Caveolin-1 Confers Resistance of Hepatoma Cells to Anoikis by Activating IGF-1 Pathway

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Key Words
Anoikis resistance • Caveolin-1 • Hepatocellular carcinoma • IGF-1 pathway

Abstract
Background/Aims: Anoikis resistance is a prerequisite for hepatocellular carcinoma (HCC) metastasis. The role of Caveolin-1 (CAV1) in anoikis resistance of HCC remains unclear. Methods: The oncogenic effect of CAV1 on anchor-independent growth and anoikis resistance was investigated by overexpression and knockdown of CAV1 in hepatoma cells. IGF-1 pathway and its downstream signals were detected by immunoblot analysis. Caveolae invagination and IGF-1R internalization was studied by electron microscopy and 125I-IGF1 internalization assay, respectively. The role of IGF-1R and tyrosine-14 residue (Y-14) of CAV1 was explored by deletion experiment and mutation experiment, respectively. The correlation of CAV1 and IGF-1R was further examined by immunochemical analysis in 120 HCC specimens. Results: CAV1 could promote anchor-independent growth and anoikis resistance in hepatoma cells. CAV1-overexpression increased the expression of IGF-1R and subsequently activated PI3K/Akt and RAF/MEK/ERK pathway, while CAV1 knockdown showed the opposite effect. The mechanism study revealed that CAV1 facilitated caveolae invagination and 125I-IGF1 internalization. IGF-1R deletion or Y-14 mutation reversed CAV1 mediated anchor-independent growth and anoikis resistance. In addition, CAV1 expression was positively related to IGF-1R expression in human HCC tissues. Conclusion: CAV1 confers resistance of hepatoma cells to anoikis by activating IGF-1 pathway, providing a potential therapeutic target for HCC metastasis.

W. Tang and X. Feng contributed equally to this work.
Introduction

Hepatocellular carcinoma (HCC) is the third most deadly type of cancer globally [1]. The high mortality rate of HCC is related mainly to late diagnosis at advanced stages, frequent tumor metastasis, and tumor recurrence after surgical resection [2]. Metastasis is the principal cause of tumor recurrence but promising therapy for HCC metastasis is not available. Thus, it is of crucial significance to unveil the molecular mechanisms underlying HCC metastasis.

Metastasis represents the last process of a multistep cell-biological process, including dissemination of cancer cells to anatomically distant organ and their subsequent adaptation to foreign tissue microenvironments [3]. As a barrier to metastasis, cells normally undergo an apoptotic process known as anoikis, a form of programmed cell death induced by loss of contact with the extracellular matrix or neighboring cells. Cancer cells acquire anoikis resistance to survive after detachment from the primary sites and travel through the circulatory and lymphatic systems to disseminate throughout the body. Anoikis resistance is a critical step in tumor metastasis [4].

Caveolin-1 (CAV1), a 21-24 kDa lipid raft-associated membrane protein and an essential constituent of caveolae, could play critical roles in several cellular processes including vesicular transport, cholesterol homeostasis and anoikis [5, 6]. CAV1 belongs to a highly conserved gene family named caveolins and is coexpressed with caveolin-2 in the cells and tissues of various origins including mesenchymal, endo/epithelial, and neuronal/glial [7]. Recently, substantial evidence has indicated that CAV1 is implicated in progression and metastasis of diverse human tumors such as meningiomas [8], bladder cancer [9], esophageal squamous cell carcinoma [10], mammary cancer [11], colon cancer [12] and osteosarcomas [13].

In HCC, CAV1 was reported to be related to vascular endothelial growth factor, microvessel density and unpaired artery [14]. Increased expression of CAV1 promoted carcinogenesis and metastasis of HCC in vitro and in vivo [15]. CAV1 could facilitate metastasis of HCC cells via Wnt/β-catenin pathway and induce epithelial–mesenchymal transition process [16]. However, the precise molecular mechanism of CAV1 in anoikis resistance of HCC has not been fully clarified. In the present study, we aimed to investigate the role of CAV1 in anoikis resistance of HCC and the underlying mechanism.

Materials and Methods

Ethics statement
Ethical approval for human subjects was obtained from the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from each patient.

