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Survivin: A novel marker and potential therapeutic target for human angiosarcoma

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Human angiosarcoma is a rare malignant vascular tumor associated with extremely poor clinical outcome and generally arising in skin of the head and neck region. However, little is known about the molecular pathogeneses and useful immunohistochemical markers of angiosarcoma. To investigate the mechanisms of angiosarcoma progression, we collected 85 cases of human angiosarcoma specimens with clinical records and analyzed ISO-HAS-B patient-derived angiosarcoma cells. As control subjects, 54 cases of hemangioma and 34 of pyogenic granuloma were collected. Remarkably, consistent with our recent observations regarding the involvement of survivin expression following Hippo pathway inactivation in the neoplastic proliferation of murine hemangioendothelioma cells and human infantile hemangioma, nuclear survivin expression was observed in all cases of angiosarcoma but not in hemangiomas and pyogenic granulomas, and the Hippo pathway was inactivated in 90.3% of yes-associated protein (YAP) -positive angiosarcoma cases. However, survivin expression modes and YAP localization (Hippo pathway activation modes) were not correlated with survival. In addition, we confirmed that survivin small interference RNA (siRNA) transfection and YM155, an anti-survivin drug, elicited decreased nuclear survivin expression and cell proliferation in ISO-HAS-B cells which expressed survivin consistently. Conclusively, these findings support the importance of survivin as a good marker and critical regulator of cellular proliferation for human angiosarcoma and YM155 as a potential therapeutic agent.

Angiosarcomas are among the rarest malignant soft tissue tumors. They typically originate from vascular endothelial cells, and generally arise in the skin of the head (face and scalp) and superficial soft tissues, although they potentially occur at any location. [(1)] However, it is difficult to prove their true cellular origin. Angiosarcoma exhibits an aggressive malignancy that is characterized by poor prognosis. Median survival was reported as approximately 30–50 months.[(2–4)] Angiosarcoma patients have a 5-year survival between 10% and 50%[(3–5)] and tend to die of the results of tumor metastases to lung and liver.[(3)] In general, aged patients have a poorer prognosis than younger patients.[(2,4,6)] As for gene alterations, CIC gene abnormalities,[(7)] mutations of PTPRB and PLCG1,[(8)] fusion genes of NUP160-SLC43A3[(9)] and EWSR1-ATF1,[(10)] high-level gene amplification and overexpression of MYC,[(11,12)] and FLT4[(13)] have been reported. These preceding studies would provide invaluable insights into the molecular targets for angiosarcomas. Thus, to establish the effective and highly specific treatment protocols for angiosarcoma, an inveterate and rare cancer, the molecular pathogeneses and specific markers and therapeutic targets should be elucidated.

Recently, we demonstrated a possible molecular pathogenesis of neoplastic proliferation of vascular endothelial cells. Briefly, vascular endothelial cells lacking cell adhesion molecules (CD31 and CD44) escaped from contact inhibition and exhibited aberrant proliferation, which was caused by survivin expression following Hippo pathway inactivation.[(14)] Interestingly, this feature mimics neoplastic proliferation in murine hemangioendothelioma (EOMA) cells[(15)] and human infantile hemangioma, which exhibits robust proliferative potential.[(16)] Furthermore, YM155, a small molecule survivin suppressant,[(17)] has been

proposed to be a novel potential anti-proliferative agent for the neoplastic vascular endothelial cells.[15]

From a series of our investigations regarding survivin expression and the Hippo pathway,[14–16,18] we noticed that these findings could be applied to understand the molecular pathogenesis for malignant progression of human angiosarcoma. For the present study, we collected 85 cases of human angiosarcomas with clinical records and analyzed survivin and yes-associated protein (YAP), an important marker of Hippo pathway activation, and expression modes. We also examined the function of survivin through RNA interference and the effect of YM155 on cell proliferation using a representative human angiosarcoma cell line (ISO-HAS-B).[19]

Materials and Methods

Clinical cases. For the present study, 85 cases of human angiosarcoma, 54 of hemangioma and 34 of pyogenic granuloma surgical specimens were collected from the Surgical Pathology files of the National Cancer Center Hospital and University of the Ryukyus Hospital after histopathological review of those specimens by surgical pathologists. Of the 54 cases of hemangioma, there were 15 of capillary, 22 of cavernous and 17 of venous type. Signed informed consent forms were obtained from all patients prior to the surgical procedure. Following immunohistochemical studies, results were reviewed by pathologists. Additional information regarding the cases is shown in Table 1 and Tables S1 and S2. The experimental protocol was approved by the ethical board of the National Cancer Center (No. 2015-118) and the University of the Ryukyus (No. 843).

Antibodies. Rabbit monoclonal antibodies against survivin (clone: 71G4B7) and YAP (D24E4) were purchased from Cell Signaling Technology (Danvers, MA, USA). A rabbit monoclonal antibody against c-Myc (MYC) (Y69) was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibodies against CD31 (JC70A) and Ki67 (MIB-1) were purchased from DAKO (Carpinteria, CA, USA). A mouse monoclonal antibody against β-actin (AC-15) was purchased from Abcam.

Immunohistochemistry. For antigen retrieval, sections were autoclaved in citric acid buffer (pH 6.0) at 121°C for 10 min.[16] The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and incubated with 5% BSA in 50 mM TBS (pH 7.4) containing 0.05% Triton X-100 (T-TBS) for 1 h at room temperature. The sections were then incubated at 4°C with the primary antibodies diluted at 1:40 (anti-CD31), 1:50 (anti-MYC and Ki67), 1:200 (anti-YAP) and 1:400 (anti-survivin) in T-TBS. After overnight incubation, the sections were incubated with EnVision+Dual Link System-HRP reagents (DAKO) for 1 h at room temperature and treated with 0.02% 3,3′-diaminobenzidine-tetrahydrochloride (DAB) (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide. Finally, sections were counterstained with Dako REAL Hematoxylin (DAKO). Regarding survivin and YAP immunopositivities, all cases were scored as Score 0 (no staining), 1 (faint/barely staining in tumor cells), 2 (weak to moderate staining in 10–50% of the tumor cells) or 3 (uniform intense staining in over 50% of the tumor cells). The positive control for survivin and YAP was human infantile hemangioma[16] and the negative control was normal human adipocytes. Regarding MYC positivity, nuclear immunoreactivity in more than 10% of angiosarcoma cells was deemed positive[20] and no nuclear staining (cytoplasmic staining only) was considered as not positive.[13]

Cell culture. Human angiosarcoma cells (ISO-HAS-B)[19] were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. ISO-HAS-B cells were cultured on non-coated or gelatin (Thermo Fisher Scientific, Waltham, MA, USA)-coated plates in endothelial cell media (DMEM with High Glucose [Wako Pure Chemical Industries, Osaka, Japan] containing 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES [pH 7.4], 100 U/mL penicillin and 100 μg/mL streptomycin [Thermo Fisher Scientific])[14,15] in 5% CO_2 at 37°C under normoxic (20% O_2) condition. Mycoplasmal infection was not detected in the ISO-HAS-B cell culture using the Mycoplasma PCR ELISA Kit (Roche Life Science, Indianapolis, IN, USA).

