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Tetraspanin 8 (TSPAN8) mediates AEG-1-induced invasion and metastasis in hepatocellular carcinoma (HCC) cells

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Abstract

Astrocyte elevated gene-1 (AEG-1) positively regulates tumor progression and metastasis. Here we document that AEG-1 upregulates transcription of the membrane protein tetraspanin8 (TSPAN8). Knocking down TSPAN8 in AEG-1-overexpressing human hepatocellular carcinoma (HCC) cells markedly inhibited invasion and migration without affecting proliferation. TSPAN8 knockdown profoundly abrogated AEG-1-induced primary tumor and intrahepatic metastasis in an orthopic xenograft model in athymic nude mice. Co-culture of TSPAN8 knockdown cells with human umbilical vein endothelial cells (HUVEC) markedly inhibited HUVEC tube formation indicating that inhibition of angiogenesis might cause reduction in primary tumor size. TSPAN8 inhibition might be a potential therapeutic strategy for metastatic HCC.

Keywords

AEG-1; TSPAN8; invasion; metastasis; angiogenesis

1. Introduction

Hepatocellular carcinoma (HCC), arising from hepatocytes, accounts for ~80% of liver malignancies. HCC is usually asymptomatic and nearly all cases are diagnosed at an advanced, metastatic stage by which conventional therapeutic approaches are ineffective [1]. Identification of molecules mediating HCC metastasis is mandatory to develop targeted and effective therapeutic strategy.

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Conflict of interest

All authors declare that no conflict of interest exists.

Author contribution:

MAA: designed, performed, analyzed the experiments and wrote the paper. PKS, RGM, AS: performed experiments and analyzed data. PBF: wrote the paper. DS: conceived and coordinated the study, performed experiments, analyzed data and wrote the paper.

Astrocyte elevated gene-1 (AEG-1) is an oncogene that is overexpressed in all cancers studied so far, especially in the advanced stages [2]. AEG-1 is also known as metadherin (MTDH) since it was identified to regulate lung metastasis of breast cancer cells [3]. In HCC, AEG-1 gene is amplified and AEG-1 overexpression is detected in >90% cases [4]. Overexpression of AEG-1 in human HCC cells strongly promotes proliferation, invasion, migration, angiogenesis, chemoresistance and *in vivo* metastasis and knockdown of AEG-1 reverses these phenotypes [4-6]. AEG-1 positively regulates several pro-tumorigenic signaling pathways, such as PI3K/Akt, MEK/ERK, Wnt/β-catenin and NF-κB, interacts with a variety of proteins and profoundly modulates global gene expression to exert its oncogenic function [4-8]. AEG-1 is a scaffold protein that may not be amenable to small molecule mediated inhibition. As such understanding the molecular mechanism by which AEG-1 promotes invasion and metastasis will facilitate development of novel strategies to counteract advanced, metastatic HCC.

TSPAN8 is a member of a family of tetraspanins that cross the plasma membrane four times. Tetraspanins participate in a wide range of physiological phenomena within the context of cell membrane and cell-cell contact such as fertilization and synaptic contacts at neuromuscular junctions [9]. TSPAN8 is overexpressed in a variety of cancers and promotes metastasis [9]. However, the role of TSPAN8 in HCC has not been adequately explored. Overexpression of TSPAN8 showed correlation with poorly differentiated HCC [10]. Additionally, TSPAN8 was observed to be overexpressed in metastatic liver tumors compared to non-metastatic tumors [11]. In the present manuscript we describe an important role of TSPAN8 in mediating AEG-1-induced invasion and metastasis thus suggesting TSPAN8 inhibition as a potential strategy to counteract metastatic HCC.

2. Materials and Methods

2.1. Cell lines, cell proliferation and colony formation assays

Hep-AEG-1-8, stable AEG-1 overexpressing clone, and Hep-AEG-1si, stable AEG-1 shRNA expressing clone, in human HCC cell HepG3 were generated and characterized previously [4, 8]. QGY-7703 and HEK-293 cells were cultured as described [7]. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and maintained in endothelial cell growth medium-2 (EGM-2). Immortal primary human hepatocytes (Hc3716-hTERT) is a kind gift from Dr. Tahara and were cultured as described [12]. Cell proliferation was measured by standard MTT assay as described [4]. For colony formation assay, cells (1×10^3) were cultured for two weeks, fixed with formaldehyde and stained with 10% giemsa [4]. Colonies of more than 50 cells were counted.

