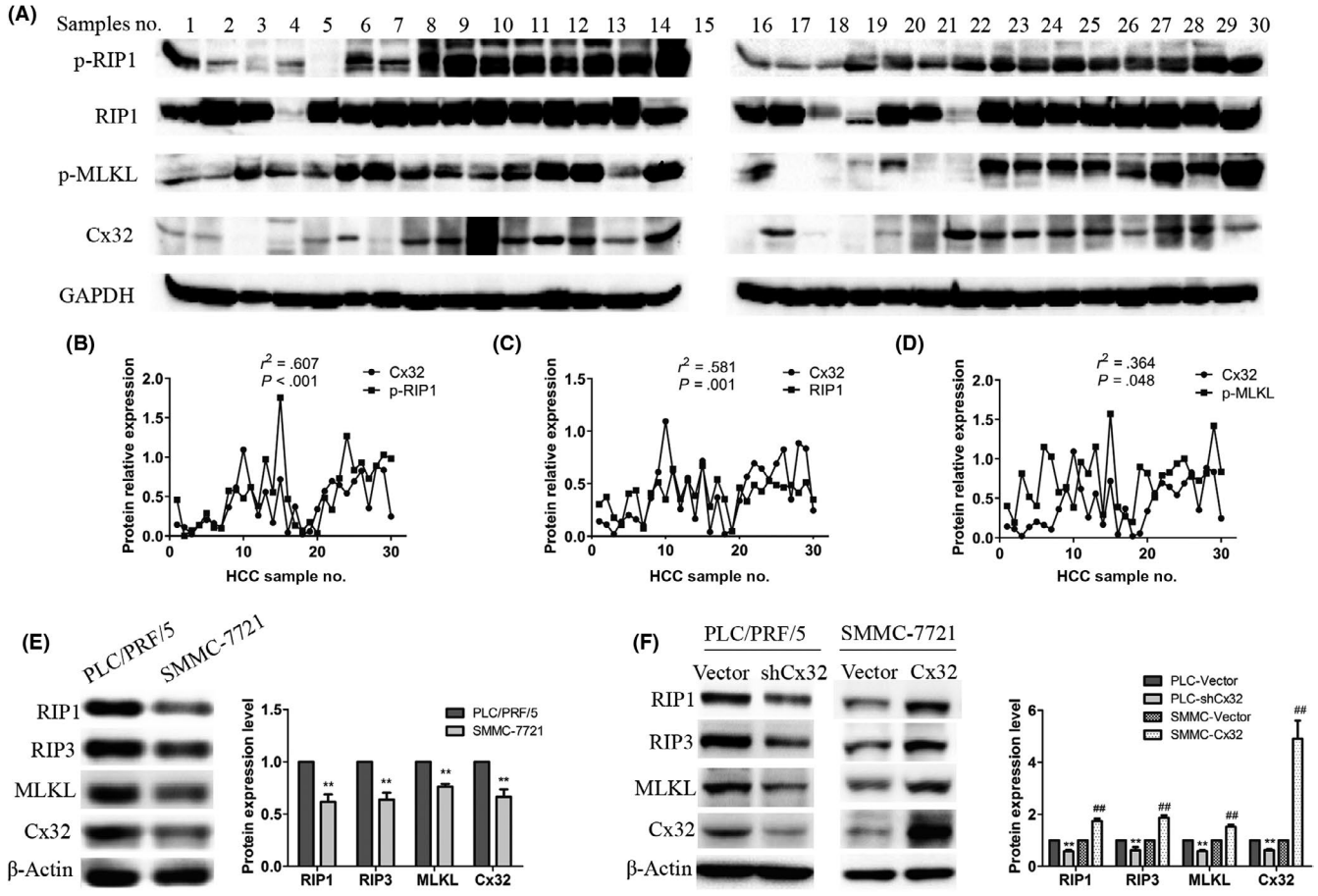

FIGURE 1 Correlation between connexin32 (Cx32) and necroptosis biomarkers in hepatocellular carcinoma (HCC) specimens and cell lines. A-D, Representative western blot (WB) images (A) and correlation analysis (B-D) showed that Cx32 expression was positively associated with the expression of phosphorylated receptor-interacting serine-threonine protein kinase 1 (p-RIP1), RIP1, and mixed lineage kinase domain-like (p-MLKL) in the 30 HCC specimens. E, F, Expression of Cx32, RIP1, RIP3, and MLKL were detected by WB in HCC cell lines (E, n = 3; $^{**}P < .01$) and stable cell lines (F, n = 3; $^{**}P < .01$ vs PLC-Vector; $^{\#\#}P < .01$ vs SMMC-Vector)