RNA interference. SignalSilence Survivin siRNA I (siSurvivin-1) and siRNA II (siSurvivin-2) were purchased from Cell Signaling Technology. The SignalSilence Control siRNA (unconjugated) (Cell Signaling Technology) (siNC) and sterile Milli-Q water (mock) were used as negative controls. The siRNA transfection was performed using Lipofectamine RNAiMAX reagents (Thermo Fisher Scientific) and a forward transfection method. Efficiencies of RNAi-mediated human survivin protein knockdown were evaluated by western blotting and immunofluorescence staining.

YM155 treatment. ISO-HAS-B cells were cultured in endothelial cell media containing YM155 (4,9-dihydro-1-(2-methoxyethyl)2-methyl-4,9-dioxo-3-(2-pyrazinylmethyl)-1H-naphth[2,3-d]imidazolium, bromide)[17] (CAS registry no. 781661-94-7) (Cayman Chemical, Ann Arbor, MI, USA) dissolved in DMSO at different final concentrations (0, 100 and 500 nM).[15]

Cell proliferation analysis. ISO-HAS-B cells were plated at 5.0×10^5 cells per 60-mm dish. In our recent observation, murine hemangioendothelioma cells exhibited overriding morphology and expressed survivin and YAP on gelatin-coating plates.[15] To study the effects of gelatin coating and media replacement on ISO-HAS-B cell proliferation, we examined several culture conditions as follows: non-coating dishes and without media replacement; 1.5% gelatin coating and without media replacement; 4.5% gelatin coating and without media replacement; non-coating dishes and media replacement; and 1.5% gelatin coating and media replacement. Endothelial cell media were replaced with fresh media every 48 h. At 48, 96, 144, 192 and 240 h after plating, the cells were collected by trypsinization and the viable cells were counted under a microscope using a hemocytometer. To study the role of survivin in human angiosarcoma cell proliferation, untreated and mock, siNC, siSurvivin-1 and siSurvivin-2-treated ISO-HAS-B cells were cultured on 1.5% gelatin-coated dishes with media replacement every 48 h, and counted at 48, 96, 144 and 192 h after plating. To examine the YM155 drug efficacy in cell proliferation, ISO-HAS-B cells were cultured on 1.5% gelatin-coated dishes in endothelial cell media containing 0, 100 and 500 nM YM155 and counted at 48, 96, 144 and 192 h after plating. Furthermore, ISO-HAS-B cells cultured on 1.5% gelatin-coated dishes in endothelial cell media until 96 h after plating were cultivated in endothelial cell media containing 0 and 500 nM YM155 and counted at 96, 144, 192 and 240 h after plating.

Immunofluorescence. Low-density and high-density cultures of untreated, mock, siNC, siSurvivin-1 and siSurvivin-2-treated ISO-HAS-B cells on 12-well plates were fixed with 4% paraformaldehyde in 50-mM HEPES buffer (pH 7.3) for 20 min, permeabilized for 20 min with 0.2% Triton X-100 in TBS (pH 7.4), and treated with 5% BSA in T-TBS for 1 h at

Table 1. Summary of the human angiosarcoma clinical cases assayed

Case	Gender	Age	Site	Size (mm)	Nodal metastasis	Distant metastasis	Recurrence	Chemotherapy	Radiotherapy	Outcome	Survivin	YAP	Hippo
A01	Male	82	Scalp	15	+	+ (lung)	Stationary	+ (DTX)	+ (70 Gy)	Death (7 months)	2	0	
A02	Male	61	Right forehead	10	–	–	Stationary	+ (DTX)	+ (70 Gy)	Alive (24 months)	2	0	
A03	Male	89	Scalp	15	–	+ (lung)	Unknown	–	+ (70 Gy)	Death (7 months)	2	0	
A04	Male	88	Left forehead-calvaria	15	+	+ (bone)	Stationary	–	+ (70 Gy)	Death (27 months)	2	0	
A05	Male	73	Calvaria	30	–	+ (lung, parotid gland)	+	+ (DTX)	+ (60 Gy)	Death (60 months)	3	1 (N+cyto)	OFF
A06	Female	91	Forehead-calvaria	200	–	–	–	–	+ (70 Gy)	Death (11 months)	2	2 (N)	OFF
A07	Female	78	Calvaria	30	–	–	–	+ (DTX)	+ (70 Gy)	Death (6 months)	3	2 (N+cyto)	OFF
A08	Female	57	Left forehead	60	–	–	–	+ (DTX)	+ (70 Gy)	Alive (38 months)	3	2 (N+cyto)	OFF
A09	Female	67	Left temple	40	–	–	–	+ (DTX)	+ (70 Gy)	Alive (38 months)	2	0	
A10	Female	73	Calvaria	50	–	+ (lung)	Stationary	–	+ (70 Gy)	Death (6 months)	1	1 (N)	OFF
A11	Male	80	Scalp	60	–	+ (lung)	–	+ (DTX)	+ (70 Gy)	Death (8 months)	2	1 (N)	OFF
A12	Female	80	Right chest	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	3	3 (N+cyto)	OFF
A13	Male	63	Left forehead	30	–	+ (lung)	–	+ (DTX)	+ (70 Gy)	Death (9 months)	2	2 (N)	OFF
A14	Male	85	Calvaria-occiput	20	+	+ (lung, parotid gland)	+	–	+ (70 Gy)	Death (25 months)	3	3 (N+cyto)	OFF
A15	Male	64	Right submandibular	10	+	+ (lung, liver)	+	+ (DTX)	+ (70 Gy)	Death (45 months)	3	2 (N+cyto)	OFF
A16	Male	74	Right forehead	30	+	+ (lung, parotid gland, bone)	+	–	+ (70 Gy)	Death (21 months)	3	3 (N+cyto)	OFF
A17	Male	73	Calvaria	39	–	+ (lung, liver, adrenal gland, bone)	+	+ (DTX)	+ (60 Gy)	Death (21 months)	1	1 (N)	OFF
A18	Female	92	Right cheek	34	Unknown	+ (lung)	+	–	+ (60 Gy)	Death (15 months)	2	1 (N)	OFF
A19	Female	73	Left calvaria	25	Unknown	+ (lung)	unknown	+ (DTX)	–	Death (9 months)	2	2 (N+cyto)	OFF
A20	Male	79	Left temple	50	+	+ (lung, liver)	stationary	–	–	Death (10 months)	2	2 (N+cyto)	OFF
A21	Male	73	Calvaria	35	Unknown	unknown	stationary	–	–	Unknown	2	2 (cyto)	ON
A22	Female	81	Calvaria	80	–	–	stationary	–	+ (20 Gy)	Death (2 months)	2	2 (N+cyto)	OFF
A23	Male	85	Occiput	20	–	+ (lung)	+	–	+ (178 Gy)	Death (82 months)	2	0	
A24	Male	78	Calvaria	26	Unknown	+ (lung)	+	–	+ (70 Gy)	Death (17 months)	3	3 (N+cyto)	OFF
A25	Male	85	Calvaria	40	+	+ (lung)	Unknown	–	+ (60 Gy)	Death (14 months)	3	2 (N+cyto)	OFF
A26	Male	75	Calvaria	37	Unknown	+ (lung, liver, spleen)	Unknown	+ (DTX)	+ (60 Gy)	Death (4 months)	3	3 (N+cyto)	OFF
A27	Male	75	Calvaria	24	–	+ (lung, liver, spleen)	Unknown	–	–	Death (11 months)	2	2 (N)	OFF
A28	Female	87	Calvaria	30	Unknown	+ (lung)	Unknown	+ (DTX)	–	Death (3 months)	3	1 (cyto)	ON
A29	Male	75	Forehead	150	–	+ (lung)	+	+ (DTX)	+ (70 Gy)	Death (26 months)	2	1 (cyto)	ON
A30	Female	78	Calvaria	120	Unknown	+ (lung, bone)	+	+ (DTX)	+ (70 Gy)	Death (21 months)	2	2 (N)	OFF
A31	Female	75	Posterior auricle	50	–	+ (parotid gland)	+	+ (DTX)	+ (70 Gy)	Death (92 months)	2	2 (N+cyto)	OFF
A32	Male	58	Posterior auricle	50	Unknown	+ (lung)	Unknown	+ (DTX)	+ (70 Gy)	Death (49 months)	2	3 (N+cyto)	OFF
A33	Male	74	Forehead	140	–	+ (lung)	Stationary	–	+ (60 Gy)	Death (9 months)	3	3 (cyto)	ON

