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Authors

Jia, Jinjing
Li, Changji
Luo, Suju
et al.

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Yes-Associated Protein Contributes to the Development of Human Cutaneous Squamous Cell Carcinoma via Activation of RAS

Jinjing Jia[1], Changji Li[1], Suju Luo[2], Feng Liu-Smith[3], Jiao Yang[1], Xin Wang[1], Nanping Wang[4], Baochang Lai[4], Ting Lei[4,5], Qiongyu Wang[1], Shengxiang Xiao[1], Yongping Shao[6,7] and Yan Zheng[1]

Cutaneous squamous cell carcinoma (cSCC) is one of the most common skin malignant tumors with an increasing incidence. Studies have shown that Yes-associated protein (YAP) participates in the development of a variety of tumors as an oncogene, but to our knowledge its role in cSCC has not been reported. In this study, we used immunohistochemistry to show that YAP expression was elevated in cSCC samples of different stages versus in normal skin and that it was well correlated with the progression of the disease. Down-regulation of YAP in cSCC cell lines A431 and SCL-1 inhibited cell proliferation by inducing growth arrest during the G1/S phase transition, promoted apoptosis, and reduced invasion and migration abilities in vitro. Conversely, overexpression of YAP promoted cell proliferation and protected cells against basal and chemotherapy-induced apoptosis. These oncogenic effects of YAP were associated with activation of the RAS protein and its downstream AKT and ERK. Using a mouse xenograft model, we further showed that YAP depletion inhibited cSCC tumor growth in vivo. Our results suggested that YAP is involved in the carcinogenesis and development of cSCC and that it may serve as a biomarker or therapeutic target of this disease.

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a kind of malignant tumor derived from keratinocytes of the epidermis or appendages. It is the second most common nonmelanoma skin cancer; it accounts for 15—20% of all cutaneous malignancies, and its incidence rate is rapidly increasing (Allen and Stolle, 2015; Brougham and Tan, 2014). Although most cSCC cases are curable by surgical removal, a small percentage of cSCCs can develop into metastases and potentially threaten patients' lives. Therefore, mechanistic understanding, prevention, diagnosis, and treatment options for cSCC are still in demand.

Yes-associated protein (YAP) was originally identified in *Drosophila* species as a key component of the Hippo pathway, which regulates tissue growth and organ size during development (Sudol, 1994; Zeng and Hong, 2008). When phosphorylated by the upstream Mst1/2/Lats1/2 kinase cascade at Ser 127, YAP is inactivated and sequestered in the cytoplasm (Oka et al., 2008; Zhao et al., 2008; Zhao et al., 2011). Upon dephosphorylation, YAP is activated and translocates to the nucleus, where it functions as a coactivator of many transcription factors such as TEAD1-4, SMADs, p73, RUNX, and TBX5 and regulates cell proliferation, apoptosis, and migration (Yu and Guan, 2013; Zhao et al., 2008). As an oncogene, YAP has been implicated in the carcinogenesis of a wide variety of human cancers including hepatocellullar carcinoma, ovarian cancer, lung cancer, gastric cancer, colon cancer, pancreatic cancer, melanoma, oral squamous cell carcinoma, and head and neck carcinoma (Chan et al., 2011; Diep et al., 2012; Huo et al., 2013; Jerhammar et al., 2014; Kang et al., 2011; Lorenzetto et al., 2014; Nallet-Staub et al., 2014; Steinhardt et al., 2008; Wu et al., 2010; Yoshikawa et al., 2015; Zhang et al., 2011). However, to our knowledge, its role in cSCC has not been investigated. In this study, we examined YAP

[1]Department of Dermatology, the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, China; [2]Department of Dermatology, Tianjin Medical University General Hospital, Tianjin, China; [3]Departments of Epidemiology and Medicine, University of California Irvine, Irvine, CA, USA; [4]Cardiovascular Research Center, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, Xi'an, China; [5]Department of Pathology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, Xi'an, China; [6]Key Laboratory of Biomedical Information Engineering of the Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, China; and [7]Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an, China