Patients and tissue specimens
Tumor specimens were obtained from 120 consecutive patients with HCC who underwent curative resection at the Liver Cancer Institute, Zhongshan Hospital, Fudan University in 2009.

Antibodies
Primary antibodies against Caveolin-1 (D46G3, #3267), Akt (pan) (C67E7, #4691), phospho-Akt (Ser473) (D9E, #4060), Erk1/2 (137F5, #4695), phospho-Erk1/2 (D13.14.4E, #4370), Bcl-2 (50E3, #2870), Bcl-xl (54H6, #2764) and Mcl-1 (D35A5, #5453) were purchased from Cell Signaling Technology (Beverly, MA). Caveolin-1 (Y14) antibody antibody was produced by immunization of rabbits with recombinant Y14 peptide (SEGHIYVP1, residues 9–18) as described previously [17]. Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (0411, sc-47724) was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with HRP were from Jackson ImmunoResearch Laboratories (West Grove, PA).
Cell culture

Hep3B was obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MHCC97H was obtained from Liver Cancer Institute of Zhongshan Hospital, Fudan University. Hep3B was cultured in Eagle’s Minimal Essential Medium supplemented with 10% fetal bovine serum. MHCC97H was routinely maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. Cells were maintained at 37°C in a humidified incubator under 5% CO₂. All the cell lines have been authenticated by short tandem repeat (STR) profiling analysis in 2014.

Lentiviral production and establishment of stable CAV1 overexpression and knockdown clones

Lentiviral vectors containing CAV1 and pCDH-CMV-MCS-EF1-Puro vector control vector were obtained from System Biosciences (Mountain View, California, USA). Lentiviral vectors containing CAV1 shRNA (TRCN0000088800 and TRCN0000088902) and pLKO.1-puro empty control vector were obtained from Sigma Aldrich (St Louis, MO, USA). The plasmids were packaged into VSV-G pseudotyped lentiviral particles following transfection of 293T cells using the pPACKH1 packaging plasmid mix (System Biosciences, Mountain View, California, USA) according to the manufacturer’s instructions. For lentiviral transduction of target cells, lentiviral-containing supernatants were collected from packaging cells at 48 hours after transfection and added to subconfluent cultures of cells with 8 μg/ml polybrene for 24 hours. Forty-eight hours after infection, infected cells were enriched by selection with puromycin (1 μg/mL for Hep3B and 2 μg/mL for MHCC97H) for five days. Confirmation of overexpression and knockdown was performed by Western blot analysis.

Western blot, colony formation and apoptosis assay

Western blot, soft agar colony formation and apoptosis assay were performed as described previously [18, 19].

Anoikis assay

Cell culture dishes were coated with a film of poly (2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma Aldrich) as described previously [20]. Briefly, a 12% solution of poly-HEMA in 95% ethanol was mixed overnight, centrifuged at 2,500 rpm to remove undissolved particles, and diluted 1:10 with 95% ethanol. 100-mm dishes were coated with 4 ml of poly-HEMA solution and left to dry at room temperature. Dishes were washed twice with PBS and once with HBSS before use. Then the cells were cultured for 48 hours before in vitro assays.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed by using in situ cell death detection kit (Roche Applied Science, Indianapolis, IN, USA) according to manufacturer’s instructions.

Caspase-3 and caspase-9 activity assay

The cells were assayed for caspase-3 and caspase-9 activity using the Apo-ONE homogeneous caspase-3/7 assay kit and Caspase-Glo 9 assay kit (Promega, WI, USA) according to the manufacturer’s instructions.

Transmission electron microscopy

Electron microscopy was conducted as described previously [21, 22]. Cells were immediately fixed with 2.5% glutaraldehyde with 0.1 mol/L sodium cacodylate and stored at 4°C until embedding. Samples were postfixed with 1% osmium tetroxide, followed by dehydration with an increasing concentration gradient of ethanol and propylene oxide. Samples were then embedded and ultrathin (50-60 nm) sections were cut using an ultramicrotome. Images were examined using a JEM-1200 electron microscope at 80 kV after the samples were stained with 3% uranyl acetate and lead citrate.