Table 1 (Continued)

Case	Gender	Age	Site	Size (mm)	Nodal metastasis	Distant metastasis	Recurrence	Chemotherapy	Radiotherapy	Outcome	Survivin	YAP	Hippo
A34	Female	93	Calvaria	32	+	–	+	–	+ (60 Gy)	Death (13 months)	3	2 (N)	OFF
A35	Male	81	Calvaria	30	+	+ (lung)	Stationary	–	+ (7 Gy)	Death (5 months)	3	2 (N+cyto)	OFF
A36	Female	96	Calvaria	30	–	–	Unknown	–	+ (70 Gy)	Unknown	3	3 (cyto)	ON
A37	Male	76	Calvaria	15	–	+ (spleen)	Stationary	+ (DTX)	+ (70 Gy)	Death (22 months)	2	0	
A38	Male	86	Forehead	20	–	–	Stationary	+ (DTX)	+ (70 Gy)	Unknown	2	0	
A39	Male	75	Scalp	60	–	+ (lung)	–	+ (DTX)	+ (70 Gy)	Death (11 months)	2	0	
A40	Male	75	Forehead	20	+	–	–	+ (DTX)	–	Death (17 months)	2	0	
A41	Female	86	Forehead	70	Unknown	unknown	Unknown	unknown	unknown	Death (19 months)	1	2 (N)	OFF
A42	Male	71	Calvaria	35	–	+ (lung, small intestine)	Unknown	–	+ (28 Gy)	Death (10 months)	2	3 (N+cyto)	OFF
A43	Male	70	Forehead	150	–	+ (lung, bone)	Stationary	+ (DTX)	+ (60 Gy)	Death (4 months)	1	2 (N)	OFF
A44	Male	75	Forehead	143	+	+ (lung)	+	+ (DTX)	+ (60 Gy)	Death (16 months)	1	2 (N+cyto)	OFF
A45	Male	78	Calvaria	15	–	+ (lung)	+	+ (DTX)	+ (70 Gy)	Death (16 months)	1	2 (N)	OFF
A46	Male	78	Forehead	80	–	+ (lung, liver)	Stationary	+ (DTX)	+ (70 Gy)	Death (7 months)	1	1 (N)	OFF
A47	Male	59	Scalp	20	–	–	–	–	–	Alive	1	1 (cyto)	ON
A48	Male	65	Neck	29	+	+ (liver)	–	+	–	Alive	1	1 (N)	OFF
A49	Male	60	Scalp	210	–	–	–	–	–	Alive	3	2 (N)	OFF
A50	Male	78	Face	Unknown	–	–	–	+	+	Alive	3	0	
A51	Male	71	Occiput	40	–	–	+	–	+ (50 Gy)	Alive	3	2 (N+cyto)	OFF
A52	Male	80	Forehead	100	+	+ (lung, liver)	+	–	+ (60 Gy)	Death (15 months)	3	1 (N)	OFF
A53	Male	79	Face	Unknown	–	–	Stationary	–	–	Death (6 months)	3	2 (N+cyto)	OFF
A54	Male	75	Left temple-neck	Unknown	+	+ (lung)	Stationary	+ (DTX)	+	Death (2 months)	3	0	
A55	Female	87	Left forehead	80	Unknown	+ (lung, bone)	Stationary	+ (DTX)	+ (60 Gy)	Death (6 months)	1	1 (N)	OFF
A56	Male	63	Left temple	25	–	–	+	+ (DTX)	+	Alive	1	2 (N)	OFF
A57	Male	76	Occiput	35	–	+ (lung)	Stationary	+ (DTX)	+	Death (18 months)	2	1 (N)	OFF
A58	Male	87	Left forehead	60	–	–	–	–	+	Alive	2	1 (N)	OFF
A59	Female	62	Scalp	25	–	+ (lung, femur)	–	–	+	Death (6 months)	2	1 (N)	OFF
A60	Male	82	Scalp	165	–	–	–	–	–	Alive	2	1 (N+cyto)	OFF
A61	Male	66	Left forehead	100	–	–	+	+ (DTX)	–	Alive	3	3 (N+cyto)	OFF
A62	Male	77	Forehead	40	–	–	+	+ (DTX)	+	alive	2	1 (N+cyto)	OFF
A63	Male	77	Left calvaria	60	–	–	+	+ (DTX)	+	Alive	3	2 (N)	OFF
A64	Male	76	Calvaria	220	–	+ (bone)	+	+ (DTX)	+	Death (24 months)	3	2 (N+cyto)	OFF
A65	Male	77	Right calvaria	100	+	+ (lung)	+	+ (DTX)	+	Death (36 months)	3	0	
A66	Male	60	Left calvaria	50	+	–	Stationary	+ (DTX)	+	Death (18 months)	3	2 (N)	OFF
A67	Male	72	Left calvaria	37	–	–	+	+ (DTX)	+	Death (48 months)	2	2 (N)	OFF
A68	Female	50	Occiput	22	+	+ (lung, bone, pancreas, peritoneum)	+	+ (DTX)	+	Death (48 months)	3	1 (N)	OFF
A69	Male	86	Calvaria	80	–	–	–	+ (DTX)	+	Alive	2	2 (N+cyto)	OFF
A70	Female	61	Left temple	37	–	+ (bone, liver)	+	+ (DTX)	+	Death (24 months)	3	1 (N)	OFF
A71	Female	64	Left temple	40	+	–	Stationary	+ (DTX)	+	Alive	3	2 (N+cyto)	OFF
A72	Female	62	Scalp	20	–	–	–	–	–	Alive	3	1 (N)	OFF

Table 1 (Continued)