Correspondence: Yan Zheng, Department of Dermatology, the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, 157 Xiwu Road, Xi'an 710004, China. E-mail: zenyan66@126.com or Yongping Shao, Key Laboratory of Biomedical Information Engineering of the Ministry of Education, Frontier Institute of Science and Technology, Xi'an Jiaotong University, 28 Xianning West Road, Xi'an 710049, China. E-mail: yongpings@gmail.com

expression in cSCC patient samples of different disease stages and analyzed its correlation with disease progression. We also investigated the biological function of YAP by a combination of knockdown and overexpression approaches. Our results showed that YAP expression positively correlates with the progression of cSCC and that YAP contributes to the carcinogenesis of cSCC by activating RAS signaling.

RESULTS

Up-regulation of YAP protein expression in cSCC

We used immunohistochemistry to investigate YAP protein expression in tissue samples from patients with different cSCC stages, including normal skin tissues (n = 30), pre-cancerous actinic keratosis lesions (n = 31), carcinoma in situ Bowen's disease (n = 34), well-differentiated cSCC (n = 35), and moderately/poorly differentiated cSCC (n = 30). The results showed both nuclear and cytoplasmic staining of YAP (Figure 1a−e). For normal skin tissues, YAP was weakly expressed in the basal layer of the skin in 26.7% (8 of 30) of samples; in actinic keratosis, YAP was weakly expressed in the disordered cells and some of the parakeratosis columns, and the positive result rate was 45.2% (14/31); in Bowen's disease, YAP was expressed in the atypical cells of the whole epidermis with a positive result rate of 70.6% (24 of 34, $P < 0.001$); in well-differentiated cSCC, YAP was expressed in poorly differentiated cells but was weaker in squamous

nests and keratin pearls, and 94.3% of samples stained positive (28 of 35, $P < 0.001$); in moderately/poorly differentiated cSCC, YAP was strongly expressed in almost all tumor cells, with a positive result rate of 96.7% (29 of 30, $P < 0.001$). Staining of consecutive tissue sections with antibodies specific for YAP, leukocyte marker LCA, tumor cell marker p63, or epithelium cell marker panCK showed that YAP staining is tumor-cell specific (see Supplementary Figure S1 online). Subsequent semiquantitative analysis of the immunohistochemistry results allowed us to perform multiple comparisons of YAP expression among cSCC samples of different stages and normal skin. YAP expression was significantly higher in Bowen's disease, well-differentiated cSCC, and moderately/poorly differentiated cSCC than in normal skin and correlated well with disease progression (Table 1 and Figure 1f). In addition, YAP protein expression is up-regulated in several cSCC cell lines including A431, HSC-1, and SCL-1 compared with the primary human epidermal keratinocytes (Figure 1g).