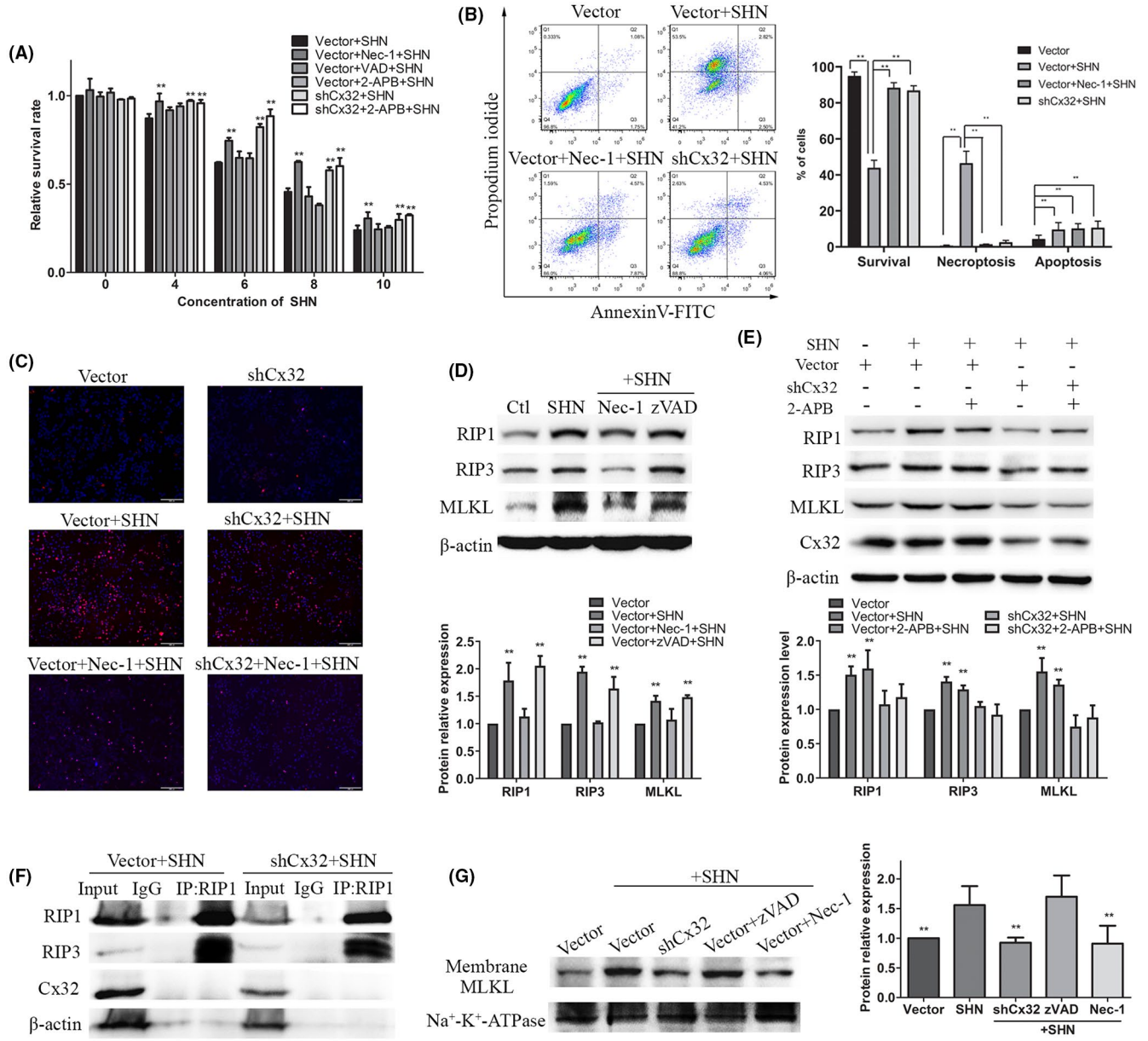

FIGURE 2 Knockdown of connexin32 (Cx32) expression alleviates shikonin (SHN)-induced necroptosis in PLC/PRF/5 cells. A, Survival rate of PLC-Vector and PLC-shCx32 cells treated with SHN was detected by CCK-8 assay (n = 3). $^{**}P < .01$ vs Vector+SHN. B, Cell rate of survival ($FITC^-/$propidium iodide $[PI]^-$), necroptosis ($FITC^-/PI^+$), and apoptosis ($FITC^+/PI^-$ and $FITC^+/PI^+$) was analyzed by annexin V-FITC/PI flow cytometry (n = 3). $^{**}P < .01$. C, Hoechst 33342/PI staining was used to detect surviving cells (dark blue), apoptotic cells ($Hoechst^+$ cells, light blue), and necroptotic cells (PI^+ cells, red). Scale bar = 200 μm. D, Western blot analysis for necroptosis pathway after treatment with 6 μM SHN for 7 h (n = 3). $^{**}P < .01$ vs Vector. E, Necroptosis pathway activation induced by SHN was suppressed by the knockdown of Cx32 expression in PLC/PRF/5 cells (n = 3). $^{**}P < .01$ vs Vector. F, Formation of necrosomes induced by SHN in PLC-shCx32 cells was significantly reduced compared with that in PLC-Vector cells. G, Cytomembrane translocation of mixed lineage kinase domain-like (MLKL) induced by SHN in PLC-shCx32 cells was markedly lower than that in PLC-Vector cells (n = 3). $^{**}P < .01$ vs SHN. 2-APB, 2-aminoethoxydiphenyl-borate; IP, immunoprecipitant; Nec-1, necrostatin-1; RIP, receptor-interacting serine-threonine protein kinase; zVAD, z-VAD-FMK

3.3 | Connexin32 aggravates SHN-induced necroptosis in SMMC-7721 cells

As shown in Figure 3A, treatment with 6-14 μM SHN for 7 hours significantly suppressed the survival rate of SMMC-7721 cells, which was reversed by pretreatment with Nec-1 but aggravated by Cx32 overexpression. We chose 12 μM SHN for 7 hours for subsequent experiments in SMMC-7721 cells. The results of flow cytometry (Figure 3B) and Hoechst/PI staining (Figure 3C) confirmed that the overexpressed Cx32 further enhanced SHN-induced necroptosis (Vector+SHN vs Cx32+SHN). The results of western blotting (Figure 3D) also indicated that overexpressed Cx32 accelerated