Case	Gender	Age	Site	Size (mm)	Nodal metastasis	Distant metastasis	Recurrence	Chemotherapy	Radiotherapy	Outcome	Survivin	YAP	Hippo
A73	Female	63	Calvaria	20	+	–	–	–	+	Alive	3	1 (N+cyto)	OFF
A74	Male	80	Calvaria	40	–	–	+	+ (DTX)	+	Alive	3	1 (N+cyto)	OFF
A75	Male	74	Left forehead	90	–	+ (bone)	Stationary	+ (DTX)	+	Death (12 months)	2	1 (N+cyto)	OFF
A76	Female	74	Chest	90	–	+ (liver)	+	–	+	Death	2	3 (N+cyto)	OFF
A77	Female	55	Forearm	15	Unknown	–	+	–	–	Death	1	1 (N)	OFF
A78	Female	67	Groin	67	+	+ (lung, peritoneal cavity, intestine)	+	+ (DTX)	–	Death	2	2 (N+cyto)	OFF
A79	Female	70	Foot	12	+	+ (scalp, retroperitoneum)	–	–	+	Death	3	2 (N+cyto)	OFF
A80	Female	64	Lower leg	10	+	+ (femur)	–	–	–	Death	2	2 (N+cyto)	OFF
A81	Male	61	Unknown	61	–	+ (bone)	–	+ (wPTX)	–	Unknown	2	2 (cyto)	ON
A82	Female	36	Dorsal skin	42	–	+ (bone)	–	–	+	Alive	3	3 (N+cyto)	OFF
A83	Female	42	Femur	35	–	–	–	–	–	Alive	2	2 (N+cyto)	OFF
A84	Male	77	Shoulder	85	+	+ (scalp)	+	–	+	Death	3	3 (N+cyto)	OFF
A85	Male	16	Lower leg	20	+	+ (femur, skin)	+	+ (DXR)	+	Death	3	1 (N)	OFF

DTX, docetaxel; DXR, doxorubicin (Adriamycin); outcome, months after diagnosed; survivin, nuclear positivity; YAP, nuclear (N) and cytoplasmic (cyto) positivity; wPTX, weekly paclitaxel. Score 0 (no staining), 1 (faint/barely staining in tumor cells), 2 (weak to moderate staining in 10–50% of the tumor cells) or 3 (uniform intense staining in over 50% of the tumor cells).

room temperature. Cells were incubated overnight at 4°C with the primary antibodies diluted at 1:200 (anti-YAP) and 1:400 (anti-survivin) in T-TBS and further incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG [H+L] and anti-mouse IgG [H+L] [Thermo Fisher Scientific]) diluted at 1:100 and Alexa Fluor 594 phalloidin (Thermo Fisher Scientific) diluted at 1:40 in T-TBS for 1 h at room temperature. Finally, cells were counterstained with Hoechst 33342 (Dojindo Laboratories) diluted at 1:100 in T-TBS for 20 min at room temperature.

Preparation of cell lysates. High-density cultures of untreated, mock, siNC, siSurvivin-1 and siSurvivin-2 treated ISO-HAS-B cells on 60-mm dishes were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with 0.1% SDS and protease inhibitor cocktail (Roche Life Science). Cell lysate samples were placed on ice for 30 min and centrifuged at 20 000 *g* for 15 min to remove insoluble materials. An aliquot of 10 μg of protein samples was suspended in SDS-sample buffer and boiled for 5 min.

Western blotting. Cell lysate samples suspended in sample buffer were subjected to 15% SDS-PAGE under reducing conditions. The gel was transferred onto the Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated with 1% BSA in TBS containing 0.1% Tween 20 (TTBS) for 1 h at room temperature to block nonspecific protein binding, followed by overnight incubation at 4°C in TTBS containing primary antibody diluted at 1:1000 (anti-survivin) and 1:5000 (anti-β-actin). After washing with TTBS, the membrane was incubated with HRP-conjugated secondary antibodies (donkey anti-mouse IgG-HRP or donkey anti-rabbit IgG-HRP [Santa Cruz Biotechnology, Dallas, TX, USA]) diluted at 1:10 000 in TTBS for 1 h at room temperature. Target protein bands were detected by using Western Lightning Plus-ECL (PerkinElmer, Waltham, MA, USA).

Statistical analysis and preparing graphs. All statistical analyses and graph preparations in this study were performed using GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla, CA, USA). Kaplan–Meier curves and log-rank (Mantel–Cox) tests were used to evaluate overall survival differences between the two groups. Fisher's exact test analysis evaluated the significance of correlation between survivin expression modes or Hippo pathway activations and survival time. The Pearson correlation coefficient was calculated for correlation analysis between Ki67 and survivin labeling indices. *P*-values of <0.05 were considered significant.

Results

Clinicopathological features. The clinicopathological findings are summarized in Table 1 (85 cases of angiosarcoma), Table S1 (54 cases of hemangioma) and Table S2 (34 cases of pyogenic granuloma). Angiosarcomas occurred in 56 male (65.9%) and 29 female (34.1%) patients. The mean age at diagnosis of the angiosarcoma patients was 72.8 years (73.7, male; 71.2, female), with a range from 16 to 96 years old. Of the 84 cases with available data, angiosarcomas occurred in the skin of the head/neck (74 cases, 88.1%), leg (4 cases, 4.8%), chest (2 cases, 2.4%), arm (1 case, 1.2%), back (1 case, 1.2%), groin (1 case, 1.2%) and shoulder (1 case, 1.2%). Of the 81 cases with available data, the average long diameter of angiosarcomas was 54.9 mm, ranging from 10 to 220 mm. Of the 73 cases with available data, there were 24 patients (32.9%) with nodal metastasis. Of the 82 cases with available data, there were 52 patients (63.4%) with distant metastasis.

Among the 52 patients with distant metastasis, there were 38 (73.1%) with pulmonary metastasis. Of the 74 cases with available data, 31 patients (41.9%) suffered local recurrence after surgery. Of the 83 cases with available data, 47 patients (56.6%) received chemotherapy and 65 patients (78.3%) received radiotherapy.

As for hemangiomas, there were 22 male (40.7%) and 32 female (59.3%) patients in 54 examined cases. The mean age at diagnosis of hemangioma patients was 46.6 years (50.0, male; 44.2, female), with a range from 5 to 85 years old. As for pyogenic granulomas, there were 16 male (47.1%) and 18 female (52.9%) patients in 34 cases examined. The mean age at diagnosis of pyogenic granuloma patients was 43.6 years (63.9, male; 25.7, female), with a range from 4 to 85 years old.

Survivin and YAP expression in human angiosarcoma. Survivin was expressed in the nucleus of all human angiosarcoma cases and definite survivin immunostaining was detected in 85.9% (Score 2, 43.5%; Score 3, 42.4%) of cases (Fig. 1a). In contrast, there were a few survivin faint/barely positive (Score 1) hemangioma (total, 1.9%; capillary, 6.7%; cavernous, 0%; venous, 0%) (Fig. S1a–c) and pyogenic granuloma (5.9%) (Fig. S1d) cases, indicating that the survivin expression is specific to angiosarcoma (Fig. 1a). As shown in Figure 1b, YAP was positive in 84.7% (Score 1, 29.4%; Score 2, 40.0%; Score 3, 15.3%) of the present series of angiosarcoma cases, whereas it was not detected in hemangiomas (Score 0, 100%) (Fig. S1a–c) and pyogenic granulomas (Score 0, 97.1%) (Fig. S1d). Survivin was localized in the nucleus of angiosarcomas (Fig. 2a–c). By contrast, there were three patterns of YAP localization: in the nucleus (Fig. 2d), in the cytoplasm (Fig. 2e) and not positive (Fig. 2f). YAP is one of the established markers to distinguish the Hippo pathway status as follows: nuclear YAP indicates Hippo pathway inactivation (Hippo-OFF) and cytoplasmic YAP indicates Hippo pathway activation (Hippo-ON).[16,17,21] In this viewpoint, the Hippo pathway tends to be inactivated (OFF, 90.3%) in 72 YAP