2.2. Cloning of TSPAN8 promoter and luciferase assay

Human male genomic DNA (20 μg; Promega) was digested with *Xho*I, purified by phenol:chloform extraction and used as template to clone ~2 kb of human TSPAN8 promoter by PCR. The following primers were used: TSPAN8F**:** 5′-GCTAGCGCTAAGGCAGAGAGGAAC-3′ and TSPAN8R: 5′-CTCGAGGCTTGTCATAGCTCCTGG-3′. The promoter was cloned into the *Nhe*I and *Xho*I sites of the promoterless pGL4.10[luc2] luciferase reporter vector (Promega) to

generate TSPAN8-luc construct. Luciferase reporter plasmids were transfected into the cells as described [7] and luciferase assays were measured using Dual Luciferase Reporter Assay kit (Promega) following the manufacturer's protocol. Firefly luciferase activity was normalized by protein concentration and by the activity of the empty vector. Pharmacologic inhibitors of ERK (U0126; 10 μM), PI3K/Akt (LY294002; 10 μM) or p38 MAPK (SB203580; 2 μM) were used as described [7].

2.3. Construction of TSPAN8 knockdown stable clones

Plasmids expressing short hairpin RNA (shRNA) for TSPAN8 and control, scrambled shRNA were obtained from Santa Cruz Biotechnology, Inc. and transfected into Hep-AEG-1-8 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Stable clones were generated by selection in 10 μg/mL of puromycin for two weeks. Single clones were isolated and cultured in maintenance medium containing 5 μg/mL of puromycin.

2.4. Preparation of whole cell lysates and Western blot analysis

Whole cell lysates were prepared using a lysis buffer from Cell Signaling containing phosphatase and protease inhibitors (Roche) [6]. Western blotting was performed using 30 μg of protein as described [4]. The primary antibodies used were: AEG-1 (chicken; 1:500; in-house), TSPAN8 (rabbit; 1:1000; Sigma-Aldrich), GAPDH, E-cadherin, N-cadherin, Snail, Slug and Vimentin (rabbit; 1:1000; Cell Signaling) and EF1α (mouse; 1:1000; Millipore).

2.5. RNA extraction and Real time PCR

Total RNA was extracted from 5×10^5 cells using QIAGEN miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Two μg of RNA was used for cDNA synthesis using high capacity cDNA reverse transcription kit from Applied Biosystems according to the manufacturer's protocol. Quantitative real-time PCR (Q-RT-PCR) was performed using Applied Biosystems ViiA7 Fast Real-Time PCR System and TaqMan Gene Expression Assays for TSPAN8 (predesigned best coverage probe) according to the manufacturer's protocol.

2.6. Matrigel Invasion assay

Invasion assay was performed using 24-well BioCoat cell culture inserts (BD) with an 8 μ-porosity polyethylene terephthalate membrane coated with Matrigel Basement Membrane Matrix (100 $\mu g/cm^2$; BD Biosciences) according to the manufacturer's protocol [4].

2.7. Wound healing (scratch) assay

Cells (5×10^4) were plated on 6-well plates and allowed to grow till confluence. Confluent cultures were scratched with a pipet tip and the wound distance was recorded at 0 hours and 72 hours using a bright-field microscope [4].

2.8. F-actin staining

Cells (1×10^4) were plated on 4 chamber slides and allowed to grow till confluence. Confluent cultures were scratched and after 72 hours cells were fixed and stained with Rhodamine phalloidin (Invitrogen). The images were analyzed using a Zeiss confocal laser-scanning microscope.

2.9. HUVEC tube formation assay

HUVECs (4×10^4) were co-cultured with 1×10^3 Hep-AEG-1-8 and TSPAN8 knockdown clones for 6 hours on Cultrex basement membrane extract (R&D Systems). Tube formation was analyzed using a bright-field microscope [5].