YAP promotes cSCC cell growth via regulation of the G1/S progression

Because YAP expression is elevated in cSCC samples and correlates with disease stages, we speculated that YAP may contribute to the development of cSCC. To test the potential oncogenic effects of YAP, we first knocked down YAP using
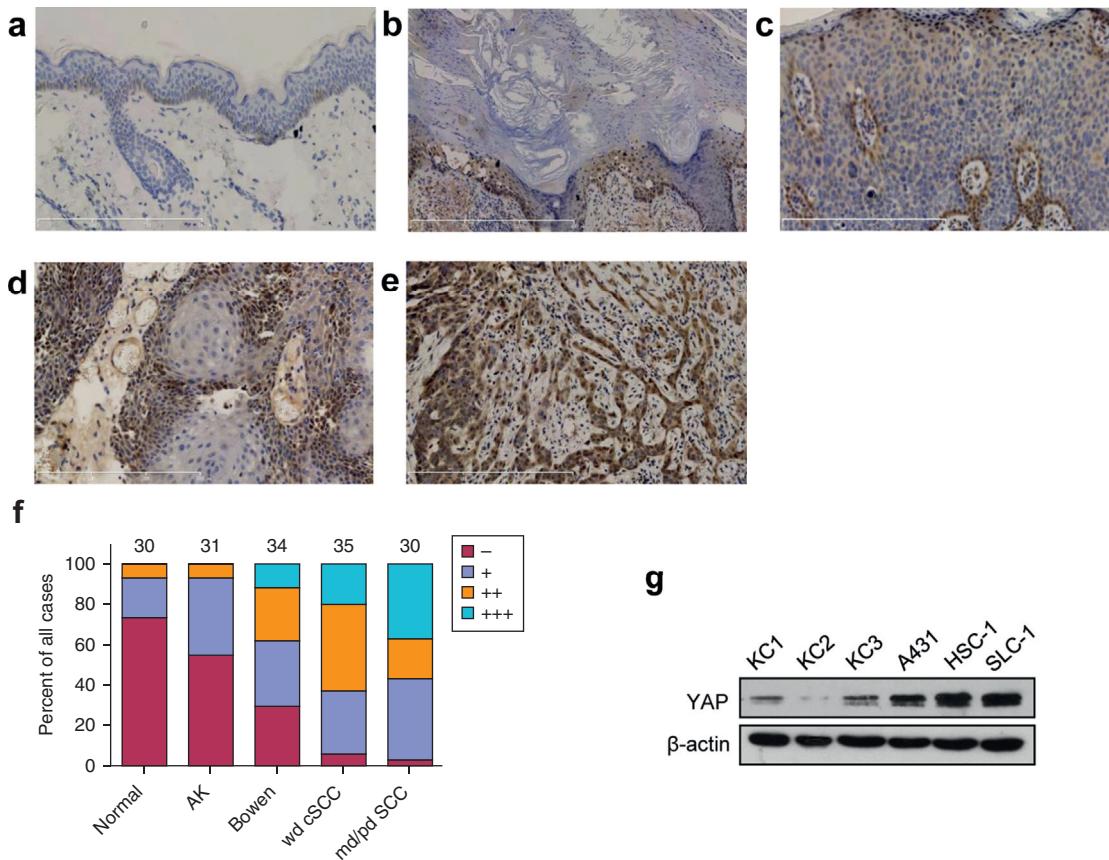
Figure 1. Immunohistochemical analysis of YAP expression in different tissues. (a) Normal skin (n = 30). Bar length = 600 μm. **(b)** Premalignant cSCC (AK, n = 31). Bar length = 600 μm. **(c)** cSCC in situ (Bowen's disease, n = 34). Bar length = 300 μm. **(d)** Well-differentiated cSCC (n = 35). Bar length = 300 μm. **(e)** Moderately and poorly differentiated cSCC (n = 30). Bar length = 300 μm. **(f)** Semiquantitative analysis of YAP staining. **(g)** Western blot analysis of YAP protein expression in primary human epidermal keratinocytes and three cSCC cell lines: A431, HSC-1 and SCL-1. AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; md, moderately differentiated; pd, poorly differentiated; SCC, squamous cell carcinoma; wd, well-differentiated.

Table 1. Expression of YAP in normal skin, actinic keratosis, Bowen's disease, well-differentiated cutaneous squamous cell carcinoma, and moderately/poorly differentiated cutaneous squamous cell carcinoma

Group	n	Expression Grade, n				% Positive Expression
		−	+	++	+++	
Normal skin	30	22	6	2	0	26.67
Actinic keratosis	31	17	12	2	0	45.16
Bowen's disease	34	10	11	9	4	70.59***
Well-differentiated cutaneous squamous cell carcinoma	35	2	11	15	7	94.29***
Moderately/poorly differentiated cutaneous squamous cell carcinoma	30	1	12	6	11	96.67***

−, negative, score 0; +, weakly positive, score 1−4; ++, moderately positive, score 5−8; +++, strong positive, score 9−12.
***$P < 0.001$ compared with normal skin.

small-interfering RNAs (siRNAs) in two cSCC cell lines, A431 and SCL-1 (Figure 2a) and examined the impact on cell growth. Depletion of YAP reduced cell growth in both cell lines as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 2b). Propidium iodide (PI) cell cycle analysis showed that YAP knockdown led to growth arrest in the G1/S stage transition (Figure 2c), which was accompanied by reduction in the expression of multiple cell cycle regulators including cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2 and CDC25A (Figure 2d, and see Supplementary Figure S2a and b online).

