



Original Articles

ZNF32 induces anoikis resistance through maintaining redox homeostasis and activating Src/FAK signaling in hepatocellular carcinoma

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ABSTRACT

Tumor cells need to attain anoikis resistance to survive prior to metastasis making it a vital trait of malignancy. The molecular mechanism by which hepatocellular carcinoma (HCC) cells resist anoikis remains not fully understood. Here, we report that ZNF32 expression is markedly upregulated in HCC cells upon detachment. Enforced ZNF32 expression significantly promotes the anchorage-independent growth capability of HepG2 and Huh7 cells, whereas knockdown of ZNF32 results in increased apoptosis of HCC cells after detachment. Mechanistically, we demonstrate that ZNF32 overexpression suppresses the reactive oxygen species (ROS) accumulation and maintains mitochondrial membrane potential, leading to ATP, GSH and NADPH elevation and promoting HCC cell survival in response to suspension. Moreover, ZNF32 enhances the phosphorylation and activation of Src/FAK signaling. Src and FAK inhibitors effectively reverse ZNF32-induced anoikis resistance in HCC cells. Collectively, our findings not only reveal a novel and important mechanism by which ZNF32 contributes to anoikis resistance through maintaining redox homeostasis and activating Src/FAK signaling, but also suggest the potential therapeutic value of ZNF32 in HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer and the second leading cause of cancer related death in the world [1,2]. The standard treatments for HCC include surgical resection, liver transplantation, local ablation therapy, transhepatic arterial chemotherapy and embolization [3,4]. Recent advances in HCC diagnosis and therapeutics have led to improved survival in patients with HCC, however, almost all of HCC patients eventually develop recurrence and metastasis, which are the underlying causes of poor long-term survival

after clinical treatment in HCC [5]. Thus, a better understanding the potential molecular mechanisms during HCC metastasis provides an opportunity for preventing the initiation of metastasis in early HCC patients and developing therapeutic strategies in advanced HCC patients.

HCC metastasis is a multistep, multifactorial process, including adhesion of tumor cells to the extracellular matrix (ECM), remodeling and degradation of ECM, invasion through local tissue, intravasation into blood or lymph vessels, and forming new tumors at distant sites [6,7]. Loss of adhesion or adhesion-mediated signaling results in

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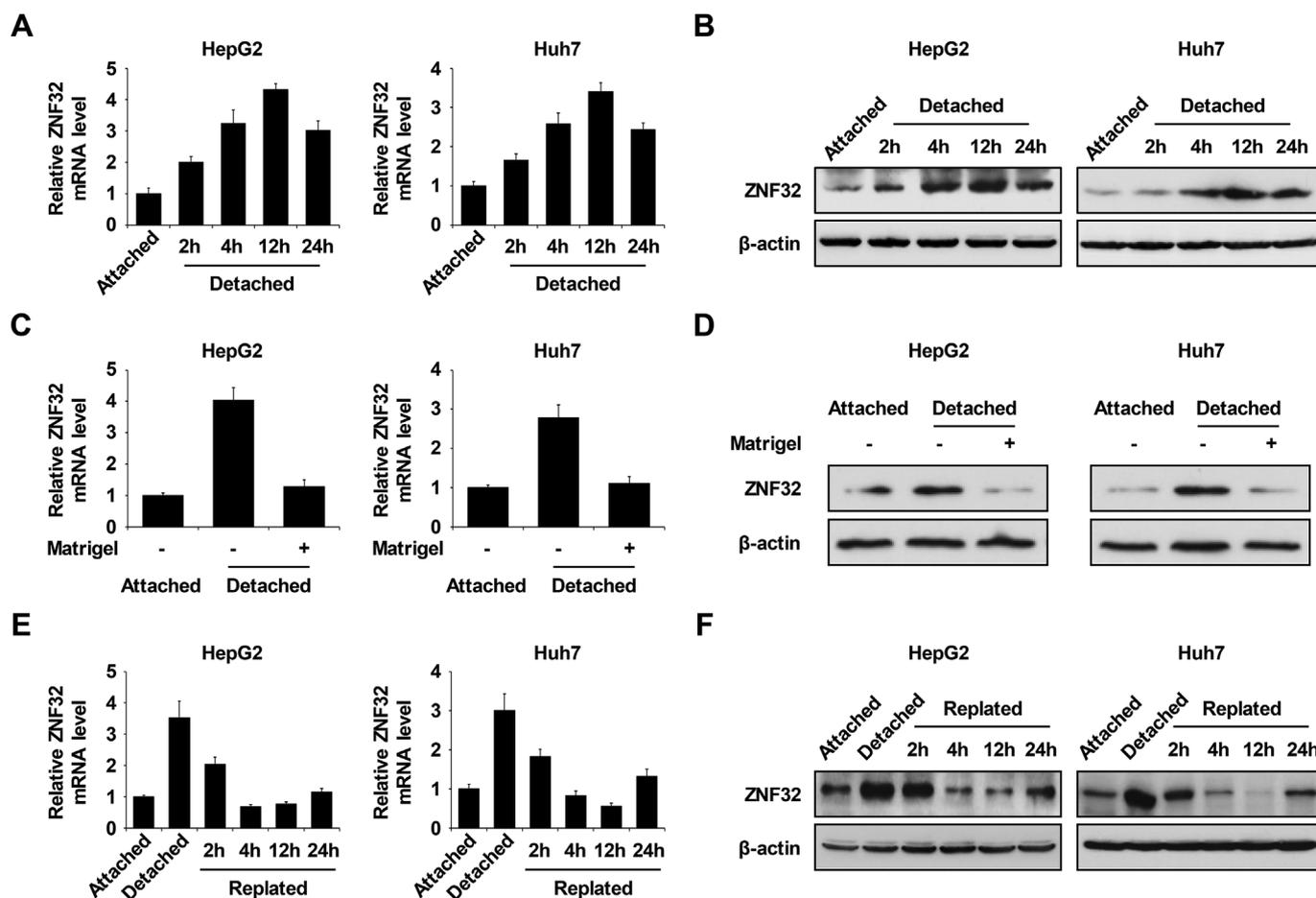


Fig. 1. ZNF32 was upregulated in detached HCC cells. Quantitative real-time PCR (A) and immunoblot analysis (B) of ZNF32 expression in attached HepG2 and Huh7 cells, or detached HCC cells cultured in suspension for indicated time. Quantitative real-time PCR (C) and immunoblot analysis (D) of ZNF32 expression in attached HepG2 and Huh7 cells, or detached HCC cells cultured in suspension for 24 h and treated in the presence or absence of Matrigel. HepG2 and Huh7 cells were left adherent, detached for 4 h, or detached and replated onto ECM for the indicated time periods, and then subjected to quantitative real-time PCR (E) and immunoblot analysis (F).

programmed cell death, referred to as anoikis. Anoikis has been suggested to act as a physiological barrier to metastasis and is an early step in preventing cancer metastasis [8,9]. However, anoikis resistance endues malignant tumor cells with anchorage-independent growth, which has a crucial role in tumor metastasis. The cancer cells exploit many mechanisms to confer anoikis resistance, including deregulation of integrin, aberrant constitutive activation of several anti-apoptotic or pro-survival pathways, undergoing epithelial–mesenchymal transition (EMT), and altered metabolism [10,11]. However, the mechanisms responsible for anoikis resistance of HCC are still not fully understood.