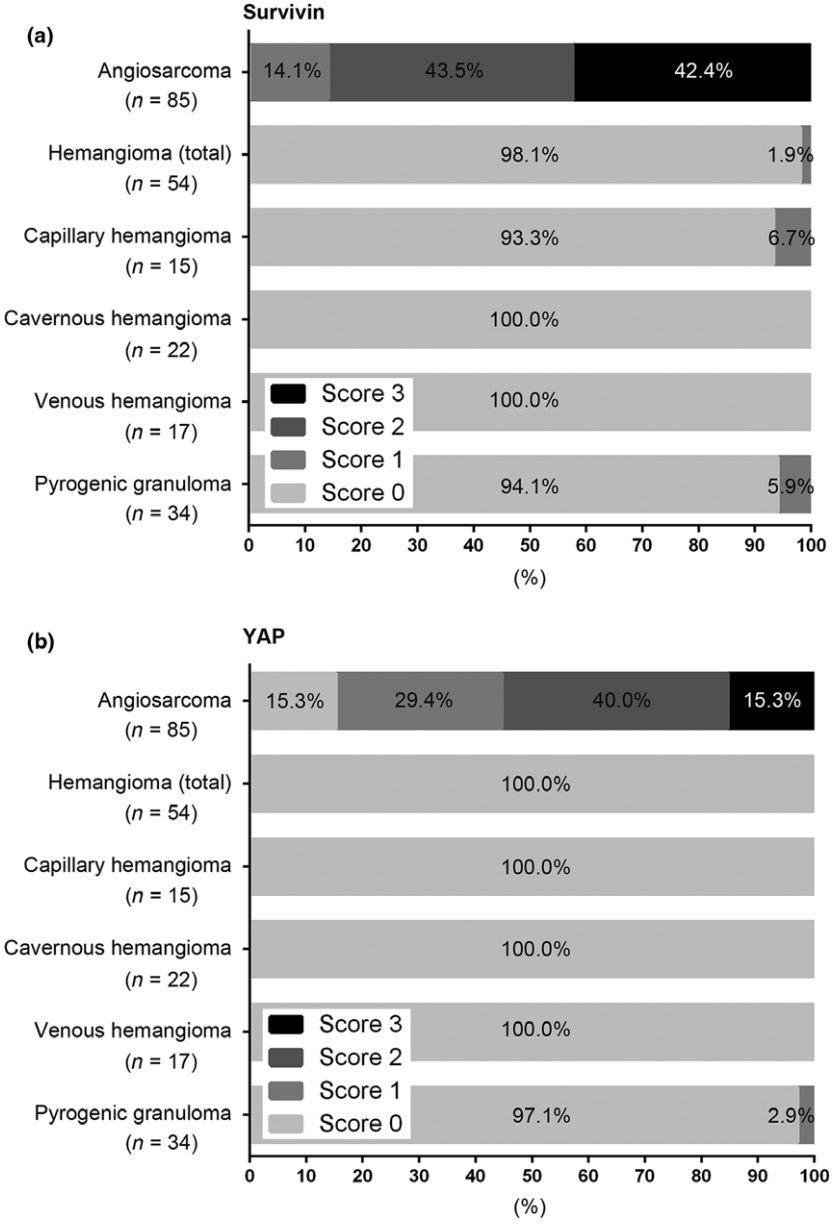

Fig. 1. The proportion of survivin and of yes-associated protein (YAP) positivity in human angiosarcoma, hemangioma and pyogenic granuloma cases. Survivin (a) and YAP (b) are characteristically expressed in human angiosarcoma. The percentage of survivin (a) and YAP (b) immunopositivity in human angiosarcoma (85 cases), hemangioma (total 54 cases: capillary, 15; cavernous, 22; and venous, 17 cases) and pyogenic granuloma (34 cases) is summarized. The scores on each of the survivin (a) and YAP (b) immunopositivity were as follows: Score 0 (no staining), 1 (faint/barely staining in tumor cells), 2 (weak to moderate staining in 10–50% of the tumor cells), or 3 (uniform intense staining in over 50% of the tumor cells).