2.10. Orthotopic xenograft in nude mice

Cells were orthotopically implanted by intrahepatic injection in athymic nude mice (6-8 weeks of age) [13]. The mouse was placed into a plexiglass chamber for induction of anesthesia with 2% isoflurane and 2 liters/min oxygen flow for a mouse of 25 g body weight. After anesthetization, the animal was transferred onto a Styrofoam pad and anesthesia was maintained by isoflurane inhalation through a suitable mouthpiece. The mouse was placed in the left lateral decubitus position and the skin was disinfected with betadine/ethanol scrub. A small skin and muscle incision (about 0.5-1 cm long) was made longitudinally (parallel to the spine) in the right flank to expose the liver. The liver was retracted and using a 30G needle 1×10^6 cells in 0.1 mL PBS was injected into the parenchyma of the left lobe of the liver. A visible pale wheal indicated a successful injection. The needle was retracted and a Q-tip was placed over the injection site for 30 seconds to prevent bleeding and spillage of material. The liver was returned to the peritoneal cavity. The peritoneum was closed with a 5-0 suture and the skin was closed by using wound clips. After closing the abdomen the skin was wiped surrounding the suture with betadine and the animal was placed on a warming pad for recovery. The animals were monitored by measuring body weight and observing posture, feeding and grooming behavior. The animals were sacrificed eight weeks after the implantation.

2.11. Statistical Analysis

Data were presented as the mean ± SD and analyzed for statistical significance using two-tailed student t-test.

3. Results

3.1. AEG-1 increases transcription of TSPAN8

We previously established stable AEG-1-overexpressing clones (such as Hep-AEG-1-8) or AEG-1 knockdown clones (Hep-AEG-1-si) in HepG3 cells and characterized them in detail [4, 7, 8]. Global gene expression analysis by Affymetrix microarray identified significant increase in TSPAN8 mRNA in AEG-1-overexpressing clones compared to control clone (Hep-PC-4) [4]. To confirm the microarray findings we checked TSPAN8 protein and mRNA levels in Hep-PC-4, Hep-AEG-1-8 and Hep-AEG-1si cells. We observed a significant increase in Hep-AEG-1-8 cells and a significant decrease in Hep-AEG-1si cells

in TSPAN8 levels when compared to Hep-PC-4 cells (Fig. 1A & B). Similarly, AEG-1 and TSPAN8 levels were substantially more in human HCC cell line QGY-7703 when compared to immortal primary human hepatocytes (Hc3716-hTERT). (Fig. 1C)

To check whether AEG-1 regulates TSPAN8 transcription, we cloned ~2 Kb promoter region of human TSPAN8 gene upstream of the transcription start site and constructed TSPAN8-luc luciferase reporter plasmid. TSPAN8-luc activity was significantly more in Hep-AEG-1-8 cells *versus* Hep-PC-4 and Hep-AEG-1si cells (Fig. 1D). QGY-7703 is a highly aggressive HCC cell expressing abundant AEG-1 [4]. Transient knockdown of AEG-1 by siRNA in QGY-7703 cells markedly decreased TSPAN8-luc activity when compared to control siRNA (Fig. 1E). To further check transcriptional regulation of TSPAN8 by AEG-1, we co-transfected increasing doses of AEG-1 expression plasmid and TSPAN8-luc plasmid into HEK-293 cells. A dose-dependent increase in TSPAN8-luc activity was observed upon overexpression of AEG-1 (Fig. 1F). Overexpression of AEG-1 activates diverse signaling pathways, including PI3K-AKT and RAS-Raf-MAPK pathways [4, 5]. TSPAN8-luc activity was markedly inhibited upon treatment with a MEK/ERK inhibitor U0126 in both Hep-AEG-1-8 and HEK-293 cells. PI3K/Akt inhibitor LY294002 or p38 MAPK inhibitor SB203580 did not affect TSPAN8-luc activity in Hep-AEG-1-8 cells but showed small but significant inhibition in HEK-293 cells. These data indicate that activation of MEK/ERK signaling by AEG-1 is the major mechanism promoting transcription of TSPAN8 (Fig. 1G & H).

3.2. TSPAN8 mediates AEG-1-induced invasion, migration and metastasis

To analyze the functional consequence of TSPAN8 overexpression by AEG-1, we established stable clones expressing control scrambled shRNA (CON-si) or TSPAN8 shRNA (TS8-si) in Hep-AEG-1-8 cells. We identified five clones with significant downregulation of TSPAN8 (Fig. 2A & B) and selected Cl-9-TS8-si and Cl-10-TS8-si for subsequent experiments because these clones presented with the most efficient knockdown of TSPAN8. Knockdown of TSPAN8 did not affect cell proliferation analyzed by MTT and colony formation assays compared to parental Hep-AEG-1-8 and CON-si clones (Fig. 2C & D) indicating that TSPAN8 does not regulate proliferation in human HCC cells.