Zinc finger protein 32 (ZNF32) belongs to the Krüppel-like family of transcription factors that are critical for normal biologic processes as well as cancer development [12,13]. Our previous study demonstrates that knockdown of ZNF32 promotes the regeneration of the lateral line system in zebrafish [14]. ZNF32 protects against oxidative stress-induced apoptosis by modulating C1QBP transcription [15]. Furthermore, ZNF32 dysregulation is associated with multidrug resistance in lung adenocarcinoma [16]. However, the role of ZNF32 in HCC progression remains unknown. In the present study, we provided the first evidence that ZNF32 was up-regulated in HCC cells upon detachment. ZNF32 overexpression significantly promoted the anchorage-independent growth capability of HCC cells. Mechanistically, we demonstrated that ZNF32 maintained mitochondrial function through inhibition of excess ROS accumulation and promoted the phosphorylation of Src/FAK signaling. Taken together, our study indicated that ZNF32 induced the anoikis resistance of HCC cells by maintaining redox homeostasis and

activating Src/FAK signaling, implicating ZNF32 as a potential therapeutic target of advanced HCC.

2. Materials and methods

2.1. Cell culture and reagents

The human HCC cells HepG2 and Huh7 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, and 100 mg/mL streptomycin. Both cell lines were authenticated by short tandem repeat DNA profiling from the cell banks. FAK Inhibitor (Y15), Src inhibitor (PP2) and Trypan Blue were purchased from Sigma. The primary antibodies included, cleaved caspase-3 (Cell Signaling Technology, #9664), cleaved PARP (Cell Signaling Technology, #5625), β -actin (Santa Cruz Biotechnology, sc-47778), Src (Cell Signaling Technology, #2123), p-Src (Cell Signaling Technology, #6943), FAK (Cell Signaling Technology, #13009), p-FAK (Cell Signaling Technology, #8556).

2.2. Stable cell line generation for ZNF32 knockdown or overexpression

The ZNF32 lentiviral expression vector was constructed by inserting expanded ZNF32 cDNA (NM_006973.2) fragments into a lentiviral shuttle vector. Silencing of ZNF32 was accomplished using a specific

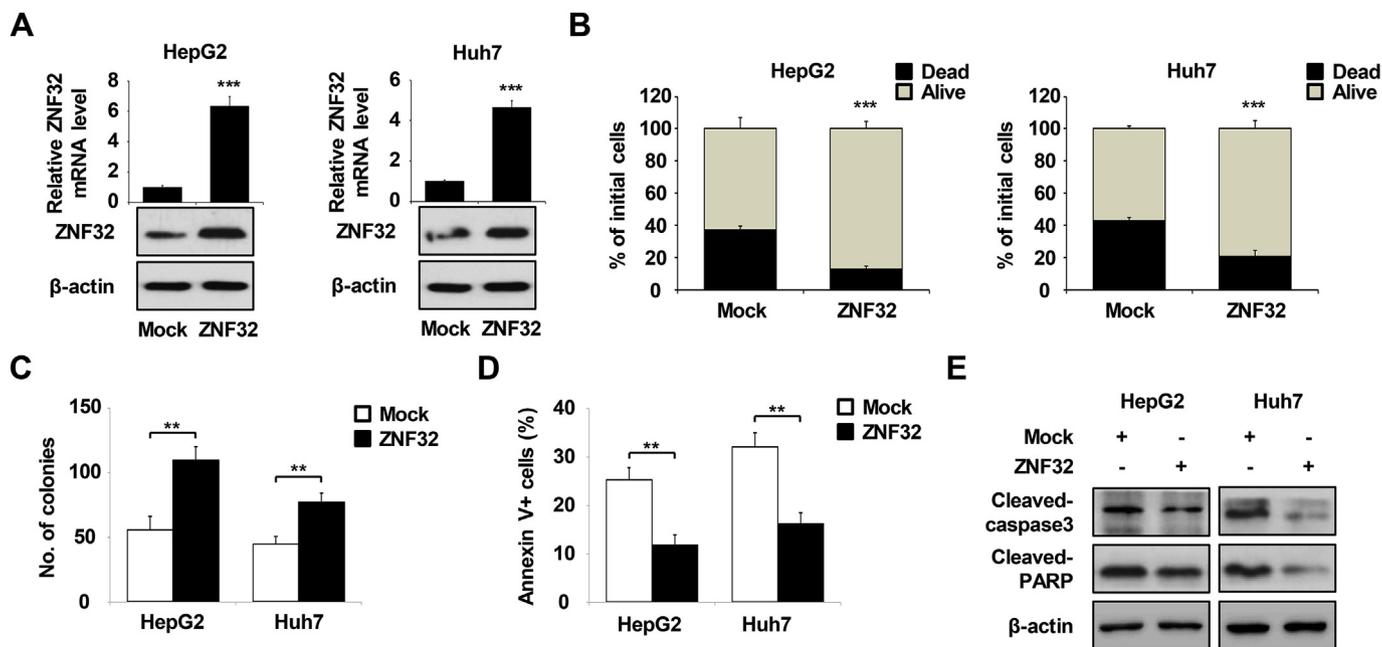


Fig. 2. ZNF32 enhanced the anoikis resistance of HCC cells. (A) Quantitative real-time PCR and immunoblot analysis of ZNF32 expression in the indicated HepG2 and Huh7 cells. (B) ZNF32-overexpression and mock HCC cells were cultured in the plates coated with poly-HEMA to avoid the adhesion of cells. Forty-eight hours later, the cell viabilities were determined by trypan blue exclusion assay. (C) HCC cells transfected with mock or ZNF32 were subjected to the soft agar assay. (D) FACS assay for measuring apoptosis of HCC cells transfected with mock or ZNF32 in suspension culture 48 h (E) HCC cells transfected with mock or ZNF32 were subjected to immunoblot analysis using the indicated antibodies. Data represent the mean \pm standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. ** $P < 0.01$, *** $P < 0.001$.