To validate the results from loss-of-function studies, we overexpressed YAP in A431 and SCL-1 cells (Figure 2e), and the effects on cell cycle were similarly analyzed. Ectopic expression of YAP promoted the growth of A431 and SCL-1 cells, although the effects were not as prominent as those observed in the knockdown experiment (presumably because of high basal levels of YAP expression in cSCC cells) (Figure 2f). In line with the growth stimulating effect, YAP overexpression increased the population of S phase cells (Figure 2g) and induced the expression of the same group of cell cycle regulators in both cell lines (Figure 2h, and see Supplementary Figure S2a and b). Together, our loss-of function and gain-of-function studies consistently showed that YAP promotes the G1/S progression in cSCC cells.

YAP protects cSCC cells against apoptosis
To test whether YAP played a role in regulating apoptosis, we monitored apoptosis via annexin V/PI staining. Non-transfected or control siRNA-transfected cells showed a low basal apoptosis rate of 6−7% in A431 and SCL-1 cells. YAP knockdown, however, increased the apoptosis rate to 10−13% (Figure 3a and b). The increased apoptosis was associated with a stronger cleavage of caspase-3, indicative of the activation of the apoptosis pathway. The expressions of previously reported YAP-regulated apoptosis factors such as the Bcl-2, Bax, and p53 pathways (Bai et al., 2013; Basu et al., 2003; Ma et al., 2014; Zagurovskaya et al., 2009) were not affected by YAP depletion (Figure 3c and see

Supplementary Figure S2c). To evaluate the effects of YAP overexpression on apoptosis, we first treated cells with 5-fluorouracil (5-Fu) to induce apoptosis, because the basal apoptosis levels were low in these two cell lines. As expected, 5-Fu treatment induced apoptosis in the blank (31.13% in A431 and 27.18% in SCL-1) and the vector (36.70% in A431 and 27.28% in SCL-1) control groups. Of note, overexpression of YAP reduced the apoptosis rate to 22.43% in A431 and 20.60% in SCL-1 cells and inhibited the cleavage of caspase-3 (Figure 3d−f). Therefore, YAP protects cSCC cells against basal or 5-Fu−induced apoptosis.

YAP promotes the migration/invasion ability of cSCC cells
In vitro Transwell assays (EMD Millipore, Billerica, MA, USA) were performed to investigate the impact of YAP on the migration and invasion of A431 or SCL-1 cells. Depletion of YAP significantly decreased the migration and invasion ability of A431 and SCL-1 cells (Figure 4a−d). However, overexpression of YAP had little effect (see Supplementary Figure S3 online). It is possible that the effects of the ectopically expressed YAP were masked by the high levels of endogenous YAP in A431 and SCL-1 cells. The matrix metallopeptidase (MMP) family plays an important role in tumor invasion and metastasis (Sani et al., 2015). Therefore, we further analyzed the expression of MMP-2 and MMP-9, two major MMPs implicated in migration and invasion, in YAP-depleted cells. The results showed that down-regulation of YAP led to decreased expression of MMP-2 and MMP-9 (Figure 4e), consistent with the results of Transwell assays. Therefore, YAP promotes the migration/invasion ability of cSCC cells, possibly by regulating the expression of MMP-2 and MMP-9.