We next checked the effect of TSPAN8 knockdown on invasion and migration properties by Matrigel invasion and wound healing (scratch) assays, respectively. A marked reduction in invasion and a significant reduction in migration was observed in TSPAN8 knockdown clones *versus* Hep-AEG-1-8 and CON-si clones (Fig. 3A & B). Actin cytoskeleton plays an important role in cell migration. After establishing the scratch, the cells were stained for F-actin after 72 hours and the morphology of the wound edges was analyzed. Cells with serrated edges containing lamellipodia were observed in Hep-AEG-1-8 and CON-si clones (arrows in Fig. 3C) while Cl-9-TS8-si and Cl-10-TS8-si cells presented with blunted edges (Fig. 3C) indicating perturbation of acting cytoskeleton organization in TSPAN8 knockdown cells contributing to inhibition in migration and invasion. Epithelial-mesenchymal transition (EMT) plays an important role in regulating invasion and migration. Indeed, we observed increase in epithelial marker E-cadherin and decrease in mesenchymal marker N-cadherin in TSPAN8 knockdown clones when compared to control clone indicating that inhibition of

TSPAN8 might confer mesenchymal-epithelial transition (Fig. 3D). No change was observed in the levels of transcriptional regulators of EMT, such as Snail and Slug, and the intermediate filament protein Vimentin suggesting that TSPAN8 knockdown predominantly modulates membrane-associated molecules.

To check whether the inhibition in *in vitro* invasion and migration upon TSPAN8 knockdown confers inhibition in *in vivo* metastasis, we established orthotopic xenografts in athymic nude mice by injecting Hep-AEG-1-8, CON-si, Cl-9-TS8-si and Cl-10-TS8-si cells in the left lobe of the liver. The primary tumor at the injection site grew in size and multiple tumors in other lobes of the liver were observed in case of Hep-AEG-1-8 and CON-si cells indicating intrahepatic metastasis (Fig. 4A). For Cl-9-TS8-si and Cl-10-TS8-si cells a small tumor at the primary injection site was observed. However, no secondary tumor was detected in other lobes of the liver indicating that intrahepatic metastasis has not taken place in these cases (Fig. 4A). Liver weights in Hep-AEG-1-8- and CON-si-injected mice were significantly more than that in Cl-9-TS8-si- and Cl-10-TS8-si-injected mice indicating robust tumor loads in the former (Fig. 4B).

Although TSPAN8 knockdown did not modulate *in vitro* proliferation, the primary tumor was substantially smaller in TSPAN8 knockdown clones compared to controls. We hypothesized that inhibition of tumor angiogenesis upon TSPAN8 knockdown might result in smaller primary tumor size. We checked tube formation by human umbilical vein endothelial cells (HUVEC) co-cultured with Hep-AEG-1-8, CON-si, Cl-9-TS8-si and Cl-10-TS8-si cells. HUVEC tube formation was significantly abrogated when TSPAN8 was knocked down while robust tubes were observed in the controls suggesting that TSPAN8 facilitates neo-angiogenesis induced by HCC cells (Fig. 4C).

4. Discussion

Hepatocellular carcinoma (HCC) has a dismal prognosis with five year survival rate of 10.9% with regional metastasis and only 2.8% with distant metastasis [1]. Patients with early detection and localized disease might be treated with liver transplantation or radio-frequency ablation that might significantly prolong life and five year survival rates of up to 60 to 70% can be achieved in well-selected patients. However, no therapy exists for metastatic disease and the only FDA-approved drug for non-resectable advanced HCC, sorafenib, provides a survival benefit of only ~2.8 months compared to placebo [14]. Therapeutic modalities targeting the metastatic component of the disease thus has a profound clinical significance.

AEG-1 plays a pivotal role in regulating invasion and metastasis. It has been included in Mammaprint, the only FDA-approved 70 gene signature analysis to measure breast cancer metastasis risk. AEG-1 exerts its oncogenic function by interacting with a diverse array of proteins, such as NF-κB, SND1, RXR and Akt2, in different subcellular compartments employing distinct regions of the protein [15]. Multiple small molecules or peptidomimetics will be required to block these interactions to mitigate the oncogenic function of AEG-1. Antibodies directed against one epitope may not interfere with all these interactions and may not adequately neutralize intracellular AEG-1. As such identification of a key molecule

mediating AEG-1-induced metastasis might facilitate bypassing the complexity of inhibiting AEG-1 and developing strategies to target the molecule.