125I IGF1 internalization assay

125I IGF1 internalization assay was conducted as previously described [23]. Cells (2×10⁵) plated on 40-mm gelatinized plates were incubated with Krebs-Ringer phosphate HEPES binding buffer (KRPBB), pH
7.5, containing 5.2 mM KCl, 1.4 mM CaCl₂, 128 mM NaCl, 30 mM HEPES, 10 mM Na₂HPO₄, 1.4 mM MgSO₄, and 1% bovine serum albumin for 1 h at 37°C. KRPHBB was replaced with KRPHBB (pH 7.5) containing 1:1000 dilution of ¹²⁵I IGF1 (IM172, Amersham Lifesciences). Cells were incubated at 37°C with ¹²⁵I IGF1 for 0 to 120 min, rinsed, and washed in KRPHBB (pH 3.5) for 10 min at 37°C to remove surface-bound IGF1. This supernatant was analyzed for surface-bound IGF1 by γ-counting. Cells were rinsed, and then solubilized with 0.4 N NaOH for 30 min on ice. Cell lysates were analyzed by scintillation counting to determine internalized IGF1. All values were standardized against protein content. The internalization rate constant Kᵣ represented by the slope of the line correlating the internalized to surface bound IGF, was calculated by linear regression [24, 25].

Site-directed mutagenesis and cell transfection
Transfection assays were performed using Lipofectamine LTX and Plus Reagent (Invitrogen, USA) according to the manufacturer’s protocol. Short interfering RNAs (siRNA) target sequence to human IGF-1R (GenBank: NM_000875) (si-IGF-1R, 5’- GCC GAT GTG TGA GA AGC -3’) were synthesized by GenePharma (Shanghai, China). Site-directed mutagenesis was performed to construct pCMV6-CAV1/Y14F by the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The oligonucleotide primers were as follows: 5´-G GACATCTCTCTTTCACCACTGTTCCC-3´ and 5´-GGGAACAGTGAAAGACCAAAGAGCAGGTCCTC-3´. The sequences of the mutation construct were verified by DNA sequencing analysis.

Immunohistochemistry and immunohistochemical staining
Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series diluted in distilled water. Following antigen retrieval with a 10 mM citrate buffer, HCC sections were incubated overnight at 4°C with primary antibody. Following 30 min incubation with secondary antibody, sections were developed in 3, 3'-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin.

The sections were observed under a light microscopy, for a histological review, to examine tumor microheterogeneity in antigen distribution. Five randomized microscopic views of 400-fold magnification of each section were observed and scored. A semi-quantitative scoring system was based on both the staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and the percentage of positive cells (0, 0% positive cells; 1, 1-10% positive cells; 2, 11-50% positive cells; 3, >50% positive cells). The final score of each sample was obtained by multiplying the scores of staining intensity and percentage of positive cells. Samples were classified as negative when the final scores were 0 to 3 and positive when 4 to 9 [18]. The evaluation of staining was carried out by two independent pathologists.

Statistical analysis
GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA) software was used for statistical analyses. All data are presented as mean ± standard deviation (SD) from three independent experiments. Continuous variables were compared using the Student’s t-test. All tests were two-tailed and a P value < 0.05 was considered statistically significant. The association between the expression of CAV1 and IGF-1R was determined by Pearson correlation analysis.

Results
CAV1 promoted anchorage independent growth and anoikis resistance in hepatoma cells
Our previous study showed that CAV1 was overexpressed in MHCC97H (an HCC cell line with high metastatic potential) but not in Hep3B (a non-metastatic HCC cell line) [16, 26]. In this study, to further examine the function of CAV1 in HCC development, we established one stable CAV1-expression clone in Hep3B cells and two stable CAV1-knockdown clones in MHCC97H cells. Enhanced CAV1 expression (> 2-fold) in Hep3B and suppressed CAV1 expression (> 0.85-fold) in MHCC97H were confirmed by Western blot analysis (Fig. 1A).