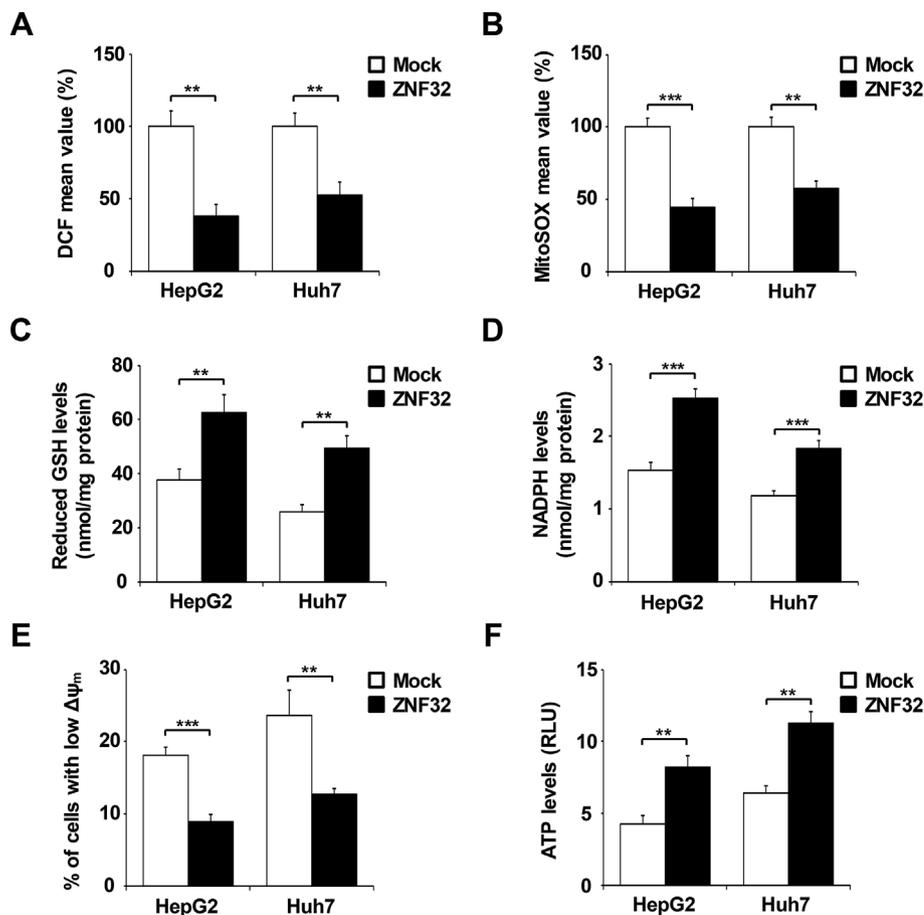


Fig. 3. ZNF32 contributed to ROS defense and the maintenance of mitochondrial membrane potential in detached HCC cells. (A) HepG2 and Huh7 cells stably expressing ZNF32 and cultured in the suspension for 48 h were stained with CM-H₂DCFDA and subjected to fluorescence intensity analysis to determine the intracellular ROS levels. (B) The indicated cells cultured in the suspension for 48 h were stained with MitoSOX Red, and ROS levels were determined. (C) The indicated cells cultured in the suspension for 48 h, and reduced GSH levels was measured. (D) The indicated cells cultured in the suspension for 48 h, and NADPH levels was measured. (E) The indicated cells cultured in the suspension for 48 h were subjected to measurement of the mitochondrial membrane potential using the JC-1 assay. (F) The indicated cells cultured in the suspension for 48 h, and ATP was measured using the ATP determination kit. Data represent the mean \pm standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. ** $P < 0.01$, *** $P < 0.001$.

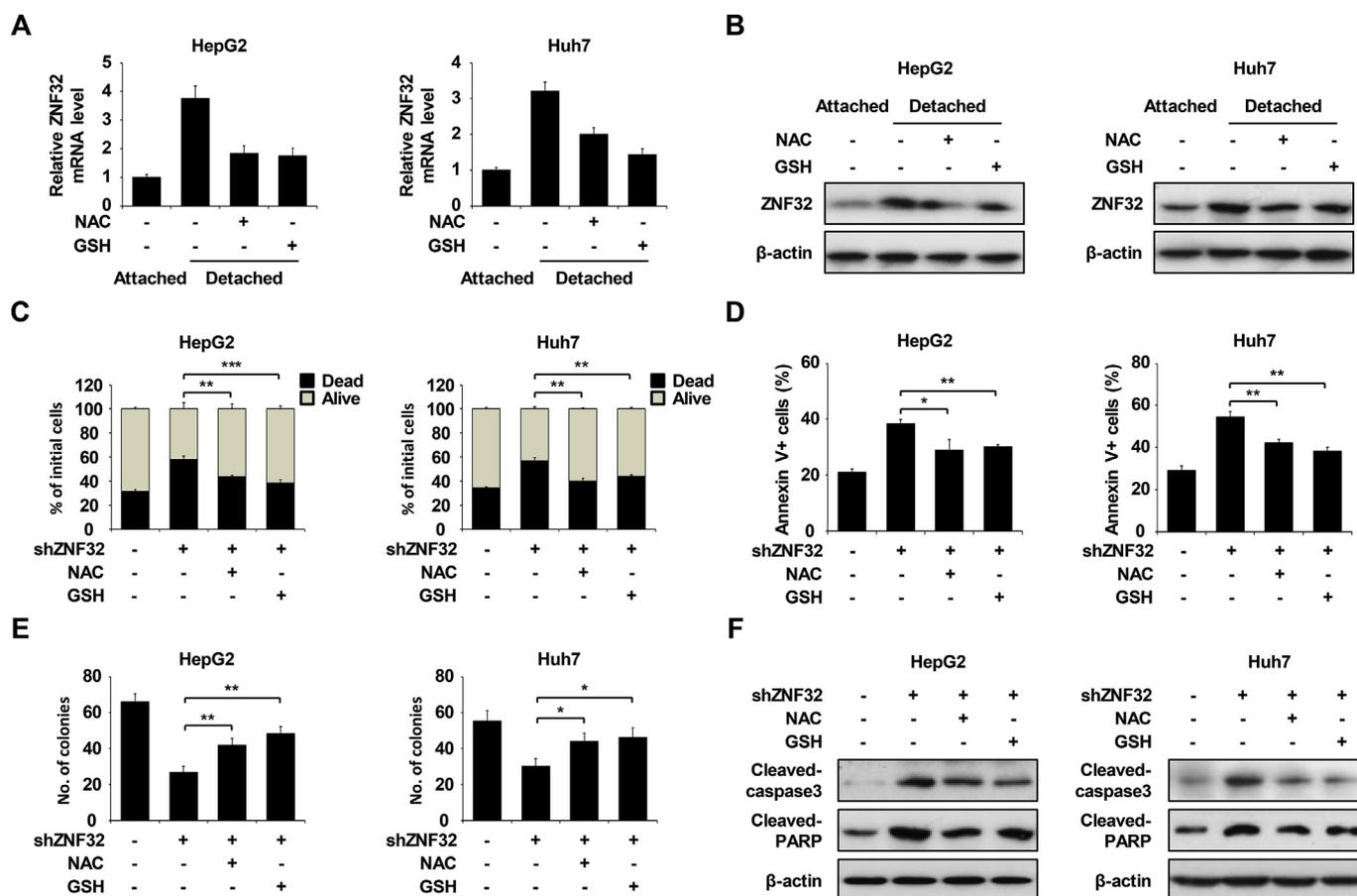


Fig. 4. Antioxidants rescued ZNF32 deficiency-induced anoikis in detached HCC cells. Quantitative real-time PCR (A) and immunoblot analysis (B) of ZNF32 expression in attached HepG2 and Huh7 cells, detached HCC cells treated with or without NAC and GSH. (C) The indicated cells were cultured in the plates coated with poly-HEMA and treated with NAC or GSH, and subjected to trypan blue exclusion assay. (D) The indicated cells were cultured and treated as in (C), and subjected to Annexin V assay. (E) The indicated cells were cultured and treated as in (C), and subjected to the soft agar assay. (F) The indicated cells were cultured and treated as in (C), and subjected to immunoblot analysis using the indicated antibodies. Data represent the mean \pm standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

shRNA targeting ZNF32. The sequences of shRNA were as follows: shRNA-ZNF32, 5'-GAA TGT AGC GTT CTT CAA TGT-3'; shRNA-NC, 5'-TTC TCC GAA CGT GTC AGG T-3'. Both lentiviral shuttle vectors expressed EGFP. The packing and purification of the recombinant lentiviral vector were performed by the GenePharma Company (Shanghai, China). The indicated HCC cells infected with the recombinant lentiviral vectors were selected with puromycin for 2 weeks.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using RNAiso Plus (TAKARA) according to the manufacturer's instructions. Real-time PCR was performed with SYBR[®] Premix Ex Taq[™] II (TAKARA) using an iCycler iQ[™] Multicolor Real-Time Detection System (BIO-RAD) as previously described [17]. The following primers were used: ZNF32, 5'-AGA ATG TAG CGT TCT TCA ATG TG-3' (forward) and 5'-CCT GTA GTG TCT TCG AAT CTG G-3' (reverse); GAPDH, 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse). The relative expression levels were determined using Gene Expression Macro Version 1.1 software (BIO-RAD).