We now identify that TSPAN8 plays a key role in mediating AEG-1-induced invasion and metastasis. Tetraspanins form complexes termed tetraspanin-enriched microdomains (TEMs) by interacting with other tetraspanins and with a variety of transmembrane and cytosolic proteins that include integrins, EpCaM, CD44 and heterotrimeric G proteins to modulate migration, cell contact, cell fusion and related processes [16]. Among the tetraspanin family members TSPAN8 is strongly associated with tumor metastasis especially in the context of HCC. Knocking down TSPAN8 not only inhibits *in vitro* invasion and metastasis but also profoundly abrogates intrahepatic metastasis thereby documenting a key role of TSPAN8 in mediating AEG-1 function. Tetraspanins in general have been shown to mediate invasion through their ability to associate matrix metalloproteinases (MMPs) in the TEM that facilitates matrix degradation [9]. AEG-1 strongly induces MMPs, such as MMP2 and MMP9, which mediate its invasive properties [17]. TSPAN8 might play a critical role in organizing AEG-1-induced MMPs into functional complexes to efficiently execute matrix-degradation activities.

Overexpression of TSPAN8 resulted in increased cell proliferation in gastric cancer and glioblastoma cells [18, 19]. We did not observe any effect on cell proliferation upon TSPAN8 knockdown. However, we observed substantial inhibition in primary tumor growth *in vivo* which could be attributed to inhibition of angiogenesis following knockdown of TSPAN8. AEG-1 potently induces angiogenesis and elucidation of a role of TSPAN8 in regulating AEG-1-induced angiogenesis further provides support to the importance of this molecule in regulating AEG-1 function [20]. TSPAN8-derived exosomes induce upregulation of pro-angiogenic factors in rat models and activation of endothelia cells [21] Although endothelia cell activation was not affected when we used conditioned media from TSPAN8 knockdown and parental cells (data not shown), co-culture of TSPAN8 knockdown cells with HUVECs markedly affected tube formation suggesting that TSPAN8 might mediate interaction between tumor cells and the endothelial cells. This cell-cell interaction hypothesis is consistent with the reported functions of TSPAN8 in development since knockdown of TSPAN8 (Tm4sf3) affected fusion of dorsal and ventral buds in *Xenopus laevis* [22].

Our studies document that AEG-1 regulates TSPAN8 transcription by activating MEK/ERK signaling pathway. AEG-1 strongly activates MEK/ERK signaling that plays a key role in regulating proliferation and invasion of human HCC cells [4]. It remains to be identified which transcription factors confer AEG-1-induced transcriptional regulation of TSPAN8. Analysis of TSPAN8 promoter identifies potential binding sites for NF-κB, a transcription factor that requires AEG-1 for its activation and function [6, 23]. Further experiments using deletion mutants of TSPAN8 promoter and AEG-1 will identify the molecular mechanism by which AEG-1 regulates TSPAN8 transcription.

Cell surface expression of TSPAN8 makes it an ideal candidate for targeting via antibody. A mouse monoclonal antibody has been generated that recognizes amino acids 140-205 of human TSPAN8 and efficiently inhibits invasion by colorectal cancer cells [24]. Another

monoclonal antibody mAb Ts29.2 recognizing human TSPAN8 significantly inhibited colorectal cancer xenografts in nude mice by decreasing mitotic index, hence cell proliferation, suggesting that the binding of the antibody might modulate response of the cancer cells to the microenvironment [25]. These findings in colorectal cancer and our orthotopic xenograft studies collectively suggest that TSPAN8 monoclonal antibody might be a potential anti-HCC strategy. In-depth *in vivo* studies need to be carried out to translate TSPAN8 inhibitory strategies for therapeutic application.