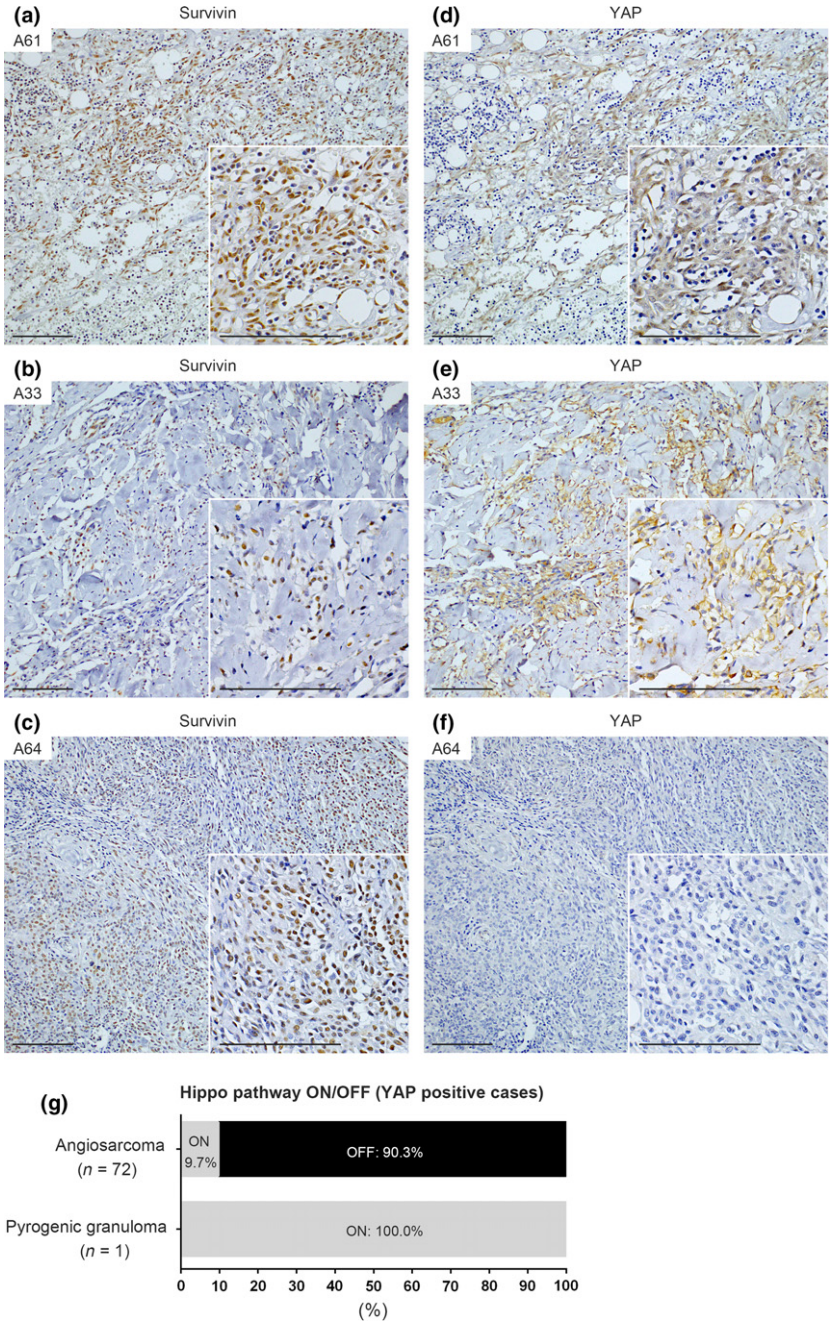

Fig. 2. Representative microscopic images of survivin and of yes-associated protein (YAP) immunolocalization and Hippo pathway dysregulation in human angiosarcoma. Immunoperoxidase (DAB) staining for survivin (a,b,c) and YAP (d,e,f), hematoxylin counterstaining. Representative examples (see Table 1): (a, d) case A61, (b, e) A33 and (c, f) A64. In human angiosarcoma, survivin was definitely localized in the nucleus of tumor cells (a–c). There were three patterns of YAP expression: (d) nuclear and cytoplasmic, (e) cytoplasmic localization and (f) not positive. Scale bars, 100 μm. (g) Of the YAP positive cases (72 cases of angiosarcoma and a case of pyogenic granuloma), the Hippo pathway was dysregulated (Hippo-OFF) in over 90% of angiosarcoma cases. The Hippo pathway activation (ON) and dysregulation (OFF) were evaluated based on confined cytoplasmic YAP localization (ON) or nuclear YAP staining (OFF).

positive angiosarcoma cases (Fig. 2g), indicating to some extent that the survivin expression could be regulated by the Hippo pathway. As for the known marker for angiosarcoma, MYC[11,12,20] labeled 45.9% (39/85 cases) of angiosarcoma in the nucleus (Fig. S2). Thus, survivin is a potential novel marker for angiosarcoma.

Relationships between survivin expression modes or Hippo pathway activations and the survival or cell proliferation status in human angiosarcoma. To evaluate correlations of the human angiosarcoma patient survival with survivin expression and Hippo pathway activations, patients were grouped into two categories according to the survivin or YAP expression modes as follows: Survivin Score 1 (n = 7) versus Scores 2 and 3 (n = 48) (Fig. S3a); Survivin Score 1 and 2 (n = 34) versus Score 3 (n = 21) (Fig. S3b); cytoplasmic YAP (Hippo-ON) (n = 3) versus nuclear YAP (Hippo-OFF) (n = 41) (Fig. S3c). Kaplan–Meier survival analysis demonstrated that there was no significant difference in survival between groups (Fig. S3). Moreover, regarding survivin expression modes and Hippo pathway activation, there was no difference between long-term (over 30 months) and short-term (<30 months) survivors (Table S3). There was a low level of correlation (r = 0.3400) between survivin and Ki67 labeling indices (Fig. S4).

Survivin modulates human angiosarcoma cell proliferation. Using a human angiosarcoma cell line (ISO-HAS-B), we examined the role of survivin in cell proliferation. ISO-HAS-B cells grew most efficiently and exhibited overriding morphology at high cell density on 1.5% gelatin-coating plates with media replacement every 48 h (Fig. 3a, red diamond and Fig. S5) as compared with the control (non-coating plates