2.4. Soft agar colony formation assay

A total of 5×10^4 cells were suspended in DMEM containing 0.35% agar and seeded onto a 0.6% agar layer in 6-well plates. The cells were maintained at 37 °C in a 5% CO₂ incubator for 2 weeks and then cells

were stained with crystal violet. Images were taken and analyzed with ImageJ software.

2.5. Induction of anoikis

To prevent cell adhesion, 6-well plates were coated with a solution of poly-HEMA (Sigma-Aldrich), dissolved at 10 mg/mL in ethanol. To coat 6-well plates, 2 mL of poly-HEMA solution was added to each well. Plates were kept at 37 °C for at least 3 days until the solvent had completely evaporated. To induce anoikis, 1×10^5 cells resuspended in DMEM medium containing 10% FBS were plated on the poly-HEMA plates. At the indicated times, the cells in suspension were recovered and submitted to trypan blue exclusion assay.

2.6. Annexin V-PE analysis

Apoptotic cells were quantified via Annexin V-PE apoptosis detection kit from KeyGEN Biotech (Nanjing, China). Briefly, cells were trypsinized with trypsin, washed twice with PBS, resuspended in 500 μ l of binding buffer containing 1 μ l of Annexin V-PE and incubated at room temperature for 15 min. Then, the cell mixture was immediately analyzed using a flow cytometer (FACS Aria, Becton Dickinson).

2.7. Assays of reactive oxygen species

The intracellular levels of H₂O₂ were measured with CM-H₂DCFDA

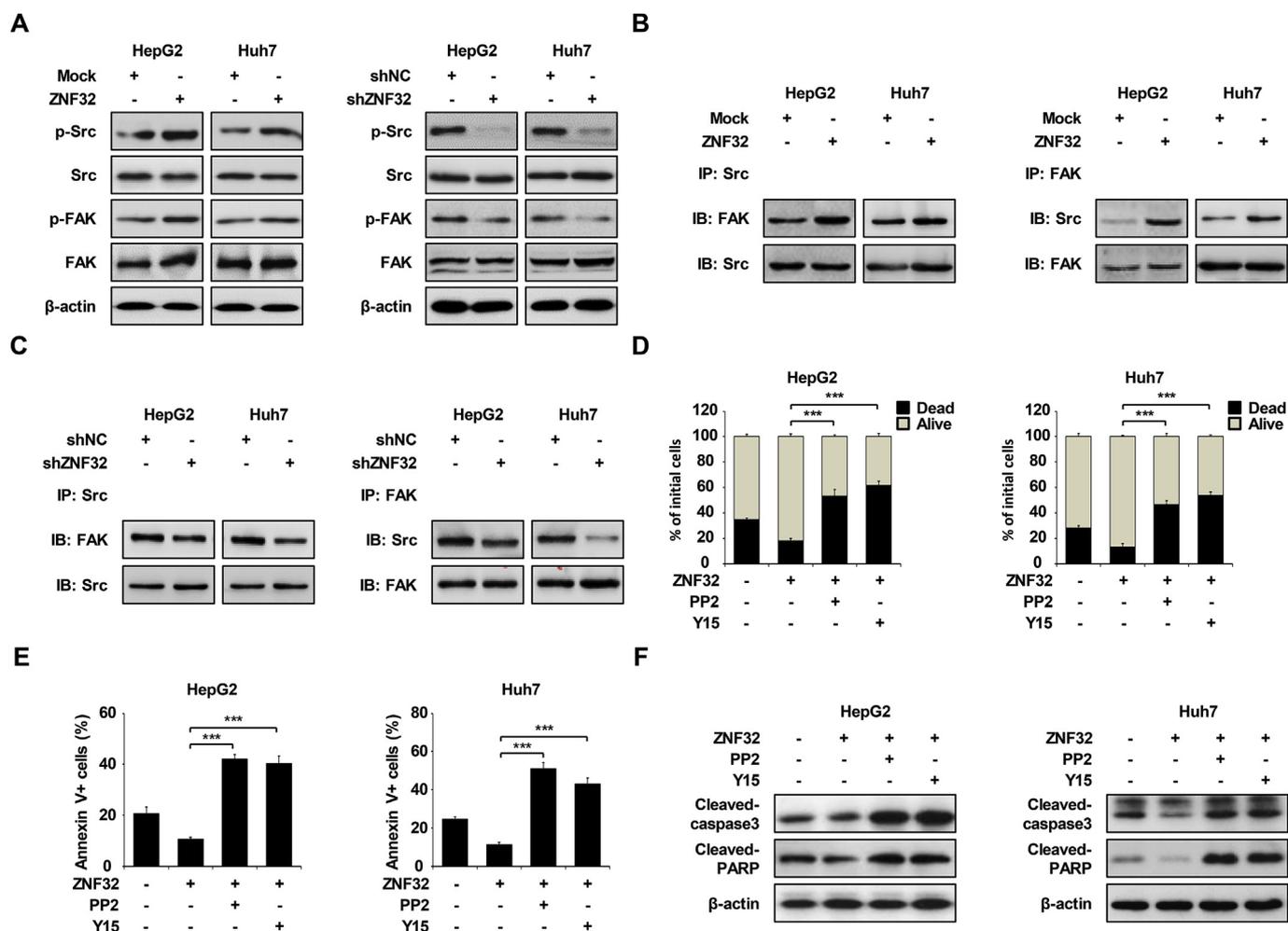


Fig. 5. The activation of Src/FAK signaling was required for ZNF32-mediated anoikis resistance. (A) The indicated HepG2 and Huh7 cells were cultured in the suspension and subjected to immunoblot analysis using the indicated antibodies. (B) and (C) The effect of ZNF32 on the association of Src with FAK was detected by immunoprecipitation for Src or FAK, followed by immunoblotting for FAK and Src. (D) ZNF32-overexpressing HCC cells were cultured in suspension and treated with PP2 or Y15, and subjected to trypan blue exclusion assay. (E) The indicated cells were cultured and treated as in (D), and subjected to Annexin V assay. (F) The indicated cells were cultured and treated as in (D), and subjected to immunoblot analysis using the indicated antibodies. Data represent the mean ± standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. ****P* < 0.001.

(Thermo). Mitochondrial ROS production was detected by the MitoSOX Red assay (Thermo). Fluorescence was detected by flow cytometry.