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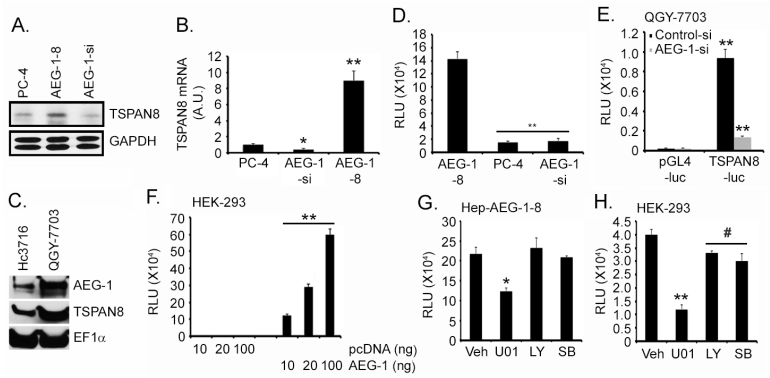

Figure 1.

AEG-1 increases transcription of TSPAN8. A. TSPAN8 and GAPDH protein levels were analyzed by Western blotting in Hep-PC-4, Hep-AEG-1-8 and Hep-AEG-1si cells. B. TSPAN8 mRNA expression was analyzed in the indicated cells. C. AEG-1, TSPAN8 and EF1α levels were analyzed by Western blotting in Hc3716-hTERT and QGY-7703 cells. D. TSPAN8-luc reporter plasmid was transfected into the indicated cells and luciferase activity was measured. E. QGY-7703 cells were transfected with TSPAN8-luc plasmid along with control siRNA or AEG-1 siRNA and luciferase activity was measured. F. HEK-293 cells were transfected with TSPAN8-luc plasmid and indicated amounts of empty vector (pcDNA3.1) or AEG-1 expression plasmid and luciferase activity was measured. G-H. Hep-AEG-1-8 (F) and HEK-293 (G) cells were transfected with TSPAN8-luc plasmid and treated with U0126 (10 μM), LY294002 (10 μM) or SB203580 (2 μM) and luciferase activity was measured. For luciferase assays, activity was measured 48 h after transfection and luciferase activity was normalized by protein concentration and by activity of promoter-less pGL4.10[luc2] plasmid. Data represent mean ± SD of at least triplicate experiments. *: $p < 0.0002$; **: $p < 0.0004$; #: $p < 0.05$.

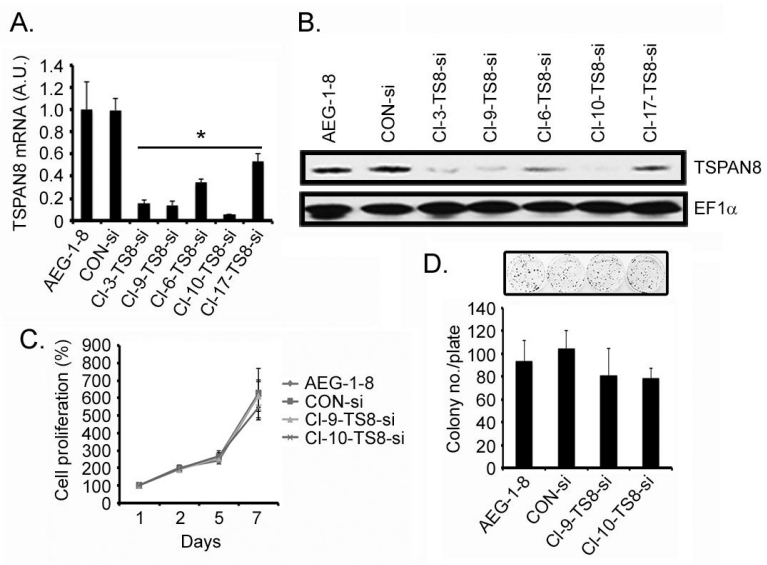

Figure 2.

Knockdown of TSPAN8 in Hep-AEG-1-8 cells does not affect proliferation.. TSPAN8 mRNA (A) and protein (B) levels were measured in parental Hep-AEG-1-8 cells and stable clones of Hep-AEG-1-8 cells expressing control scrambled shRNA (CON-si) or TSPAN8 shRNA (TS8-si). C. Cell proliferation of the indicated cells were measured by standard MTT assay. D. Colony formation by the indicated cells were measured after 2 weeks. Data represent mean ± SD of at least triplicate experiments. *: $p < 0.0002$.