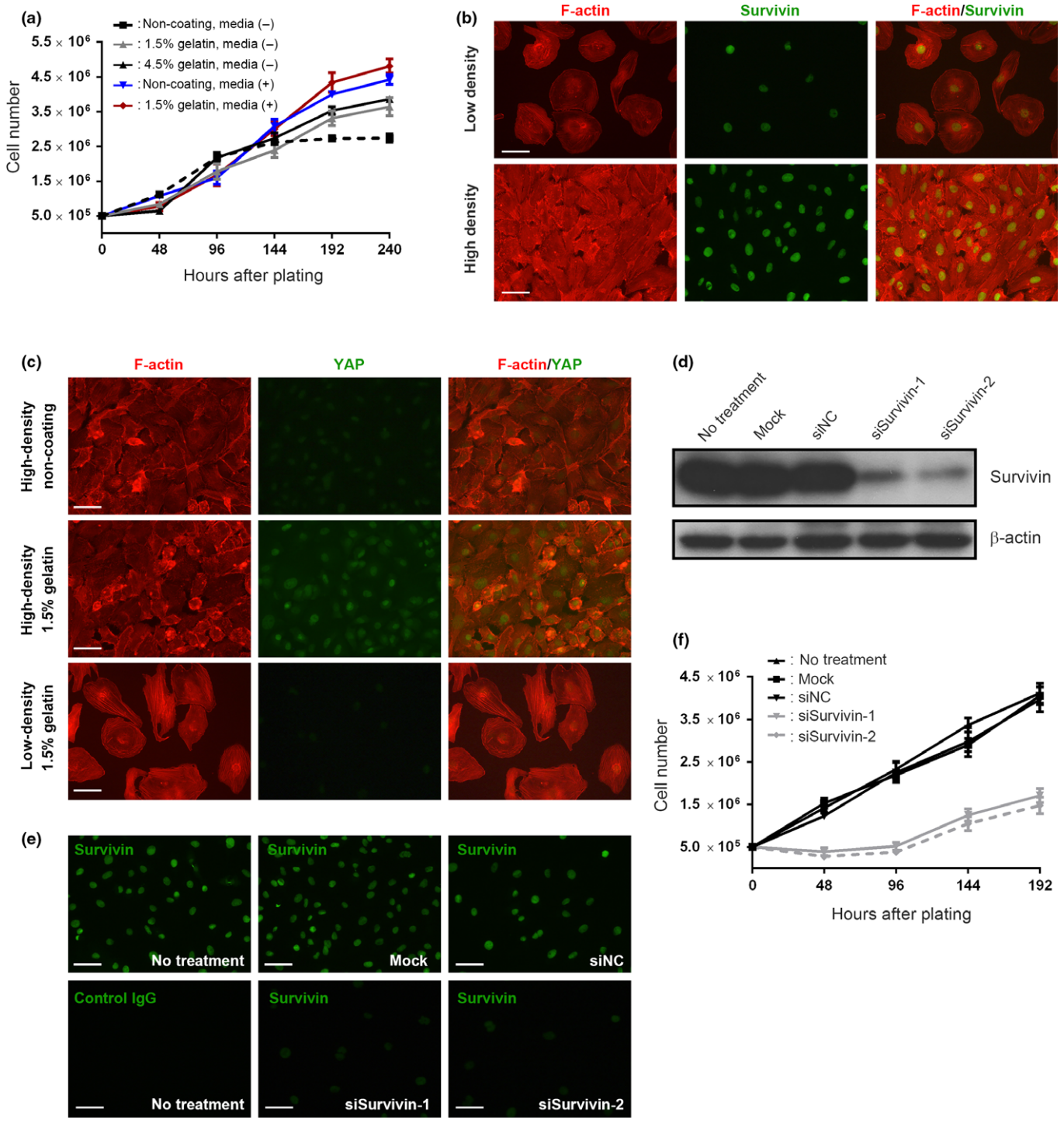

Fig. 3. Survivin plays a key role in human angiosarcoma cell proliferation. (a) Growth curves of ISO-HAS-B human angiosarcoma cells cultured at different conditions: on non-coating plates without media replacement (black square); on 1.5% gelatin-coating plates without media replacement (gray triangle); on 4.5% gelatin-coating plates without media replacement (black triangle); on non-coating plates with media replacement every 48 h (blue inverted triangle); and on 1.5% gelatin-coating plates with media replacement at every 48 h (red diamond). All data are means ± SD from triplicate experiments. (b) Representative F-actin, survivin and merged immunofluorescence images of ISO-HAS-B cells at low (top row) and high (bottom row) cell density on 1.5% gelatin-coating plates with media replacement, illustrating robust nuclear Survivin labeling. Scale bars, 100 μm. (c) Representative F-actin, of yes-associated protein (YAP), and merged immunofluorescence micrographs of ISO-HAS-B cells at high-density culture on non-coating plates (top row), high-density culture on 1.5% gelatin-coating plates (middle row) and low-density culture on 1.5% gelatin-coating plates (bottom row) with media replacement. Predominant nuclear YAP staining was observed at high cell density on 1.5% gelatin coating (middle row). Scale bars, 100 μm. (d) Western blotting revealed robust decreased survivin protein levels in ISO-HAS-B cells transfected with human survivin siRNA constructs (siSurvivin-1 and siSurvivin-2) as compared with control cells (no treatment, mock and control siRNA construct [siNC] transfected cultures). (e) Immunofluorescence microscopy revealed that survivin nuclear labeling was abrogated by survivin siRNA constructs transfected cells as compared with control cells. Scale bars, 100 μm. (f) Growth curves of control cells (no treatment, black triangle; mock, black square; siNC, black inverted triangle) and survivin knockdown cells for 192 h. Survivin knockdown cells exhibited obvious inhibition of cell proliferation. All data are means ± SD from triplicate experiments.

without media replacement) (Fig. 3a, black square and Fig. S5). At both low and high cell density, survivin was ubiquitously expressed in the nucleus (Fig. 3b). YAP was very faintly labeled in the nucleus at high cell density on non-coating plates (Fig. 3c, upper row) and at low cell density on 1.5% gelatin-coating plates (Fig. 3c, bottom row). However, at high cell density on 1.5% gelatin-coating plates, YAP was predominantly localized in the nucleus (Fig. 3c, middle row), indicating the Hippo pathway dysregulation (Hippo-OFF). To evaluate the efficiency of survivin knockdown, ISO-HAS-B cells (no treatment), mock transfected cells (mock), control siRNA transfected cells (siNC) and two different human survivin siRNA transfected cells (siSurvivin-1 and siSurvivin-2) were cultured for 72 h after transfection and analyzed (Fig. 3d,e). The relative survivin protein expression (Fig. 3d) and nuclear localization (Fig. 3e) levels were decreased in ISO-HAS-B cells transfected with the two survivin siRNA (siSurvivin-1 and siSurvivin-2) tested. Survivin siRNA-transfected ISO-HAS-B cells (siSurvivin-1 and siSurvivin-2) exhibited robust cell growth inhibition as compared to control cells (no treatment, mock and siNC) (Fig. 3f, Fig. S6).

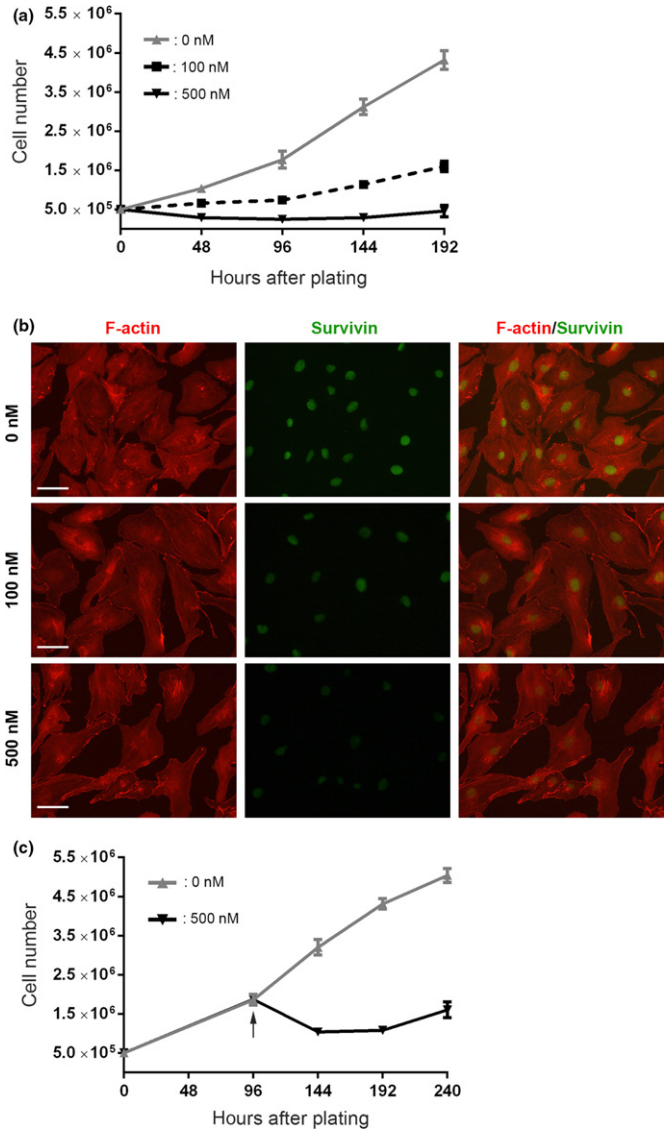

Fig. 4. YM155 modulates human angiosarcoma cell proliferation through inhibition of nuclear survivin expression. (a) ISO-HAS-B human angiosarcoma cells cultured in endothelial cell media containing YM155 (100 [black square] and 500 nM [black inverted triangle]) exhibited evidently suppressed cell proliferation as compared with control (0 nM [gray triangle]). All data are means ± SD from triplicate experiments. (b) Representative F-actin, survivin and merged immunofluorescence micrographs of ISO-HAS-B cells treated with YM155 (0 nM, top row; 100 nM, middle row; 500 nM, bottom row). ISO-HAS-B cells treated with YM155 (100 and 500 nM) showed pronounced decreased nuclear survivin expression and exhibited abnormal stellate-like morphology. Scale bars, 100 μm. (c) ISO-HAS-B cells were cultured in endothelial cell media containing YM155 (0 nM [gray triangle] and 500 nM [black inverted triangle]) from 96 (arrow) to 240 h after plating, illustrating the efficacy of YM155 in suppression of cell proliferation at sub-confluent cultures. All data are means ± SD from triplicate experiments.

Survivin inhibitor YM155 suppresses ISO-HAS-B cell proliferation. YM155-treated (100 and 500 nM) ISO-HAS-B cells exhibited robust growth suppression in a dose-dependent manner as compared to control cells (0 nM) (Fig. 4a and Fig. S7). YM155 suppressed survivin expression in the nucleus in a dose-dependent manner (Fig. 4b). Furthermore, YM155 elicited robust growth suppression of sub-confluent ISO-HAS-B cells (Fig. 4c and Fig. S8).