2.8. NADPH, GSH and ATP assays

The intracellular levels of NADPH were measured using the NADP/NADPH Quantitation Colorimetric Kit (Biovision) as described previously [18]. The intracellular levels of reduced GSH were measured using the Reduced Glutathione (GSH) Assay Kit (Biovision) according to the manufacturer's instructions. The intracellular level of ATP was measured with an ATP Colorimetric/Fluorometric Assay Kit (Biovision). All the measurements were normalized to the protein content of the cells.

2.9. Mitochondrial membrane potential analysis

The mitochondrial membrane potential was measured using a mitochondria-specific dual fluorescence probe, JC-1 (KeyGEN Biotech, Nanjing, China), as described previously [15]. Briefly, cells were washed twice with PBS and loaded with JC-1-containing solution for 30 min at 37 °C. JC-1 fluorescence was quantified by flow cytometry, in which red JC-1 aggregates were gated in the FL2 channel and green JC-1 monomers in the FL1 channel. Analysis of the multivariate data was

performed using FlowJo software.

2.10. In vivo assays for tumor metastasis and anoikis

Female BALB/c nu/nu mice (6-week-old) were raised in specific pathogen-free conditions. Animal care and experimental protocols were in accordance with guidelines established by the Institutional Animal Care and Use Committee of Sichuan University. For mouse lung metastasis model, single-cell suspensions of 5 × 10⁶ cells were injected into the tail vein of nude mice. The mice were killed 8 weeks after injection and the lungs were resected and fixed in formalin, and the visible tumor nodules were counted. The fixed lungs were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and microscopic lung metastasis was counted under a light microscope.

For mouse peritoneal cavity model, 5 × 10⁶ indicated HepG2 cells were i.p. injected into nude mice. After 72 h post injection, ascites fluid was drawn out from the peritoneal cavity. GFP positive tumor cells were selected by flow cytometry and viable cells were counted using trypan blue exclusion assay.

2.11. Statistical analysis

All the experiments were performed at least three times

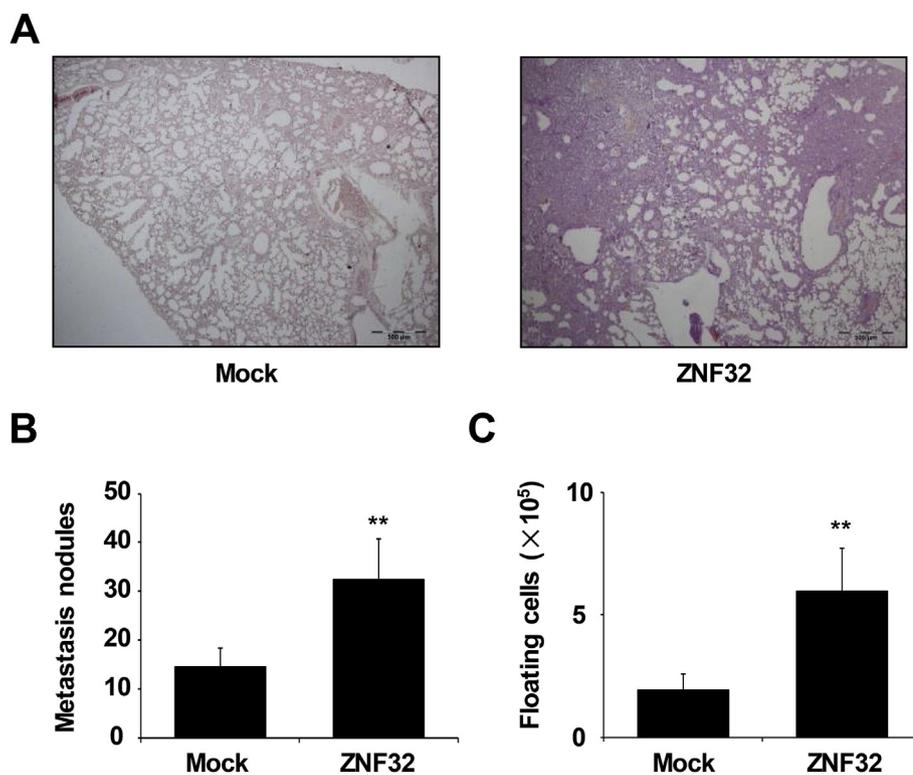


Fig. 6. ZNF32 promoted anoikis resistance of HCC cells *in vivo*. HepG2 cells transfected mock or ZNF32 were injected into the tail vein of Balb/c nude mice to establish an *in vivo* model of lung metastasis. (A) The representative images of lung tissue sections from the indicated groups obtained 8 weeks after tail vein injection. (B) The number of lung metastasis nodules was counted from mice as in (A). (C) Indicated HepG2 cells were i.p. injected into Balb/c nude mice. GFP-positive HepG2 cells isolated from ascites fluid were selected by flow cytometry and viable cells were counted using trypan blue exclusion assay. Data represent the mean \pm standard deviation (SD). Each experiment was performed at least in triplicate, producing consistent results. ** $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

independently, and all data are expressed as “mean \pm SD”. A one-way ANOVA test was used to analyse quantitative data between groups. The data were analyzed using SPSS statistical software version 22.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ZNF32 is upregulated in HCC cells following detachment

To explore the relationship between ZNF32 and anoikis in HCC cells, we first evaluated the expression of ZNF32 in HepG2 and Huh7 cells upon detachment. HCC cells were plated on poly-2-hydroxyethylmethacrylate (poly-HEMA)-coated plates to inhibit cell attachment to matrix as previously described [19]. Both the mRNA and protein levels of ZNF32 were robustly increased in a time-dependent manner following detachment (Fig. 1A and B). The addition of Matrigel basement membrane-like matrix to detached HCC cells markedly blocked the elevated levels of ZNF32 (Fig. 1C and D). Moreover, we found that if HCC cells that had been detached for 4 h were allowed to reattach to ECM, the expression of ZNF32 was decreased (Fig. 1E and F). These data suggest a potential role of ZNF32 in the anoikis of HCC cells.

3.2. ZNF32 promotes the anchorage-independent growth of HCC cells

To determine whether ZNF32 is involved in anoikis in HCC cells, we engineered stable upregulation or knockdown of ZNF32 expression in HepG2 and Huh7 cells via ectopic expression of ZNF32 cDNA or ZNF32-specific short hairpin RNA (shRNA) using lentiviral technology (Fig. 2A and Supplementary Fig. S1A). ZNF32 overexpression significantly increased the number of surviving detached HepG2 and Huh7 cells, whereas ZNF32 deficiency suppressed the anoikis resistance of HCC cells in response to detachment (Fig. 2B and Supplementary Fig. S1B). Enforced ZNF32 expression significantly promoted the anchorage-independent growth capability of HCC cells, leading to a marked increase of colonies as analyzed by soft agar assay (Fig. 2C). In contrast, ZNF32 deficiency resulted in decreased the colonies number of HepG2 and

Huh7 cells on soft agar compared with control cells (Supplementary Fig. S1C). Moreover, upregulated ZNF32 markedly reduced the percentage of apoptotic cells (Fig. 2D) and protein levels of cleaved caspase 3 and cleaved PARP (Fig. 2E). The converse results were observed in ZNF32-knockdown HCC cells (Supplementary Figs. S1D and S1E). Taken together, these results indicate that ZNF32 induces anoikis resistance of HCC cells *in vitro*.