YAP promotes cSCC cell growth by activating RAS signaling
To understand the molecular mechanisms of the oncogenic effects of YAP, we analyzed two major signaling pathways involved in the regulation of cell growth, survival, and migration: the PI3K/AKT pathway and the extracellular signal-regulated kinase (ERK) pathway. Knockdown of YAP inhibited the activities of these two pathways, as illustrated by the reduction in the level of phospho-AKT and phospho-ERK1/2 but not in total AKT and ERK1/2 (Figure 5a). Conversely, over-expression of YAP enhanced the phospho-AKT and phospho-ERK1/2 signals without altering total AKT or ERK1/2 expression (Figure 5b). Because both the PI3K/AKT and ERK pathways are located downstream of the RAS protein, we performed RAS pull-down assay to test whether YAP regulates AKT and ERK signaling by modulating RAS activity. Indeed, YAP knockdown reduced RAS activity, and overexpression of YAP enhanced it (Figure 5c and d). Previous studies in breast epithelial cells have shown that YAP transcriptionally induces the expression of an EGFR ligand, amphiregulin (AREG), which activates the EGFR/RAS signaling pathway (Zhang et al., 2009). In line with this, we found that YAP knockdown decreased both cellular and secreted AREG levels in A431 and SCL-1 cells, whereas overexpression of YAP had the opposite effects (Figure 5a, b, and e). Treatment with AREG promoted cSCC cell growth in a dose-dependent manner (see Supplemental Figure S4a online), although at a very high dosage (500 nM) the growth-stimulating effect started to diminish, likely because of
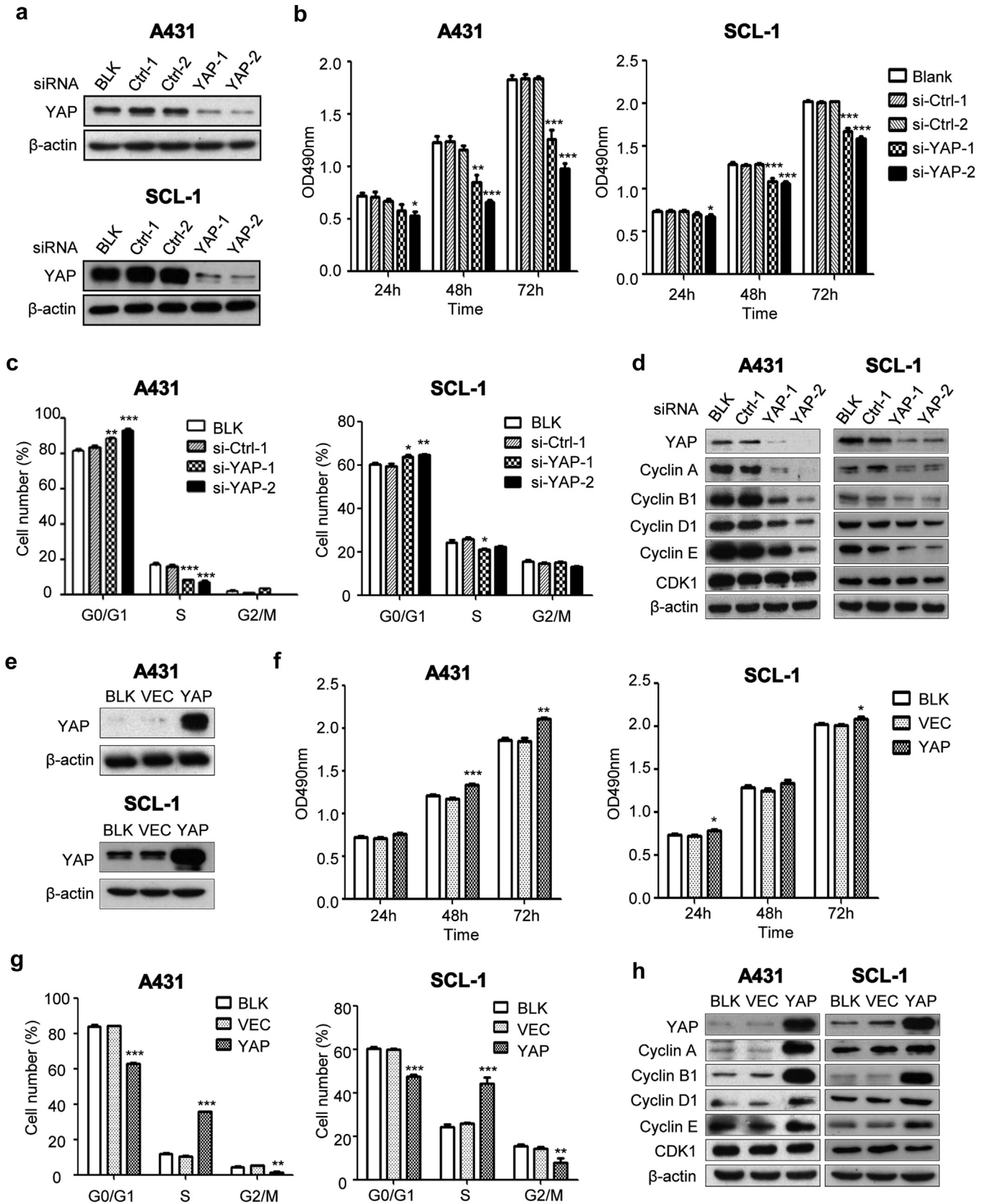
Figure 2. Effect of down-regulation and overexpression of YAP on cell proliferation. YAP protein expression (48 hours after transfection) was measured (**a**) after YAP-siRNA interference or (**e**) YAP plasmid transfection. Cells were transfected with (**b**) YAP siRNA or (**f**) YAP plasmid, and MTT assay was performed after 24, 48, and 72 hours. The cell cycle profiles were analyzed 48 hours after (**c**) siRNA or (**g**) plasmid transfection by flow cytometry. Cell cycle regulators were analyzed by Western blot analysis after (**d**) YAP knockdown or (**h**) overexpression, with β-actin being an internal control. All the quantitative data are presented as mean ± standard error of the mean (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. BLK, blank transfected group; Ctrl, control; h, hours; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; si, small interfering; VEC, vector transfected group.