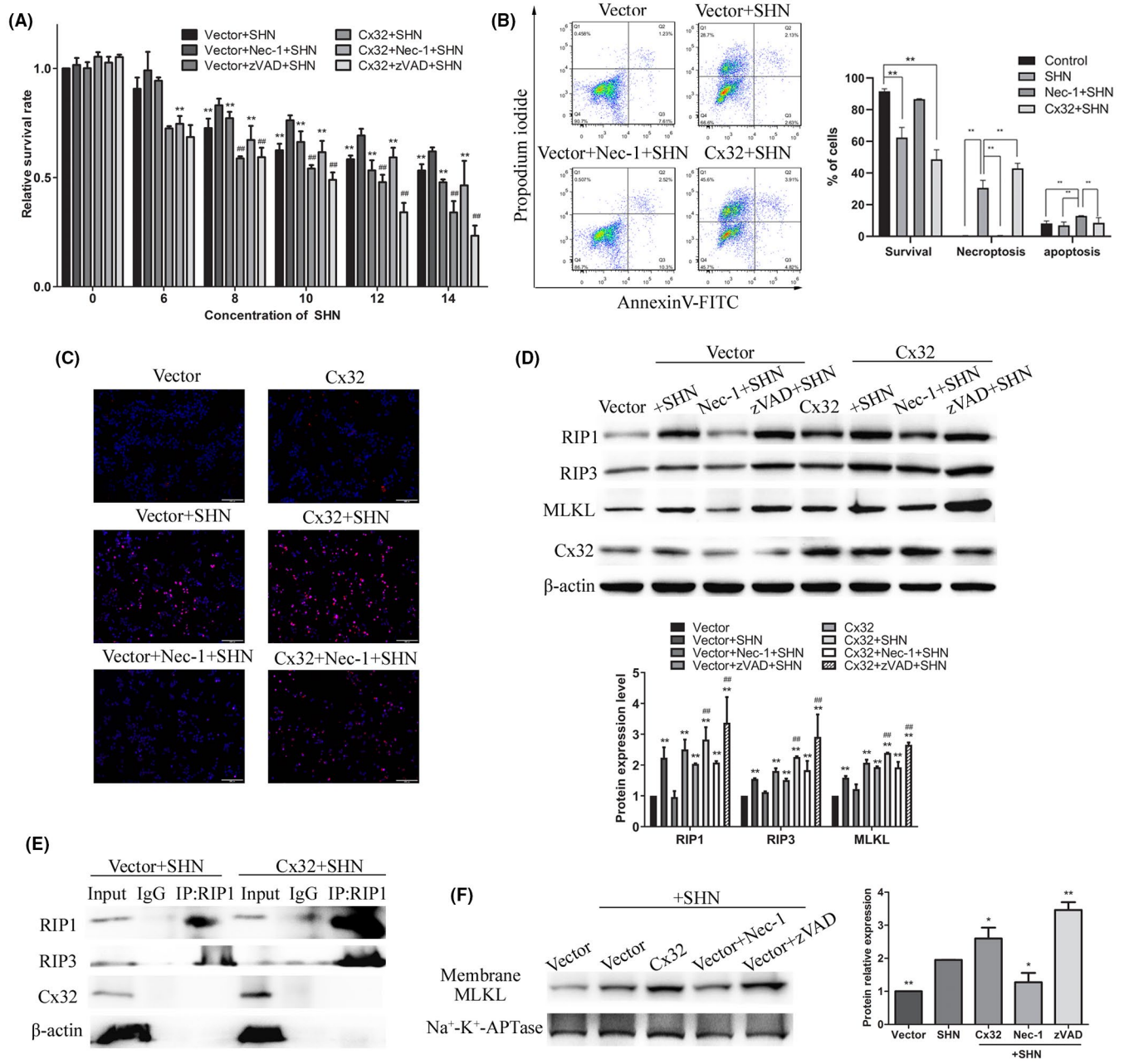

FIGURE 3 Overexpression of connexin32 (Cx32) promotes shikonin (SHN)-induced necroptosis in SMMC-7721 cells. A, Survival rate of SMMC-Vector and SMMC-Cx32 cells treated with SHN was detected by CCK-8 assay (n = 3). $^{**}P < .01$ vs Vector+necrostatin-1 (Nec-1)+SHN; $^{\#\#}P < .01$ vs Cx32+Nec-1+SHN. B, Cx32 increased SHN-induced necroptotic cells ($FTIC^{-}$/propidium iodide $[PI]^{+}$) in SMMC-7721 cells, as detected by flow cytometry. $^{**}P < .01$. C, Cx32 increased the number of SHN-induced necrotic cells (PI^{+} cells with red fluorescence). Scale bar = 200 μm. D, Overexpression of Cx32 promoted the activation of SHN-induced necroptosis by enhancing the expression of receptor-interacting serine-threonine protein kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like (MLKL) (n = 3). $^{**}P < .01$ vs Vector; $^{\#\#}P < .01$ vs Cx32. E, Overexpressed Cx32 promoted the formation of necrosomes in SMMC-7721 cells, as detected by coimmunoprecipitation experiments. F, SHN-induced membrane translocation of MLKL in SMMC-Cx32 cells was more significantly increased than that in SMMC-Vector cells (n = 3). $^{*}P < .05$ vs SHN; $^{**}P < .01$ vs SHN. IP, immunoprecipitant; zVAD, z-VAD-FMK

SHN-induced necroptosis by upregulation of RIP1, RIP3, and MLKL, which was alleviated by pretreatment with Nec-1 but not with zVAD. The overexpressed Cx32 increased the SHN-induced interaction of RIP1-RIP3 (Figure 3E) and membrane translocation of MLKL (Figure 3F), resulting in the formation of necrosome and membrane damage. In summary, Cx32 expression significantly aggravated SHN-induced necroptosis in HCC cell lines.

3.4 | Connexin32 increased expression of RIP1 and RIP3 by suppressing activity of caspase 8

Active caspase 8 mediates apoptosis by activating downstream caspase cascades and inhibits necroptosis[15] by cleaving RIP1[16] and RIP3.[17] Thus, we investigated whether caspase 8 is involved in the Cx32-mediated activation of necroptosis. Figure 4A,B shows