Anchorage-independence is an essential tumorigenic property of cancers. Here, CAV1-overexpression significantly promoted anchorage-independent growth of hepatoma cells, whereas CAV1-knockdown inhibited growth of hepatoma cells (Fig. 1B). Anoikis resistance
is a prerequisite for acquisition of anchorage-independent growth in malignant cells [27], we thus proposed that the difference of anchorage-independent growth between groups
might be attributable to CAV1-induced anoikis resistance. To test this hypothesis, we examined the effect of CAV1 on cell suspension conditions. Hepatoma cells were plated on non-adhesive poly-HEMA for 72 h. CAV1-overexpression showed marked anoikis resistance, while its knockdown promoted apoptosis as detected by Annexin V staining (Fig. 1C and Fig. 1D). Similarly, CAV1-overexpression cells were found to undergo less anoikis than the control, whereas CAV1-knockdown cells showed more apoptosis compared with the control as indicated by TUNEL assay (Fig. 1E and Fig. 1F). In accordance with this phenomenon, caspase-3 and caspase-9 activity was decreased in CAV1-overexpression cells and increased in CAV1-knockdown cells (Fig. 2A and Fig. 2B).

**CAV1 promoted anchorage-independent growth and anoikis resistance by activating IGF-1 receptor pathway**

The above results indicated that CAV1 promoted anchorage-independent growth and anoikis resistance in hepatoma cells. Since CAV1 has been reported to interact directly with insulin-like growth factor-1 receptor (IGF-1R) [28], we examined the expression of IGF1R and its two major downstream signaling pathways: the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the RAF/MEK/ERK pathway which were closely related to anoikis resistance [29]. The result of Western blotting showed that the expression of IGF-1R, phosphor-Akt (Ser473) and phosphor-ERK1/2 was remarkably increased in CAV1-overexpression cells versus the control (Fig. 3A and 3C). In addition, we detected the key molecules of apoptosis
signal pathway and found that the expression of antiapoptotic Bcl-2 and Mcl-1 was obviously decreased in CAV1-overexpression comparing to the control. However, there was no difference of antiapoptotic Bcl-xl expression between groups (Fig. 3B and 3D).

CAV1 promoted caveolae invagination and IGF-1R internalization in hepatoma cells
To further explore the reason for CAV1-induced IGF-1R activation, we detected the effect of CAV1 on caveolae internalization. The result of electron microscopy showed that CAV1-overexpression increased the number of invaginated caveolae while CAV1-knockdown

Fig. 3. CAV1 regulated anchorage independence and anoikis resistance by activating IGF-1R pathway in vitro. Hep3B and MHCC97H cells were cultured in a detached condition induced by poly-HEMA. (A) Expression of IGF-1R, phosphor-Akt (Ser473) and phosphor-ERK1/2 examined by Western blot. (B) Expression of apoptosis signal pathway molecules examined by Western blot. (C) Quantification of IGF-1R, phosphor-Akt (Ser473) and phosphor-ERK1/2 expression. (D) Quantification of Bcl-2, Bcl-xl and Mcl-1 expression.
produced the opposite effect (Fig. 4A-B). Then we detected surface IGF-1 binding and IGF-1 internalization by radiolabeled $^{125}$I-IGF-1. CAV1-overexpression significantly reduced the surface $^{125}$I-IGF-1 binding and increased the internalization of IGF-1R compared to the control (Fig. 4C). However, CAV1-knockdown showed the opposite effect (Fig. 4D). The ratio of internalized to surface bound $^{125}$I-IGF-1 was larger in CAV1-overexpression cells than the control, while less in CAV1-knockdown cells than the control (Fig. 4E-F). These data indicated CAV1 could promote caveolae internalization and $^{125}$I-IGF-1R internalization, thus activating IGF-1R pathway.