cellular toxicity. Moreover, AREG treatment led to a partial rescue of the growth arrest induced by YAP depletion (Figure 5f), which was associated with reactivation of the ERK and AKT signaling pathways (see Supplementary Figure S4). Our results suggested that YAP contributes to the growth of cSCC cells by activating the AREG/RAS/AKT and/or ERK axis.

Down-regulation of YAP inhibits cSCC cells *growth in vivo*

To address the impact of YAP on cSCC tumor growth in vivo, A431 cells that stably express control (sh-Ctrl-1 and sh-Ctrl-2) or YAP-targeting small hairpin RNAs (shRNAs) (sh-YAP-1 and sh-YAP-2) were subcutaneously implanted into the left and right flanks of nude mice, and tumor growth was monitored over time. Consistent with the in vitro results, YAP knockdown significantly inhibited the growth of tumors in vivo (Figure 6a). By the end of the experiment (day 15), tumors from the sh-YAP group had much smaller sizes and lower weights versus tumors from the control group (Figure 6b–c). The knockdown efficiency of YAP by shRNAs at the end point of the experiment was confirmed by Western blot and immunohistochemistry analyses (Figure 6d and see Supplementary Figure S5 online). As expected, AREG, phospho-AKT and phospho-ERK1/2 levels were reduced in tumor tissues of the sh-YAP group (Figure 6d).

DISCUSSION

In recent years, an increasing amount of evidence has implicated the oncogene YAP in the carcinogenesis of a wide range of human cancers. However, to our knowledge its role in the development of cSCCs has not been investigated. In this study, we provided evidence that YAP expression correlated with cSCC progression and that YAP promoted cSCC cell proliferation, survival, and migration via activation of RAS signaling.

Our data showed that YAP promoted cSCC cell growth by regulating the G1/S transition. YAP depletion reduced the expression of multiple G1/S regulators such as cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2 and CDC25A, whereas overexpression of YAP reversed this pattern. Similar regulation of cell cycle regulators by YAP has been observed in other studies. For example, in *Drosophila* species, the YAP homolog Yorkie promotes tissue expansion by activating cyclin E (Huang et al., 2005). Zender et al. (2006) showed that murine tumors harboring the 9qA1 amplicon, which contains the *Yap* gene, overexpressed cyclin E and that YAP knockdown by shRNA in 9qA1-harboring cells resulted in down-regulation of cyclin E. In human gallbladder tumor, YAP depletion using shRNAs led to a G1/S growth arrest accompanied by the decrease of CDK2, CDC25A, and cyclin A (Li et al., 2014). In breast cancer, however, YAP promotes cell growth by up-regulating cyclin D1 but not cyclin E (Wang et al., 2012). Therefore, YAP may control G1/S entry by modulating the expression of different cell cycle regulators, depending on the cellular context. To date, no solid evidence exists to support a direct transcriptional control of YAP on these proteins. How YAP regulates these cell cycle proteins is largely unknown. In our cSCC system we found that YAP activated the RAF—mitogen-activated protein kinase/ERK kinase (MEK)-ERK and the PI3K/AKT pathways,

both of which can promote cell cycle progression by regulating the aforementioned factors. Thus, it is possible that YAP may indirectly exert its mitogenic effect by activating ERK and AKT signaling.