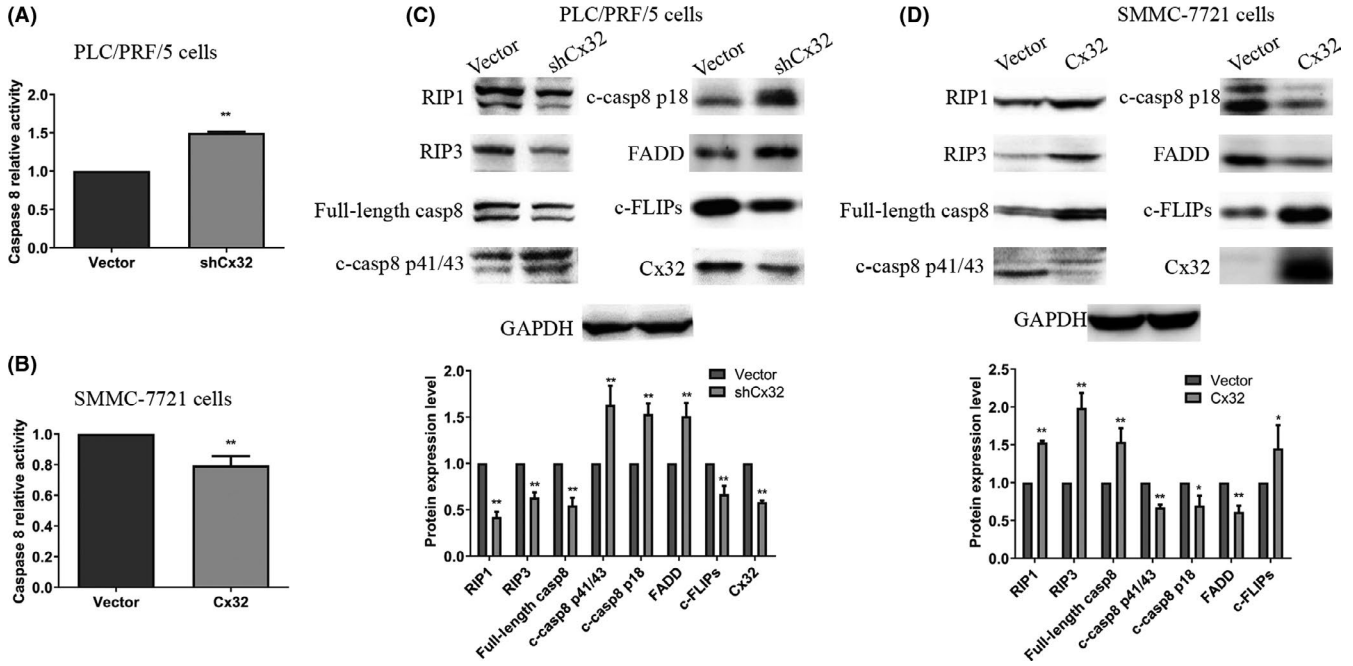

FIGURE 4 Connexin32 (Cx32) enhances the levels of receptor-interacting serine-threonine protein kinase 1 (RIP1) and RIP3 by inhibiting caspase 8 (casp8) activity. A, B, Cx32 negatively regulated the activity of caspase 8 (n = 3). $^{**}P < .01$. C, Silencing Cx32 expression decreased the levels of RIP1, RIP3, full-length caspase 8, and c-FLIP$_S$ but increased the expression of Fas-associated protein with death domain (FADD) and cleaved-caspase 8 (c-casp8; p41/43 and p18) in PLC/PRF/5 cells (n = 3). $^{**}P < .01$. D, Overexpressed Cx32 caused the increase in RIP1, RIP3, full-length caspase 8, and c-FLIP$_S$ and the decrease in FADD and cleaved-caspase 8 (p41/43 and p18) in SMMC-7721 cells (n = 3). $^{**}P < .01$

that Cx32 negatively affected the activity of caspase 8. As shown in Figure 4C, silencing Cx32 in PLC/PRF/5 cells significantly inhibited the expression of RIP1 and RIP3 but enhanced the expression of cleaved-caspase 8 (p41/43 and p18), which could be the result of the shCx32-mediated downregulation of c-FLIP$_S$, an inhibitor of caspase 8 activation, and the upregulation of FADD, an adaptor of caspase 8. The opposite results were observed in SMMC-7721 cells (Figure 4D). In summary, Cx32 enhanced the expression of c-FLIP$_S$ but downregulated the expression of FADD, resulting in the inactivation of caspase 8 and thus protecting RIP1 and RIP3 from caspase 8-mediated cleavage.

3.5 | Connexin32 contributes to caspase 8 phosphorylation by interacting with Src

Src kinase suppresses the activity of caspase 8 by phosphorylating caspase 8 (Tyr380).[18] As shown in Figure 5A-D, Cx32 positively regulated the protein and mRNA levels of Src in HCC cells. Coimmunoprecipitation experiments indicated that there was a protein interaction between Cx32 and Src in HCC cell lines (Figure 5E,F). We then investigated whether Src is involved in the Cx32-mediated suppression of caspase 8. In Figure 5G, the knockdown of Cx32 in PLC/PRF/5 cells significantly decreased the expression of Src, p-caspase 8 (Tyr380), c-FLIP$_S$, RIP1, and RIP3 but increased the expression of FADD, which were all reversed by the overexpression of Src. In contrast, silencing Src in SMMC-Cx32 cells reversed the overexpressed Cx32-induced upregulation of Src, p-caspase 8 (Tyr380), c-FLIP$_S$, RIP1, and RIP3 and the downregulation of FADD (Figure 5H). Is Src crucial for Cx32 aggravation of SHN-induced necroptosis? As shown in Figure 5I,K, knockdown of Cx32 in PLC/PRF/5 cells alleviated SHN-induced necroptosis by downregulation of RIP1, RIP3, and MLKL, which were restored by cotransfection with Src overexpressed vector (shCx32+SHN vs shCx32+Src+SHN). Moreover, downregulation of Src reduced overexpressed Cx32-enhanced necroptosis induced by SHN in SMMC-7721 cells, as shown in Figure 5J,L (Cx32+SHN vs Cx32+siSrc+SHN). In conclusion, Cx32 interacted with Src and contributed to the Src-mediated phosphorylation of caspase 8, which caused the suppression of caspase 8 and activation of the necroptosis pathway.