Discussion

Angiosarcomas are one of the rarest neoplasms and comprise <1% of sarcomas.[1] Generally, cutaneous angiosarcomas that arise in the head and neck, particularly the scalp, are the most common clinical form and usually occur in patients over 70 years of age.[1–6] Because angiosarcomas exhibit an unclear margin and tend to metastasize to distant organs such as lung and liver,[1,3] clinical outcome is extremely poor. Although surgical resection is the first-line treatment for angiosarcoma, depending on the extent of lesion size, radiation, chemotherapy (e.g. docetaxel) and immunotherapy (e.g. IL-2 and picibanil) are available, alone or in combination.[22–24] However, complete remission is rare and patients frequently achieve disease stabilization in the presence of residual angiosarcoma masses.[25] Several potential theories relating to the development of angiosarcomas have been proposed and discussed. Chronic lymphedema and therapeutic radiation are well recognized predisposing factors.[1,26] Another theory is that chronic immunosuppression due to kidney transplant presents a risk for angiosarcomas arising in non-functioning arteriovenous fistulae.[27] In addition, particularly in molecular genetics, MYC gene amplification and overexpression in the nucleus have been well documented and exploited as a useful marker for angiosarcoma.[11–13,20] Although contributing to our understanding of angiosarcomas, no single theory adequately explains the pathobiology of these lesions and highlights the complexity of these lesions because a definitive mechanism is still elusive. Hence, to identify new therapeutic targets and effective treatment protocols for complete remission, precise molecular pathogeneses for angiosarcoma should be elucidated.

In light of our recent findings demonstrating a critical role for survivin and YAP, an important component for Hippo pathway regulation,[28,29] as modulators of murine vascular endothelial and hemangioendothelioma cell proliferation *in vitro*[14,15] and human infantile hemangioma in the early proliferative phase (age < 1 year) *in situ*,[16] we embarked on an investigation of these components in human surgical specimens of angiosarcoma.

Clinicopathologically, 85 cases of angiosarcoma in the present study followed similar trends as previous reports;[1–6] angiosarcomas tend to occur in head and neck regions (88.1%)

of elderly patients (median age: 72.8 years), frequently metastasize to lung, and exhibit poor clinical outcome. Interestingly, we found that survivin was expressed in the nucleus in all cases of angiosarcoma and robustly in around half of the cases (Score 3, 42.4%), but in a few cases of hemangioma (1.9%) and pyogenic granuloma (5.9%), indicating that survivin is a potential good marker for angiosarcoma as compared to MYC (positive in 45.9% of angiosarcomas). The results for survivin expression in hemangiomas (capillary, cavernous and venous) reflected their less proliferative potential because infantile hemangiomas in the early proliferative phase were not included in the present study. We payed attention to the nuclear survivin expression in angiosarcomas. In addition to the well-recognized role for inhibiting apoptosis in cytoplasm, survivin plays a key role for cell division in the nucleus during mitosis by binding to microtubules and promoting chromosomal segregation and cytokinesis during embryonal development and carcinogenesis.[30–33] Because generally angiosarcomas exhibit extremely poor clinical outcome, our data is consistent with preceding literature revealing that nuclear survivin expression in cancer patients is associated with poor prognosis/outcome,[34–36] while there was no relationship between survivin expression modes (Score 1 to 3) and survival. Because the nuclear survivin expression was observed in all cases of human angiosarcoma, including long-term survivors, and there was a low level of correlation between survivin expression and Ki67-positive cell proliferation status, we assume that survivin plays a key role in anti-cell death and facilitates driving the oncogenic process of human angiosarcoma rather than the prognostic factor.

The Hippo pathway has emerged as a conserved cell growth regulatory signaling pathway essential for controlling organ size and three-dimensional structure by regulation of cell proliferation followed by contact inhibition.[37] Inactivation of the Hippo pathway (Hippo-OFF) is reported to lead to diverse human cancers, including soft tissue sarcoma.[38] The abnormal localization of cell adhesion molecules (e.g. CD31)[14,15] is a possible mechanism underlying the inactivation of the Hippo pathway in human angiosarcoma. YAP is one of the core components of the Hippo pathway[28,29,37] and nuclear YAP localization indicates Hippo pathway inactivation.[14,15,18,21] As a possible mechanism for survivin expression in angiosarcoma, we assumed YAP nuclear translocation, namely, Hippo pathway inactivation, because survivin is one of the YAP target genes.[14,39,40] Interestingly, our findings indicate that YAP is specifically expressed and translocated in the nucleus in angiosarcomas; namely, Hippo pathway inactivation (Hippo-OFF). However, the Hippo pathway activation modes were not related to angiosarcoma patient survival. Overall, our data are consistent with the possibility that survivin, which might be mainly upregulated by nuclear YAP (Hippo-OFF), is a key modulator of proliferation in human angiosarcomas and inhibition of survivin activity may be a potential therapeutic target. Regarding the survivin expression in angiosarcoma without inactivation of the Hippo pathway, we assume that there are other pathways involved in survivin expression (e.g. several receptor tyrosine kinases signaling pathways).[41]

As we recently reported, a small molecule survivin suppressant YM155, which inhibits survivin expression by interfering with Sp1 binding to the survivin promoter[42] and is currently under study in phase II clinical trials for breast cancer,[43] melanoma[44] and non-small-cell lung cancer (NSCLC)[45] patients, is an efficacious modulator of murine hemangioendothelioma cells *in vitro*.[15] In this report, we demonstrated that human angiosarcoma ISO-HAS-B cells[19] were not contact-inhibited and exhibited robust nuclear survivin expression regardless of cell density and nuclear YAP localization (Hippo-OFF) at high cell density; these results were consistent with our results on human angiosarcoma specimens. Importantly, ISO-HAS-B cells treated with YM155 and survivin siRNA-transfected ISO-HAS-B cells exhibited identical proliferation profiles and decreased nuclear survivin expression on gelatin.

In aggregate, our findings reveal that nuclear survivin, which is regulated in part by Hippo pathway, is a good marker for human angiosarcoma and upregulates cell proliferation in human angiosarcoma cells. Therefore, we surmise that survivin is a potential therapeutic target and selective inhibition of survivin expression by YM155[15] may have potential as another beneficial treatment for human angiosarcomas. In the next step toward the development of a diagnostic marker(s), we would demonstrate the specificity/sensitivity of survivin and YAP expression in human angiosarcoma through comparison of their expression in other types of human vascular tumors.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Survivin and YAP are not positive in hemangioma and pyrogenic granuloma.

Fig. S2. Representative nuclear MYC localization in human angiosarcoma.

Fig. S3. Correlations of the survival with survivin expression and Hippo pathway activation in human angiosarcoma.

Fig. S4. The correlation of the survivin and Ki67 labeling indices in human angiosarcoma.

Fig. S5. Morphological analysis of ISO-HAS-B cells plated on non-coating or gelatin-coating plates with or without media replacement.

Fig. S6. Morphological analysis of control and survivin knockdown ISO-HAS-B cells.

Fig. S7. Morphological analysis of control and YM155 treated ISO-HAS-B cells.

Fig. S8. Morphological analysis of control and YM155 treated ISO-HAS-B cells.

Table S1. Summary of the human hemangioma clinical cases assayed.

Table S2. Summary of the human pyogenic granuloma clinical cases assayed.

Table S3. Correlation between overall survival and survivin-Hippo pathway in human angiosarcoma cases.