3.3. ZNF32 regulates redox homeostasis in HCC cells following detachment

As ECM-detachment is now well established to cause a host of catastrophic metabolic alterations including a robust increase in ROS, diminished pentose phosphate pathway flux, and reduced cellular ATP levels [20,21], we next examined whether ZNF32 affected cellular redox balance upon detachment. Flow cytometry analysis of CM-H₂DCFDA, a ROS sensitive dye, revealed that ZNF32 significantly repressed ROS levels in the suspended HCC cells (Fig. 3A). Likewise, experiments using MitoSOX Red dye, a probe of mitochondrial superoxide production in live cells, showed that mitochondrial ROS levels were lower in ZNF32-overexpression cells compared with control cells (Fig. 3B). Conversely, knockdown of ZNF32 resulted in increased accumulation of intracellular and mitochondrial ROS levels (Supplementary Figs. S2A and S2B). The glutathione (GSH) and NADPH levels, two most abundant antioxidant to neutralize ROS in human cells, were increased in ZNF32 overexpressing HCC cells under detachment conditions (Fig. 3C and D). However, decreased GSH and NADPH contents were observed in ZNF32 knockdown cells (Supplementary Figs. S2C and S2D). Moreover, overexpression of ZNF32 reduced mitochondrial permeabilization according to the fluorescent JC-1 aggregation assay and promoted ATP generation in HCC cells following detachment (Fig. 3E and F), while ZNF32-deficient HCC cells displayed marked mitochondrial membrane potential impairment and diminished ATP production (Supplementary Figs. S2E and S2F). These results reveal that ZNF32 promotes HCC cells resistance to anoikis through enhancing the antioxidant capacity of cells to detoxify excess ROS.

3.4. Antioxidants rescue ZNF32 deficiency-induced anoikis in HCC cells following detachment

Given the evidence that ZNF32 is upregulated in detached HCC cells and that its overexpression can repress excess ROS accumulation, we investigated the effect of antioxidants on ZNF32 expression in detached cells. The antioxidant agent N-acetyl-L-cysteine (NAC) or GSH was sufficient to eliminate cellular ROS levels in HepG2 and Huh7 cells (Supplementary Figs. S3A and S3B). Treatment of detached HepG2 and Huh7 cells with NAC or GSH significantly inhibited the elevated expression of ZNF32 in detached cells (Fig. 4A and B), suggesting that ZNF32 expression was regulated by ROS levels. Next, we determined whether antioxidants addition could attenuate ZNF32 deficiency-induced anoikis in HCC cells after detachment. Indeed, silencing ZNF32 caused increased apoptotic rate of detached HCC cells that was rescued by NAC or GSH treatment (Fig. 4C and D). Consistently, NAC or GSH addition efficiently promoted anchorage-independent survival of HepG2 and Huh7 cells in the absence of ZNF32 (Fig. 4E). Moreover, ZNF32-deficient cells exhibited increased protein levels of cleaved caspase 3 and cleaved PARP that was reversed by NAC or GSH treatment (Fig. 4F). These data indicate that antioxidants can rescue the survival of ZNF32-silenced HCC cells after detachment.

3.5. Src/FAK signaling is required for ZNF32-mediated anoikis resistance

Src/FAK pathway has been reported to protect the tumor cells from anoikis [22,23]. To further understand the molecular mechanism by which ZNF32 regulated anoikis resistance, we explored the effect of ZNF32 on the activation of Src/FAK signaling. ZNF32 overexpression resulted in the increased phosphorylations of Src and FAK, whereas ZNF32 knockdown inhibited the activation of Src and FAK (Fig. 5A). We next assessed whether ZNF32 influenced the interactions between Src and FAK. Immunoprecipitation of Src followed by anti-FAK immunoblot analysis revealed an association between Src and FAK that was significantly augmented by ZNF32 overexpression (Fig. 5B). Parallel effects were observed when FAK immunoprecipitates were assayed for Src (Fig. 5B). Conversely, down-regulation of ZNF32 inhibited Src/FAK complex formation following detachment (Fig. 5C). To assess the functional roles of Src/FAK signaling in ZNF32-mediated anoikis, indicated HCC cells were treated with Src inhibitor PP2 or FAK inhibitor Y15 after detachment, respectively. Indeed, PP2 or Y15 treatment was efficiently to suppress the phosphorylations of Src and FAK, respectively (Supplementary Fig. S4). As shown in Fig. 5D, PP2 and Y15 treatment resulted in decreased number of surviving detached HepG2 and Huh7 cells in the presence of ZNF32. Consistent with this, both PP2 and Y15 treatment effectively reversed ZNF32-induced anoikis resistance in HepG2 and Huh7 cells (Fig. 5E), leading to the increased levels of cleaved caspase 3 and cleaved PARP (Fig. 5F). These results imply that the activation of Src/FAK signaling plays a critical role in ZNF32-mediated anoikis resistance in HCC cells.

3.6. ZNF32 enhanced the metastasis and anoikis resistance of HepG2 cells *in vivo*

To confirm whether ZNF32 contributes to the metastasis of HCC cells, we established a lung metastasis model by injecting HepG2 cells into the tail vein of nude mice. A representative lung metastasis is presented in Fig. 6A. Overexpression of ZNF32 significantly augmented the ability of HepG2 cells to establish lung metastases (Fig. 6B). To further determine the effect of ZNF32 on *in vivo* anoikis, the indicated HepG2 cells were implanted into the peritoneal cavity of nude mice. At 72 h after implantation, GFP positive tumor cells from peritoneal cavity were selected by flow cytometry and viable cells were counted using trypan blue exclusion assay. As shown in Fig. 6C, ZNF32 overexpression resulted in a significantly increased the number of surviving HepG2

cells in the mouse peritoneal cavity compared to control cells. These data indicate that high level of ZNF32 is associated with acquired anoikis resistance and metastasis of HCC cells *in vivo*.

4. Discussion

Anoikis, defined as detachment-induced apoptosis, exerts as a barrier to cancer metastasis. Resistance to anoikis is known as a critical step in metastasis, as it compromises tumor cells to survive in the systemic circulation and facilitates their metastasis to distant organs [24,25]. However, the molecular mechanism of anoikis resistance in HCC remains not fully understood. In the present study, we found that ZNF32 expression was significantly upregulated in HCC cells following detachment. Overexpression of ZNF32 promoted the resistance to anoikis, whereas ZNF32 suppression resulted in reduced anchorage-independent growth in HCC cells. Our data revealed a crucial role for ZNF32 in regulating HCC malignant progression.

Recent studies have demonstrated that ECM-detachment induces robustly increased basal ROS levels that can cause damage to cellular components and ultimately lead to cell death [26,27]. Strikingly, cancer cells seemed to benefit from the elimination of ECM-detachment mediated ROS production as overexpression of the ErbB2 oncogene led to luminal filling and diminished ROS levels [28]. Here, we reported that overexpression of ZNF32 significantly repressed ROS levels and enhanced antioxidant-scavenging capacity as indicated by accumulation of two intracellular antioxidants GSH and NADPH to promote survival of suspended HCC cells. Moreover, the defect of anchorage-independent growth caused by ZNF32 deficiency was restored by antioxidants NAC or GSH treatment. Consistent with our study, a recent study reported that antioxidant enhanced anchorage-independent colony formation of breast cancer cells [29]. Furthermore, additional studies have revealed that upregulated pyruvate dehydrogenase kinase 4 attenuated the production of ROS through stalling the flux of glycolytic carbon into mitochondrial oxidation to enable cell survival during ECM-detachment [30]. Based on elevated ROS levels are essential to promote tumor cell survival and malignant progression [31,32], we hypothesize that cancer cells need to both inhibit anoikis and appropriately regulate ROS levels to survive during the metastatic cascade.