3.6 | Knockdown of Cx32 suppressed necroptosis in vivo

The effect of Cx32 on HCC cell growth and necroptosis in nude mice was investigated. As shown in Figure 6A-E, knockdown of Cx32 significantly reduced the tumors' proliferative ability, which was consistent with our previous report.[5] However, loss of Cx32 significantly reduced the expression of Src and membrane levels of MLKL (Figure 6F), indicating that inhibiting Cx32 expression alleviated the spontaneous activation of necroptosis in vivo. Moreover,

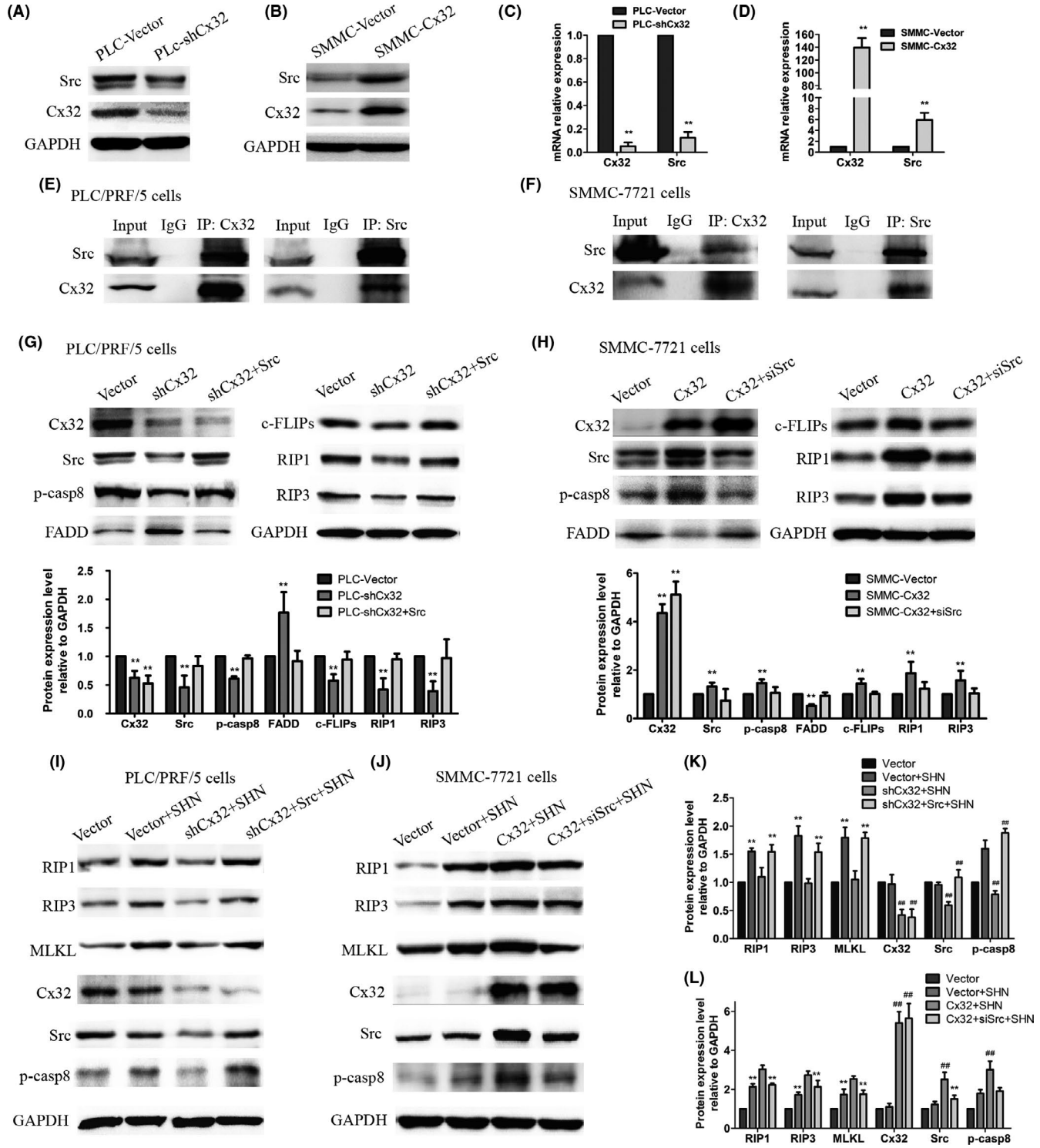

FIGURE 5 Connexin (Cx32) expression suppresses the activity of caspase 8 by binding to Src. A, B, Cx32 positively regulated the protein expression of Src in hepatocellular carcinoma (HCC) cell lines. C, D, Cx32 positively regulated the mRNA levels of Src in HCC cell lines (n = 3). $^{**}P < .01$. E, F, Cx32 interacted with Src in HCC cell lines, as determined by coimmunoprecipation experiments. G, Overexpressed Src reversed the downregulation of p-caspase 8 (p-casp8), c-FLIP$_S$, receptor-interacting serine-threonine protein kinase 1 (RIP1), and RIP3 and the upregulation of Fas-associated protein with death domain (FADD) caused by silencing Cx32 in PLC/PRF/5 cells (n = 3). $^{**}P < .01$. H, Knockdown of Src restored overexpressed Cx32-induced increased expression of p-caspase 8, c-FLIP$_S$, RIP1, and RIP3 and decreased expression of FADD in SMMC-7721 cells (n = 3). $^{**}P < .01$. I, K, Knockdown of Cx32 alleviated shikonin (SHN)-induced necroptosis and p-caspase 8 expression, which were restored by overexpressing Src (n = 3). $^{**}P < .01$ vs shCx32+SHN; $^{\#\#}P < .01$ vs Vector+SHN. J, L, Overexpressed Cx32 aggravated SHN-induced necroptosis and the level of p-caspase 8, which were reversed by knockdown of Src (n = 3). $^{**}P < .01$ vs shCx32+SHN; $^{\#\#}P < .01$ vs Vector+SHN. IP, immunoprecipitant; MLKL, mixed lineage kinase domain-like