YAP also has an anti-apoptotic effect against chemotherapeutic drugs. For example, YAP has been shown to confer resistance to cisplatin- and doxorubicin-induced apoptosis in ovarian cancer, hepatocellular carcinoma, and esophageal squamous cell carcinoma (Huo et al., 2013; Imanaka et al., 2011; Mao et al., 2014). Consistent with these reports, we found that YAP conferred resistance to 5-Fu—induced apoptosis in cSCC cells. The YAP-downstream effectors involved in apoptosis protection of cSCC cells remains unknown. Although other studies have implicated Bcl-2, Bax, and the p53 pathway in YAP-regulated apoptosis (Bai et al., 2013; Basu et al., 2003; Ma et al., 2014; Zagurovskaya et al., 2009), we didn't observe changes in these proteins upon alteration of YAP expression (Figure 3c and f and see Supplementary Figure S2c). The molecular details of YAP-mediated apoptosis protection require further investigation. However, the RAF/MEK/ERK and PI3K/AKT pathways are activated by YAP and are known to promote cell survival. Therefore, it is reasonable to postulate that YAP may protect cSCC cells against apoptosis by activating these two pathways.

Lastly, we showed that YAP regulated the migration and invasion ability of cSCC cells. Although overexpression of YAP had marginal effects on cell migration or invasion, likely because of a high basal expression level of endogenous YAP, down-regulation of YAP significantly inhibited migration and invasion, indicating the necessity of YAP for these cellular activities. YAP knockdown also reduced the expression of MMP-2 and MMP-9 in accordance with the impaired cell invasiveness. These findings are consistent with results from others showing that YAP regulates epithelial-mesenchymal transition (Diepenbruck et al., 2014; Shao et al., 2014; Zhang et al., 2014) and contributes to metastasis in many cancers (Fu et al., 2014; Lau et al., 2014; Pei et al. 2015; Piccolo et al., 2014).

The simultaneous activation of both the RAF/MEK/ERK and PI3K/AKT pathways by YAP raised the question of whether the common upstream regulator of the two pathways, RAS, is activated by YAP. Indeed, a RAS pull-down experiment confirmed that RAS activity was regulated by YAP. A previous model proposed by Zhang et al. (2009) showed that YAP promotes the transcription of an EGFR ligand, AREG, that binds to and activates EGFR and its downstream RAS signaling. Consistent with this model, we found that YAP regulated the expression of AREG in cSCC cells. Moreover, AREG stimulated cSCC cell growth and partially recued the growth inhibitory effect of YAP depletion. Thus, in cSCC cells, YAP may enhance the EGFR/RAS signaling pathway by transcriptional activation of AREG. The activated RAS protein further activates the downstream RAF/MEK/ERK and PI3K/AKT signaling, which promote the proliferation, survival, and migration/invasion of cSCC cells (see Supplementary Figure S6 online).