Mitochondrial is the main source for ROS and ATP generation, and the loss of mitochondrial membrane potential is involved in the alteration of mitochondrial metabolism and mitochondrial failure-induced cell death [33,34]. In this study, we showed that upregulated ZNF32 sustained mitochondrial membrane potential and facilitated ATP production in detached HCC cells, suggesting that ZNF32 protected detached HCC cells from anoikis by modulating mitochondrial function. In support of this, expression of SOD2, a mitochondrial protein that reduces oxidative stress-induced mitochondrial impairment caused by respiratory chain leak, was increased in more advanced and higher grade mammary tumors and positively correlated with metastasis of breast cancer cells [35]. Furthermore, the elevated expressions of ZNF32 in detached HCC cells were significantly inhibited by NAC or GSH treatment. Our previous study demonstrated that the transcription of ZNF32 was precisely regulated by SP1 upon oxidants treatment [15]. These results suggest that ZNF32 may act as a sensor of intracellular ROS levels that maintains redox homeostasis under oxidative stress.

FAK, a broadly expressed non-receptor tyrosine kinase which transduces signals from integrins, growth and hormonal factors, plays a critical role in many fundamental biological processes and functions, including cell adhesion, migration, proliferation and survival [36,37]. Src was found to induce the FAK phosphorylation at Y397, leading to the activation of FAK [22,38]. In the present study, we found that ZNF32 overexpression promoted the phosphorylations of Src and FAK, whereas knockdown of ZNF32 resulted in inactivation of Src and FAK after detachment. In line with our observation, several studies reported that the activation of Src triggered downstream pro-survival pathways to confer anoikis resistance, thus promoted tumor growth and

metastasis [39,40]. In contrast, the Src kinase inhibitor PP2 and FAK inhibitor Y15 treatment significantly inhibited anchorage-independent growth of ZNF32-overexpressing HCC cells. Therefore, our data indicate that the ZNF32 overexpression activates Src, which in turn promotes phosphorylation of FAK, and ultimately leading to anoikis resistance in HCC.

In summary, our current findings provide the first evidence that upregulated ZNF32 expression is associated with anoikis resistance in HCC cells, and the expression of ZNF32 is dependent on intracellular ROS levels. Overexpression of ZNF32 maintains mitochondrial membrane potential and augments cellular antioxidant capacity to detoxify excess ROS, and these effects promote HCC cell survival upon detachment. Taken together, our study delineates the molecular mechanisms underlying the ZNF32-dependent regulation of anoikis resistance through manipulation of redox homeostasis and activation of Src/FAK signaling, and we propose that inhibition of ZNF32 may serve as an efficient strategy to enhance the sensitivity of HCC cells to anoikis.

Conflicts of interest

The authors disclose no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2018.09.033>.