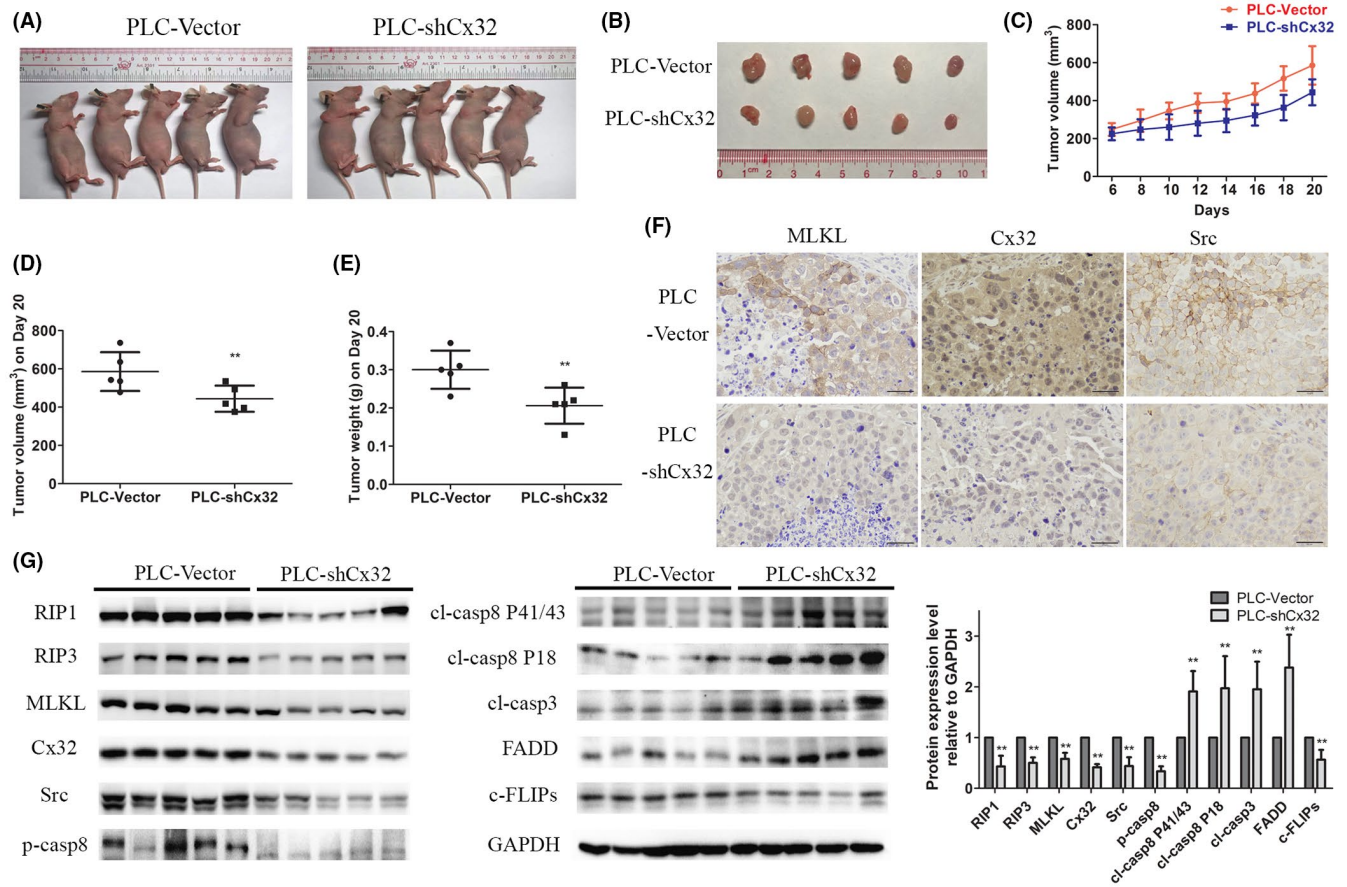

FIGURE 6 Knockdown of connexin32 (Cx32) suppressed the proliferation and necroptosis activation of PLC/PRF/5 cells in vivo. A, B, Representative images of nude mice xenograft models and tumor tissues. C, Tumor growth curves. D, E, Tumor volumes (D) and weight (E) on day 20. F, Knockdown of Cx32 decreased the expression of Src and membrane mixed lineage kinase domain-like (MLKL), detected by immunohistochemistry. Scale bar = 50 μm. G, Knockdown of Cx32 inhibited the expression of receptor-interacting serine-threonine protein kinase 1 (RIP1), RIP3, MLKL, Src, phosphorylated caspase 8 (p-casp8; Tyr380), and c-FLIP$_S$, but increased the levels of Fas-associated protein with death domain (FADD), cleaved-caspase 8 (cl-casp8; P41/43 and P18), and cl-casp3 (n = 5). $^{**}P < .01$

silencing Cx32 decreased the levels of Src, p-caspase 8 (Tyr380), and c-FLIP$_S$, but upregulated the expression of cleaved-caspase 8 (P41/43 and P18) and its adaptor FADD, indicating that knockdown of Cx32 decreased necroptosis activation by alleviating Src-mediated caspase 8 suppression. Consistent with our previous report,[9] knockdown of Cx32 promoted the expression of cleaved-caspase 3, the executor of apoptosis, suggesting that altering the expression of Cx32 changed the mode of HCC cell death by regulating the activity of caspase 8.

Does Cx32 accelerate SHN-induced necroptosis in vivo? As shown in Figure 7A-E, the tumor growth inhibition induced by treatment with SHN (3 mg/kg/d) was greater in group 3 (PLC-Vector+SHN) with high Cx32 expression than in group 4 (PLC-shCx32+SHN) with low Cx32 levels, suggesting that knockdown of Cx32 expression alleviated SHN-induced tumor growth suppression. As shown in Figure 7F,G, silencing the expression of Cx32 reduced the expression of RIP1, RIP3, and MLKL. Moreover, downregulation of Cx32 also decreased the levels of Src and p-caspase 8 (Tyr380) but increased the expression of cleaved-caspase 8. The above results suggested that loss of Cx32 expression decreased SHN-induced activation of necroptosis by alleviating Src-mediating the phosphorylation of caspase 8 in vivo.

4 | DISCUSSION

Here, we identified Cx32 as a novel positive regulator of necroptosis by promoting Src-dependent caspase 8 inhibition through the interaction between Cx32 and Src in HCC cells. Initially, Cx32 expression was positively associated with necroptosis biomarkers including RIP1, p-RIP1, and p-MLKL in 30 HCC specimens. Loss of Cx32 expression significantly alleviated SHN-induced necroptosis in vitro and in vivo through decreasing the expression of RIP1, RIP3, and MLKL, the formation of necrosomes, and the membrane translocation of MLKL. Connexin32 hindered the activation of caspase 8, the key initiator of apoptosis and inhibitor of necroptosis, by binding to Src and enhancing Src-induced caspase 8 suppression in vitro and in vivo. In conclusion, we identified the novel role of Cx32 in necroptosis and further elucidated Cx32 as a potential target for pronecroptosis therapy in HCC patients.