**Fig. 4.** CAV1 increased numbers of caveolae and promoted internalization of $^{125}$I-IGF1 in hepatoma cells. Hep3B cells were stably transfected with control vector and CAV1 plasmid (CAV1). MHCC97H cells were stably transfected with control shRNA and CAV1 shRNA (shRNA1). (A) Representative electron microscopy images of cells in each group. Arrow points, caveolae. (B) Quantification of caveolae in each group. (C) Surface bound and internalized $^{125}$I-IGF1 were measured and compared in CAV1-overexpression and the control cells. (D) Surface bound and internalized $^{125}$I-IGF1 were measured and compared in CAV1-knockdown and the control cells. (E) Ratio of internalized to surface bound $^{125}$I-IGF1 in CAV1-overexpression and the control cells at the indicated times. (F) Ratio of internalized to surface bound $^{125}$I-IGF1 in CAV1-knockdown and the control cells at the indicated times. $P$ values at 120 min were determined by Student’s t test.
CAV1 mediated anchorage-independent growth, anoikis resistance and caspases inhibition in hepatoma cells through the function of its tyrosine-14 residue and IGF-1R

Since tyrosine-14 (Y14) residue was a crucial phosphorylation site required for CAV1 functions [30, 31], we conducted a caveolin-1 mutant containing tyrosine 14 substituted with phenylalanine (CAV1/Y14F). In addition, we used Y14 antibody to examine the role of Y14 residue on CAV1-mediated oncogenic behaviors. Moreover, we used IGF-1R siRNA to study the role of IGF-1R on CAV1-mediated tumor-promoting effect. The result showed that increased anchorage-independent growth caused by CAV1-overexpression was reversed by Y14 mutation, Y14 antibody and IGF-1R interference (Fig. 5A and 5B). Similarly, suppressed anoikis and decreased caspase-3/9 activity caused by CAV1-overexpression was rescued by Y14 mutation, Y14 antibody and IGF-1R interference (Fig. 5C and 5D, Fig. 6A and 6B).

CAV1 expression was positively related to IGF-1R expression in human HCC tissues

To further explore the correlation of CAV1 with IGF-1R, we performed immunohistochemistry staining in 120 paired formalin-fixed paraffin-embedded HCC tissue samples. The result showed that CAV1 and IGF-1R was mainly located in cell membrane and cytoplasm (Fig. 7A). Survival analysis showed that CAV1 expression in tumor tissue was
positively correlated to shorter time to progression (TTP) \( P = 0.008 \) (Fig. 7B). Evaluation of immunohistochemistry staining and correlation analysis indicated that CAV1 expression level was positively related to IGF-1R expression in HCC tissues \( (R = 0.5403, P = 0.0003) \) (Fig. 7C).

**Discussion**

Anoikis, defined as detachment-induced apoptosis, is an essential characteristic of most normal epithelial and mesenchymal cells for extracellular matrix-derived signals of survival. The acquisition of anoikis-resistance is therefore regarded as a critical step during the metastatic transformation of a tumor [32, 33]. CAV1 was reported to correlate with anoikis in a variety of cancers. CAV1 expression decreased capacities of anchorage-independent growth and anoikis in MCF-7 breast cancer cells [34]. CAV1 mediated anoikis resistance through regulating Mcl-1 interaction and stabilization in lung cancer cells [35]. However, to date it has remained unclear whether CAV1 inhibited or promoted anoikis resistance in hepatoma cells. In the present study, for the first time, we explored the role of CAV1 in anoikis of hepatoma cells and underlying mechanisms. We observed that CAV1 over-

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**Fig. 6.** CAV1 inhibited detachment-induced caspase activation through tyrosine-14 residue (Y14) and IGF-1R. (A) Y14 mutation or block and IGF-1R interference abolished CAV1-induced caspase-3 inhibition. (B) Y14 mutation or block and IGF-1R interference abolished CAV1-induced caspase-9 inhibition.

**Fig. 7.** CAV1 expression was positively related to IGF-1R expression in human HCC tissues. (A) Representative immunohistochemistry staining images of CAV1 and IGF-1R in human HCC tissues \( (n = 120) \). (B) Survival analysis of CAV1 expression in tumor tissues of 120 HCC patients. (C) Correlation analysis of CAV1 and IGF-1R expression in tumor tissues.
expression could significantly protect hepatoma cells from anoikis induced by detachment and promote anchorage-independent growth. CAV1 overexpression also decreased the activity of caspases-3 and 9 during anoikis. As expected, CAV1 knockdown showed the opposite effect in vitro. Our findings identified CAV1 as a critical player in HCC metastasis, which was in accordance with our previous study [16].