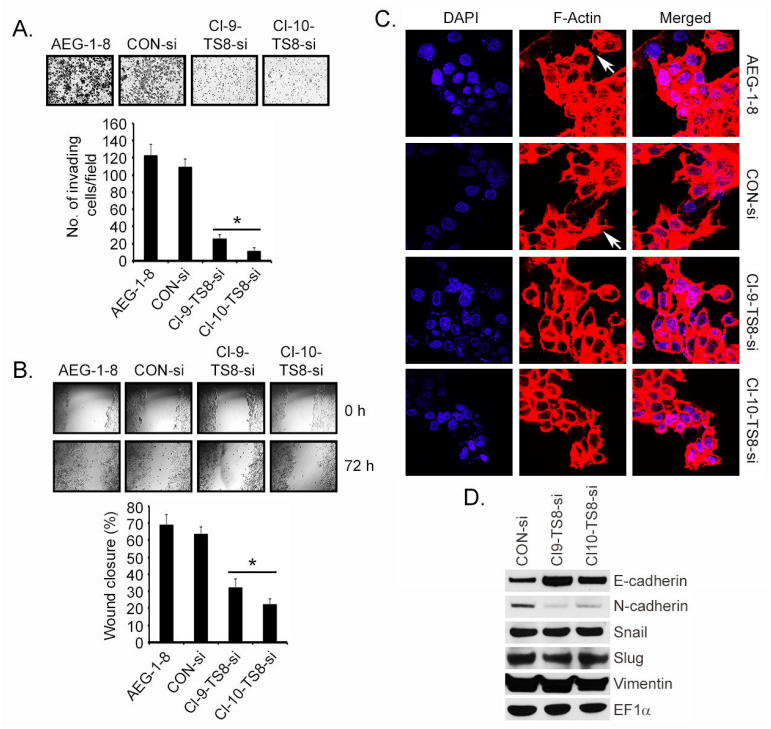

Figure 3.

Knockdown of TSPAN8 abrogates invasion and migration in Hep-AEG-1-8 cells. A. Matrigel invasion assay was performed in the indicated cells. Top, photomicrograph of the invaded cells. Bottom, graphical quantification of the invaded cells from 10 fields. B. Wound healing (scratch) assay was performed in the indicated cells. Top, photomicrograph of the wound at the time of scratch (0 h) and 72 h later. Bottom, graphical quantification of wound closure. Data represent mean ± SD. *: $p < 0.0002$. C. F-actin staining at the wound edge in the indicated cells 72 h after the wound. D. Western blot analysis for the indicated proteins in the indicated cells.

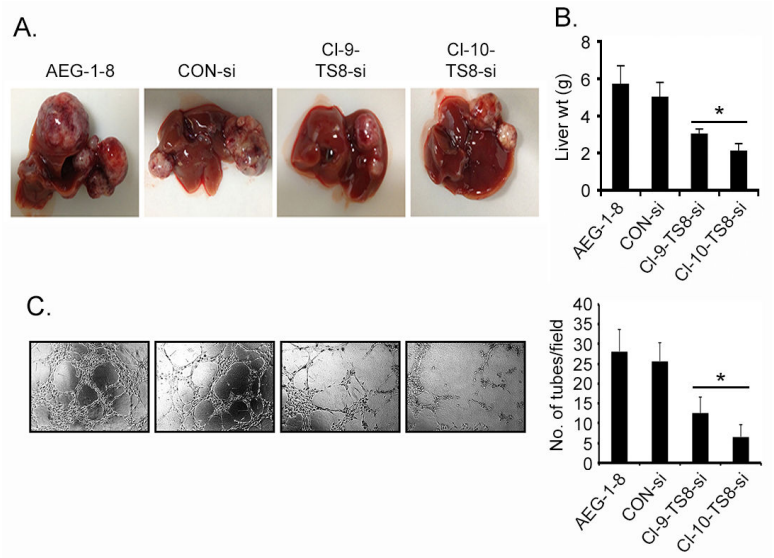

Figure 4.

TSPAN8 knockdown inhibits intrahepatic metastasis and angiogenesis. A. Photograph of the livers carrying orthotopic xenografts of the indicated cells. B. Liver weight at the end of the study (n = 5 per group). C. Tube formation assay was performed in human umbilical vein endothelial cells (HUVEC) co-cultured with the indicated cells. Left, photomicrograph of the tubes 6 h after co-culture. Right, Graphical quantification of the tubes. Data represent mean ± SD. *: $p < 0.0002$.