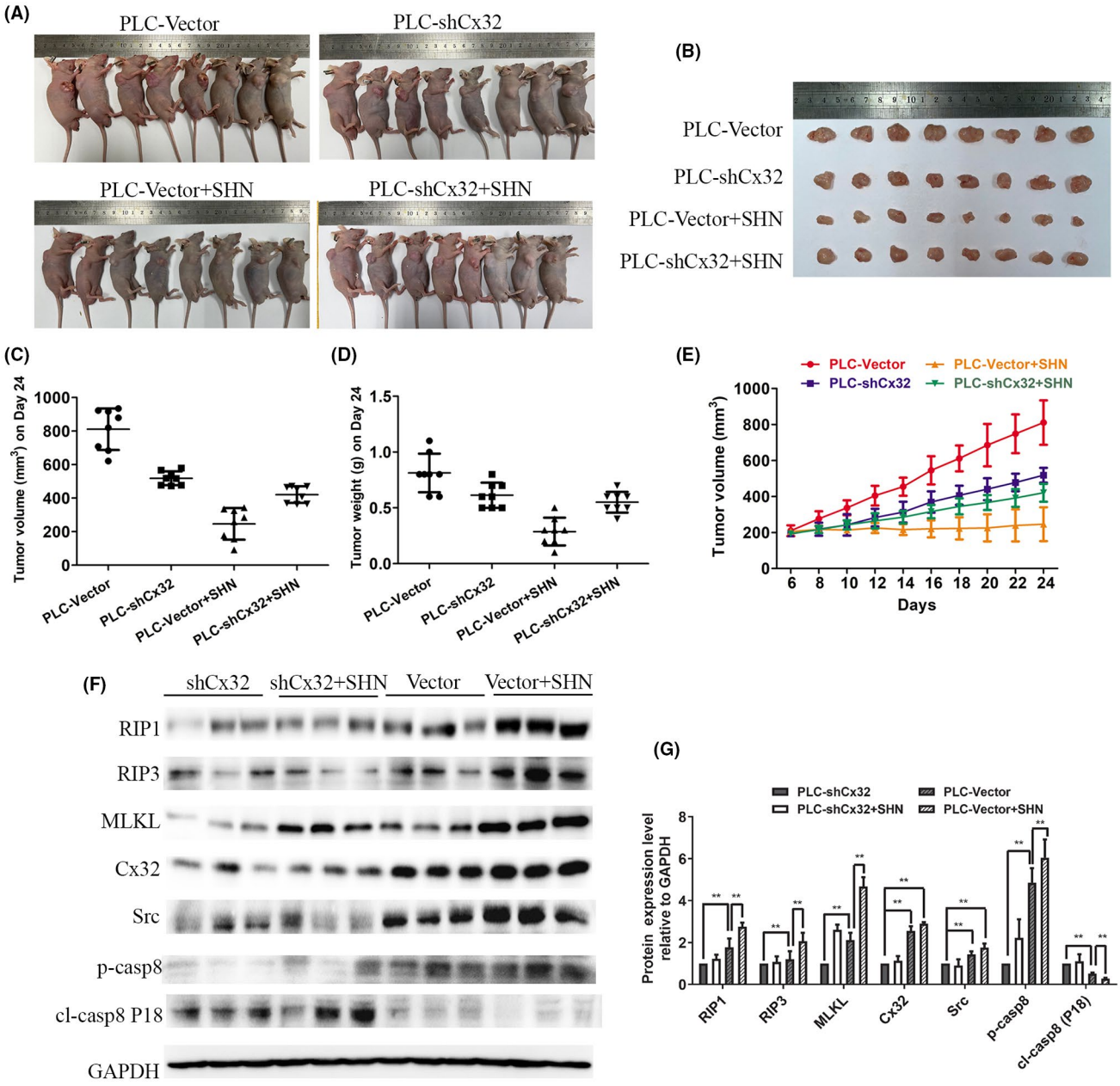

FIGURE 7 Knockdown of connexin32 (Cx32) alleviated shikonin (SHN)-induced necroptosis in vivo. A, B, Representative images of xenograft models and tumor tissues. C, D, Tumor volumes (C) and weight (D) on day 24. E, Tumor growth curves. F,G, SHN intraperitoneal injection induced more exacerbated necroptosis in tumors derived from PLC/PRF/5-Vector cells than in tumors derived from PLC/PRF/5-shCx32 cells (n = 3). $^{**}P < .01$ MLKL, mixed lineage kinase domain-like; p-casp8, phosphorylated caspase 8; RIP, receptor-interacting serine-threonine protein kinase

Gap junctions are cytomembrane protein channels composed of connexin proteins, which mediate direct cell-cell communication to maintain the homeostasis and regulate cell growth, differentiation, and death and inhibit tumorigenesis.[19] Hence, Cxs have long been considered GJ-dependent tumor suppressors.[20] However, emerging evidence has proven that Cxs are internalized in the cytoplasm of some tumors and exert proper GJ-independent roles to affect the initiation and progress of tumors.[3] For instance, cytoplasmic accumulation of Cx32 enhances the invasion and metastasis of HCC cells in vitro and in vivo,[21] and expands the cell population of cancer stem cells by elevating self-renewal.[22] Recently, our previous finding suggested that Cx32 was constantly internalized and accumulated along with hepatocarcinogenesis, and was responsible for HCC cells proliferating and avoiding apoptosis by activating the EGFR pathway.[5] Hence, effective strategies for eliminating HCC cells with high Cx32 expression, which have a greater potential to develop resistance to apoptosis inducers, are worth investigating.