IGF-1R signaling frequently mediated anoikis resistance in diverse malignant cells [36, 37]. As two major downstream signaling pathways of IGF-1R signaling, phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the RAF/MEK/ERK pathway was closely correlated with anchorage-independent growth and anoikis resistance caused by CAV1 overexpression.

Internalization is a mechanism by which receptor tyrosine kinases (RTKs) leave the plasma membrane, traveling inside the cell to activate specific signaling. The fine turning of these processes can be altered in cancer cells [39]. Regulated alternation in caveolae number and dynamics has been reported to correlate closely with the internalization of a variety of RTKs like epithelial Growth Factor and vascular endothelial growth factor [40, 41]. CAV1 is essential for the formation and stability of caveolae, which is directly involved in the internalization of RTKs [42]. Our results demonstrated that CAV1 could increase the quantity of caveolae on the invagination and facilitated IGF-1R internalization. These findings explained how CAV1 activate IGF-1R signaling pathway in hepatoma cells. Tyrosine-14 (Y14) residue of CAV1, a target site for phosphorylation by the non-receptor tyrosine kinases, played important roles in a variety of CAV1-mediated physiological and pathologic processes [43]. EGF-stimulated Y14 phosphorylation promoted caveolae formation in epithelial cells [44]. Y14 phosphorylation following LPS exposure induced the interaction between CAV1 and TLR4, thus provoking TLR4-MyD88 signaling and sepsis-induced lung inflammation in endothelial cells [45]. Y14 phosphorylation also sensitized breast cancer cells to paclitaxel through inactivating Bcl-2 and Bcl-xL [46]. Based on these studies, we supposed that Y14 site may play an important role in the anoikis resistance mediated by CAV1. In the present study, we performed rescue assay by site-directed mutagenesis or blocking Y14 with specific antibody. The data showed that anoikis resistance caused by CAV1 could be remarkably reversed by Y14 interference in hepatoma cells. The finding highlighted benefit of Y14 interference in antagonizing CAV-mediated the oncogenic behaviors and provided a new potential treatment strategy for HCC.

CAV1 has aroused great interest and has been extensively studied in cancer development and progression. With respect to HCC, CAV1 was upregulated in HCC cell lines, especially in highly invasive HCC cell lines. CAV1 overexpression has been shown to correlate with the incidence of metastasis and poor prognosis in HCC patients [14, 15, 47]. CAV1 increased cellular sensitivity to resveratrol through enhancing its internalization and trafficking in HepG2 hepatoma cells [48]. CAV1 upregulated the expression of Twist, a well-known EMT inducer of HCC, thus promoting the epithelial–mesenchymal transition of HCC cells [49]. Our previous study revealed that CAV1 could facilitate carcinogenesis and metastasis of HCC via activating Wnt/β-catenin pathway [16]. In the present study, we put forward a new concept that CAV1 could influence HCC metastasis by suppressing anoikis, which could offer new clues to understand the role of CAV1 in HCC.

In conclusion, these results suggest that CAV1 confer resistance of hepatoma cells to anoikis by activating IGF-1 pathway, via facilitating caveolae invagination and IGF-1R internalization. In our study, Y14 antibody was identified as an efficient inhibitor for CAV1-
induced anoikis resistance and anchorage-independent growth, which opens a new avenue for personalized therapeutic intervention of hepatoma metastasis in HCC patients with CAV1-overexpression.

**Abbreviations**

CAV1 (caveolin-1); CPM (counts per minute); HBBS (Hepes-buffered balanced salt solution); DAPI (DNA-binding AT-specific fluorochrome 4′-6-diamidino-2-phenylindole); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); HCC (hepatocellular carcinoma); IGF-1R (insulin-like growth factor-1 receptor); min (minute); PBS (phosphate buffered saline); PI (propidium iodide); SD (standard deviation); shRNA (small hairpin RNA); TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling); PI3K (phosphoinositide 3-kinase); poly-HEMA (poly (2-hydroxyethyl methacrylate)).

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**Disclosure Statement**

No potential conflicts of interest were disclosed.

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