References

- J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, *International journal of cancer, J. Int. Cancer* 136 (2015) E359–E386.
- L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, *Ca - Cancer J. Clin.* 65 (2015) 87–108.
- R. Jin, H. Lin, G. Li, J. Xu, L. Shi, C. Chang, X. Cai, TR4 nuclear receptor suppresses HCC cell invasion via downregulating the EphA2 expression, *Cell Death Dis.* 9 (2018) 283.
- J.A. Marrero, L.M. Kulik, C. Sirlin, A.X. Zhu, R.S. Finn, M.M. Abecassis, L.R. Roberts, J.K. Heimbach, Diagnosis, staging and management of hepatocellular carcinoma: 2018 practice guidance by the American association for the study of liver diseases, *Hepatology* 68 (2018) 723–750.
- B. Yang, M. Li, W. Tang, W. Liu, S. Zhang, L. Chen, J. Xia, Dynamic network biomarker indicates pulmonary metastasis at the tipping point of hepatocellular carcinoma, *Nat. Commun.* 9 (2018) 678.
- J.S. Desgrosellier, D.A. Cheresh, Integrins in cancer: biological implications and therapeutic opportunities, *Nat. Rev. Canc.* 10 (2010) 9–22.
- G. Giannelli, P. Koudelkova, F. Dituri, W. Mikulits, Role of epithelial to mesenchymal transition in hepatocellular carcinoma, *J. Hepatol.* 65 (2016) 798–808.
- A.P. Gilmore, Anoikis, *Cell death and differentiation* 12 (Suppl 2) (2005) 1473–1477.
- X. Hu, Y. Zhao, L. Wei, B. Zhu, D. Song, J. Wang, L. Yu, J. Wu, CCDC178 promotes hepatocellular carcinoma metastasis through modulation of anoikis, *Oncogene* 36 (2017) 4047–4059.
- C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis, *Science* 331 (2011) 1559–1564.
- C. Leng, Z.G. Zhang, W.X. Chen, H.P. Luo, J. Song, W. Dong, X.R. Zhu, X.P. Chen, H.F. Liang, B.X. Zhang, An integrin beta4-EGFR unit promotes hepatocellular carcinoma lung metastases by enhancing anchorage independence through activation of FAK-AKT pathway, *Cancer Lett.* 376 (2016) 188–196.
- C.K. Kim, P. He, A.B. Bialkowska, V.W. Yang, SP and KLF transcription factors in digestive physiology and diseases, *Gastroenterology* 152 (2017) 1845–1875.
- M.P. Tetreault, Y. Yang, J.P. Katz, Kruppel-like factors in cancer, *Nat. Rev. Canc.* 13 (2013) 701–713.
- Y. Wei, K. Li, S. Yao, J. Gao, J. Li, Y. Shang, J. Zhang, L. Zhang, Y. Li, X. Mo, W. Meng, R. Xiang, J. Hu, P. Lin, Y. Wei, Loss of ZNF32 augments the regeneration of nervous lateral line system through negative regulation of SOX2 transcription, *Oncotarget* 7 (2016) 70420–70436.
- K. Li, B. Gao, J. Li, H. Chen, Y. Li, Y. Wei, D. Gong, J. Gao, J. Zhang, W. Tan, T. Wen, L. Zhang, L. Huang, R. Xiang, P. Lin, Y. Wei, ZNF32 protects against oxidative stress-induced apoptosis by modulating CIQP transcription, *Oncotarget* 6 (2015) 38107–38126.
- J. Li, J. Ao, K. Li, J. Zhang, Y. Li, L. Zhang, Y. Wei, D. Gong, J. Gao, W. Tan, L. Huang, L. Liu, P. Lin, Y. Wei, ZNF32 contributes to the induction of multidrug resistance by regulating TGF-beta receptor 2 signaling in lung adenocarcinoma, *Cell Death Dis.* 7 (2016) e2428.
- K. Li, C.F. Mo, D. Gong, Y. Chen, Z. Huang, Y.Y. Li, J. Zhang, L. Huang, Y. Li, F.V. Fuller-Pace, P. Lin, Y.Q. Wei, DDX17 nucleocytoplasmic shuttling promotes acquired gefitinib resistance in non-small cell lung cancer cells via activation of beta-catenin, *Cancer Lett.* 400 (2017) 194–202.
- N. Chaman, M.A. Iqbal, F.A. Siddiqui, P. Gopinath, R.N. Bamezai, ERK2-Pyruvate kinase Axis permits phorbol 12-myristate 13-Acetate-induced megakaryocyte differentiation in K562 cells, *J. Biol. Chem.* 290 (2015) 23803–23815.
- A.J. Valentijn, A.P. Gilmore, Translocation of full-length Bid to mitochondria during anoikis, *J. Biol. Chem.* 279 (2004) 32848–32857.
- J.A. Mason, C.A. Davison-Versagli, A.K. Leliaert, D.J. Pape, C. McCallister, J. Zuo, S.M. Durbin, C.L. Buchheit, S. Zhang, Z.T. Schafer, Oncogenic Ras differentially regulates metabolism and anoikis in extracellular matrix-detached cells, *Cell Death Differ.* 23 (2016) 1271–1282.
- C.A. Davison, S.M. Durbin, M.R. Thau, V.R. Zellmer, S.E. Chapman, J. Diener, C. Wathen, W.M. Leevy, Z.T. Schafer, Antioxidant enzymes mediate survival of breast cancer cells deprived of extracellular matrix, *Cancer Res.* 73 (2013) 3704–3715.
- A.K. Sood, G.N. Armaiz-Pena, J. Halder, A.M. Nick, R.L. Stone, W. Hu, A.R. Carroll, W.A. Spannuth, M.T. Deavers, J.K. Allen, L.Y. Han, A.A. Kamat, M.M. Shahzad, B.W. McIntyre, C.M. Diaz-Montero, N.B. Jennings, Y.G. Lin, W.M. Merritt, K. DeGeest, P.E. Vivas-Mejia, G. Lopez-Berestein, M.D. Schaller, S.W. Cole, S.K. Lutgendorf, Adrenergic modulation of focal adhesion kinase protects human ovarian cancer cells from anoikis, *JCI (J. Clin. Invest.)* 120 (2010) 1515–1523.
- E.K. Park, M.J. Park, S.H. Lee, Y.C. Li, J. Kim, J.S. Lee, J.W. Lee, S.K. Ye, J.W. Park, C.W. Kim, B.K. Park, Y.N. Kim, Cholesterol depletion induces anoikis-like apoptosis via FAK down-regulation and caveolae internalization, *J. Pathol.* 218 (2009) 337–349.
- C.L. Buchheit, K.J. Weigel, Z.T. Schafer, Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression, *Nat. Rev. Canc.* 14 (2014) 632–641.
- J. Lu, M. Tan, Q. Cai, The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism, *Cancer Lett.* 356 (2015) 156–164.
- M.A. Hawk, C.L. Gorsuch, P. Fagan, C. Lee, S.E. Kim, J.C. Hamann, J.A. Mason, K.J. Weigel, M.A. Tsegaye, L. Shen, S. Shuff, J. Zuo, S. Hu, L. Jiang, S. Chapman, W.M. Leevy, R.J. DeBerardinis, M. Overholzer, Z.T. Schafer, RPK1-mediated induction of mitophagy compromises the viability of extracellular-matrix-detached cells, *Nat. Cell Biol.* 20 (2018) 272–284.
- A. Avivar-Valderas, E. Salas, E. Bobrovnikova-Marjon, J.A. Diehl, C. Nagi, J. Debnath, J.A. Aguirre-Ghiso, PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment, *Mol. Cell Biol.* 31 (2011) 3616–3629.
- M.A. Hawk, Z.T. Schafer, Mechanisms of redox metabolism and cancer cell survival during extracellular matrix detachment, *J. Biol. Chem.* 293 (2018) 7531–7537.
- Z.T. Schafer, A.R. Grassian, L. Song, Z. Jiang, Z. Gerhart-Hines, H.Y. Irie, S. Gao, P. Puigserver, J.S. Brugge, Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment, *Nature* 461 (2009) 109–113.
- S. Kamarajugadda, L. Stemborski, Q. Cai, N.E. Simpson, S. Nayak, M. Tan, J. Lu, Glucose oxidation modulates anoikis and tumor metastasis, *Mol. Cell Biol.* 32 (2012) 1893–1907.
- M. Idelchik, U. Begley, T.J. Begley, J.A. Melendez, Mitochondrial ROS control of cancer, *Semin. Canc. Biol.* 47 (2017) 57–66.
- V. Catalano, A. Turdo, S. Di Franco, F. Dieli, M. Todaro, G. Stassi, Tumor and its microenvironment: a synergistic interplay, *Semin. Canc. Biol.* 23 (2013) 522–532.
- S.R. Chowdhury, U. Ray, B.P. Chatterjee, S.S. Roy, Targeted apoptosis in ovarian cancer cells through mitochondrial dysfunction in response to Sambucus nigra agglutinin, *Cell Death Dis.* 8 (2017) e2762.
- C. Yang, W. Lim, F.W. Bazer, G. Song, Myricetin suppresses invasion and promotes cell death in human placental choriocarcinoma cells through induction of oxidative stress, *Cancer Lett.* 399 (2017) 10–19.
- A.V. Ivshina, J. George, O. Senko, B. Mow, T.C. Putti, J. Smeds, T. Lindahl, Y. Pawitan, P. Hall, H. Nordgren, J.E. Wong, E.T. Liu, J. Bergh, V.A. Kuznetsov, L.D. Miller, Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer, *Cancer Res.* 66 (2006) 10292–10301.
- K.G. Franchini, Focal adhesion kinase – the basis of local hypertrophic signaling domains, *J. Mol. Cell. Cardiol.* 52 (2012) 485–492.
- P. Paoli, E. Giannoni, P. Chiarugi, Anoikis molecular pathways and its role in cancer progression, *Biochim. Biophys. Acta* 1833 (2013) 3481–3498.
- J. Subramani, M. Ghosh, M.M. Rahman, L.A. Caromile, C. Gerber, K. Rezaul, D.K. Han, L.H. Shapiro, Tyrosine phosphorylation of CD13 regulates inflammatory cell-cell adhesion and monocyte trafficking, *J. Immunol.* 191 (2013) 3905–3912.
- H. Dai, Y.F. Lv, G.N. Yan, G. Meng, X. Zhang, Q.N. Guo, RanBP9/TSSC3 complex cooperates to suppress anoikis resistance and metastasis via inhibiting Src-mediated Akt signaling in osteosarcoma, *Cell Death Dis.* 7 (2016) e2572.
- B. Aslan, P. Monroig, M.C. Hsu, G.A. Pena, C. Rodriguez-Aguayo, V. Gonzalez-Villasana, R. Ruppaimoole, A.S. Nagaraja, S. Mangala, H.D. Han, E. Yuca, S.Y. Wu, C. Ivan, T.J. Moss, P.T. Ram, H. Wang, A. Gol-Chambers, O. Ozkayar, P. Kanlikilicer, E. Fuentes-Mattei, N. Kahraman, S. Pradeep, B. Ozpolat, S. Tucker, M.C. Hung, K. Baggerly, G. Bartholomeusz, G. Calin, A.K. Sood, G. Lopez-Berestein, The ZNF304-integrin axis protects against anoikis in cancer, *Nat. Commun.* 6 (2015) 7351.