Unlike apoptosis, which is regulated by the caspase family and members of the Bcl-2 family, necroptosis is critically activated by

RIP1, RIP3, and MLKL.[23] Therefore, necroptosis is an alternative mode of programmed cell death overcoming apoptosis resistance.[24] For instance, SHN induces necroptosis to kill cisplatin-resistant bladder cancer cells.[25] Smac mimic induces necroptosis in caspase-8-deficient colorectal cancer in vivo, which is resistant to apoptosis inducers.[26] In our study, we investigated whether triggering necroptosis was an efficient therapeutic strategy for high Cx32-expressing HCC cells.

Initially, we established a necroptosis model in vitro by treating HCC cells with SHN, which has been reported to induce necroptosis by increasing reactive oxygen species (ROS) levels and the expression of RIP1 and RIP3.[27,28] Shikonin induced significant cell death in HCC cells, which was verified to be necroptosis by pretreating with RIP1 specific inhibitor Nec-1,[29] measuring the necrotic cell rate, detecting the expression of RIP1, RIP3, and MLKL,[24] membrane MLKL,[30] and necrosomes.[9] Interestingly and importantly, the overexpression of Cx32 significantly accelerated SHN-induced necroptosis in SMMC-7721 cells; in contrast, the knockdown of Cx32 alleviated necroptosis in PLC/PRF/5 cells. However, pretreatment with the GJ inhibitor 2-APB had no significant influence on necroptosis, indicating that GJs were not involved in modulating necroptosis. In summary, we were the first to prove that Cx32 contributes to sensitizing HCC cells to SHN-induced necroptosis. Yuan et al reported that Cx43-containing GJs promoted acute kidney injury after liver transplantation by increasing the cell-cell transmission of ROS and ROS-dependent inflammation and necroptosis.[31] However, in our study, the mechanism by which Cx32 regulated necroptosis was the Cx32-mediated regulation of proteins rather than GJs.

How does Cx32 affect necroptosis? We further focused on caspase 8, which is a critical regulator of apoptosis and necroptosis.[32] Under normal circumstances, active caspase 8 induces apoptosis by cleaving downstream effector caspases but abolishes necroptosis by cleaving RIP1[16] and RIP3.[17] In our results, Cx32 significantly suppressed the enzymatic activity of caspase 8 and decreased the expression of cleaved-caspase 8 (p41/43 and p18). Hence, Cx32 protected RIP1 and RIP3 from caspase 8-mediated cleavage and inactivation. Additionally, we found that Cx32 downregulated the expression of FADD, which recruits pro-caspase 8 to form the death-inducing signaling complex (DISC) and promotes the activation of caspase 8,[33] and upregulated the expression of c-FLIP$_S$, which prevents DISC formation by competing with caspase 8 to bind FADD.[34] Taken together, our data indicate that the inhibition of caspase 8 activity mediated by Cx32 through an increase in c-FLIP$_S$ and a decrease in FADD might be the crucial mechanism by which the expression of RIP1 and RIP3 is elevated.

In addition, the tyrosine phosphorylation of caspase 8 is also a key mechanism of the loss of caspase 8 activity.[35] Src kinase was reported to directly phosphorylate caspase 8 on Tyr380 and prevents its activation and proapoptotic function.[36] The carboxyl-terminal (CT) domain of Cxs contains multiple protein-protein interacting sites[37] so that Cxs could exert different GJ-independent functions to regulate the tumor biological behavior. For example, Cx30.3 activated Src signaling through MET to enhance the resistance to gemcitabine and dasatinib.[38] In addition, the SH3 binding site of the CT domain of Cx40 interacted with Src and ZO-1, causing the structural changes of Cx40.[39] Our results showed that Cx32 interacted with Src in HCC cells, as detected by Co-IP experiments. Using rescue experiments, we showed that Src is the key molecule for Cx32 suppressing caspase 8 activity and facilitating necroptosis.

By establishing the PLC/PFR/5 xenograft model, we found knockdown of Cx32 reduced the expression of necroptosis biomarkers RIP1, RIP3, and MLKL, and the levels of Src and p-caspase 8 (Tyr380), but increased the expression of cleaved-caspase 8 and cleaved-caspase 3, suggesting that knockdown of Cx32 inhibited necroptosis activation but promoted apoptosis by alleviating Src-mediated caspase 8 activity suppression. We also found the SHN given intraperitoneally could activate necroptosis in tumors derived from PLC/PRF/5 cells. The growth inhibition rate achieved by SHN in tumors generated from PLC/PRF/5-Vector cells was significantly higher than that in tumors generated from PLC/PRF/5-shCx32 cells, suggesting that downregulated Cx32 ameliorated SHN-induced necroptosis in vivo. Knockdown of Cx32 also suppressed the proliferation of HCC cells, consistent with our previous report that Cx32 inhibits apoptosis and promotes proliferation by activation of the EGFR/STAT3/Erk pathway.[5] It seems to be paradoxical that Cx32 concurrently promoted both the activation of necroptosis and the proliferation ability in HCC cells. One possible explanation is that the Cx32-mediated protumor effect was stronger than the pronecroptosis effect in HCC cells without any pronecroptosis stimulation. Only when HCC cells with high Cx32 expression were exposed to necroptosis inducers, such as SHN, would the Cx32-mediated pronecroptosis function be significantly amplified and facilitate the elimination of HCC cells.

In conclusion, Cx32 interacts with Src and then promotes the phosphorylation of caspase 8, the upregulation of c-FLIP$_S$, and the downregulation of FADD, resulting in the inactivity of caspase 8. Due to the inhibition of caspase 8, Cx32 enhances the expression of RIP1 and RIP3 by protecting them from caspase 8-mediated cleavage and contributes to SHN-induced necroptosis. This discovery suggests that Cx32 is a switch protein that determines cell death execution by apoptosis or necroptosis. We identified a therapeutic strategy involving necroptosis inducers, which could be more effective anticarcinogens than apoptosis inducers in HCC patients with high Cx32 expression. The overexpression of Cx32 could be a biomarker for choosing the most appropriate pharmaceutical strategy.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval (K-201354) of clinical data and tissues was obtained from the Research Committee of Ethics in the Affiliated Cancer Hospital of Xinjiang Medical University. Written informed consent form for the experimental studies was obtained from the patients or their guardians.

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