In conclusion, this work showed that YAP plays an important role in human cSCC and therefore may serve as a new target for early prevention, treatment, and prognostic purposes.
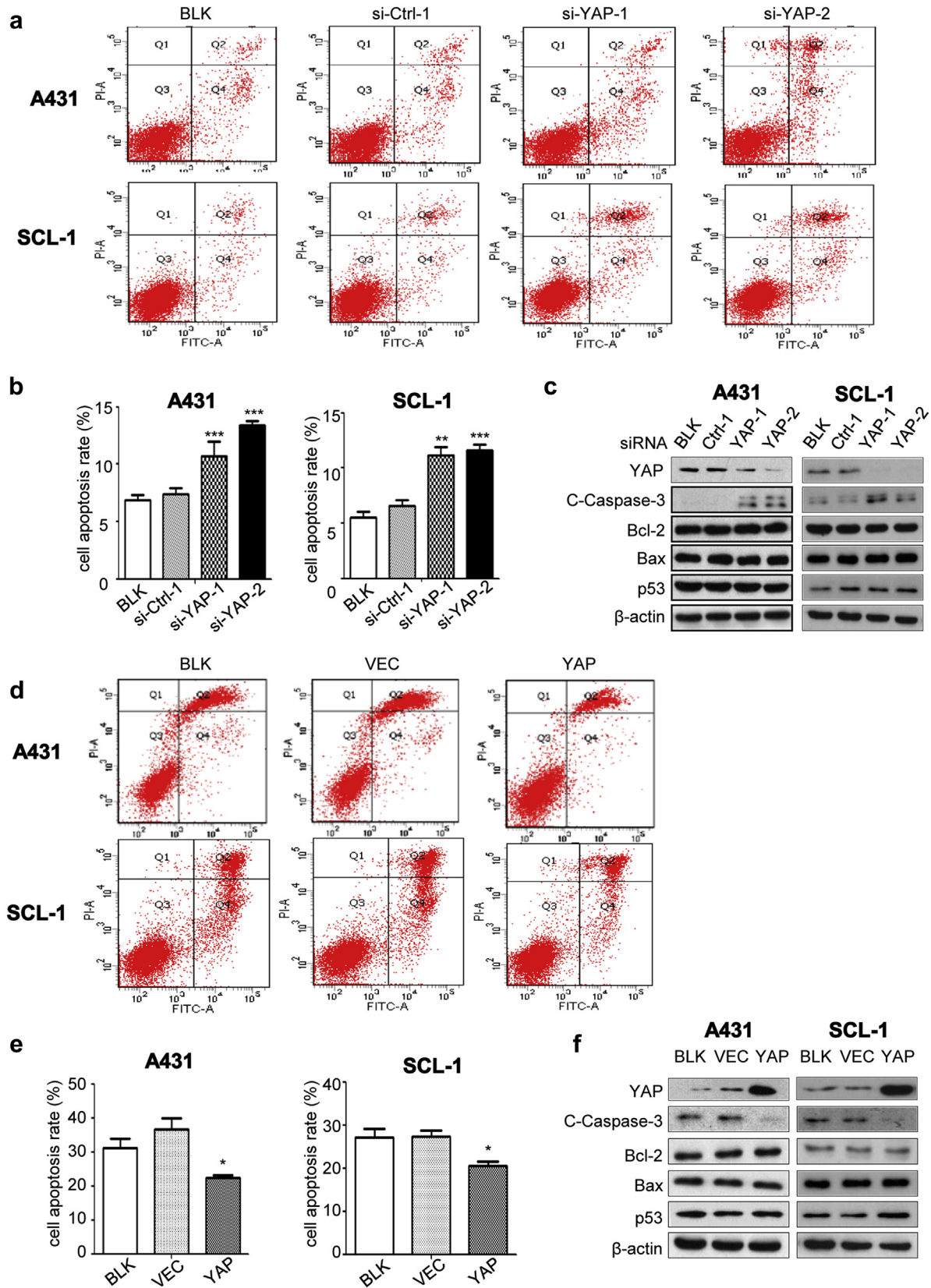
Figure 3. **Effect of down-regulation and overexpression of YAP on apoptosis.** (a) Cell apoptosis was analyzed by annexin V/propidium iodide staining and flow cytometry after 48 hours of YAP-siRNA transfection. (b) Quantification of the results from **a**. (d) Cells were transfected with YAP plasmid for 24 hours before the addition of 5 μg/ml of 5-fluorouracil. After another 24 hours, cell apoptosis was analyzed. (e) Quantification of the results from **d**. Cells were transfected with (c) YAP siRNA or (f) YAP plasmid for 48 hours and lysed for Western blot analysis on indicated proteins. β-Actin was used as an internal control. All the quantitative data were presented as mean ± standard error of the mean (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. BLK, blank transfected group; Ctrl, control; FITC, fluorescein isothiocyanate; si, small interfering; VEK, vector transfected group.
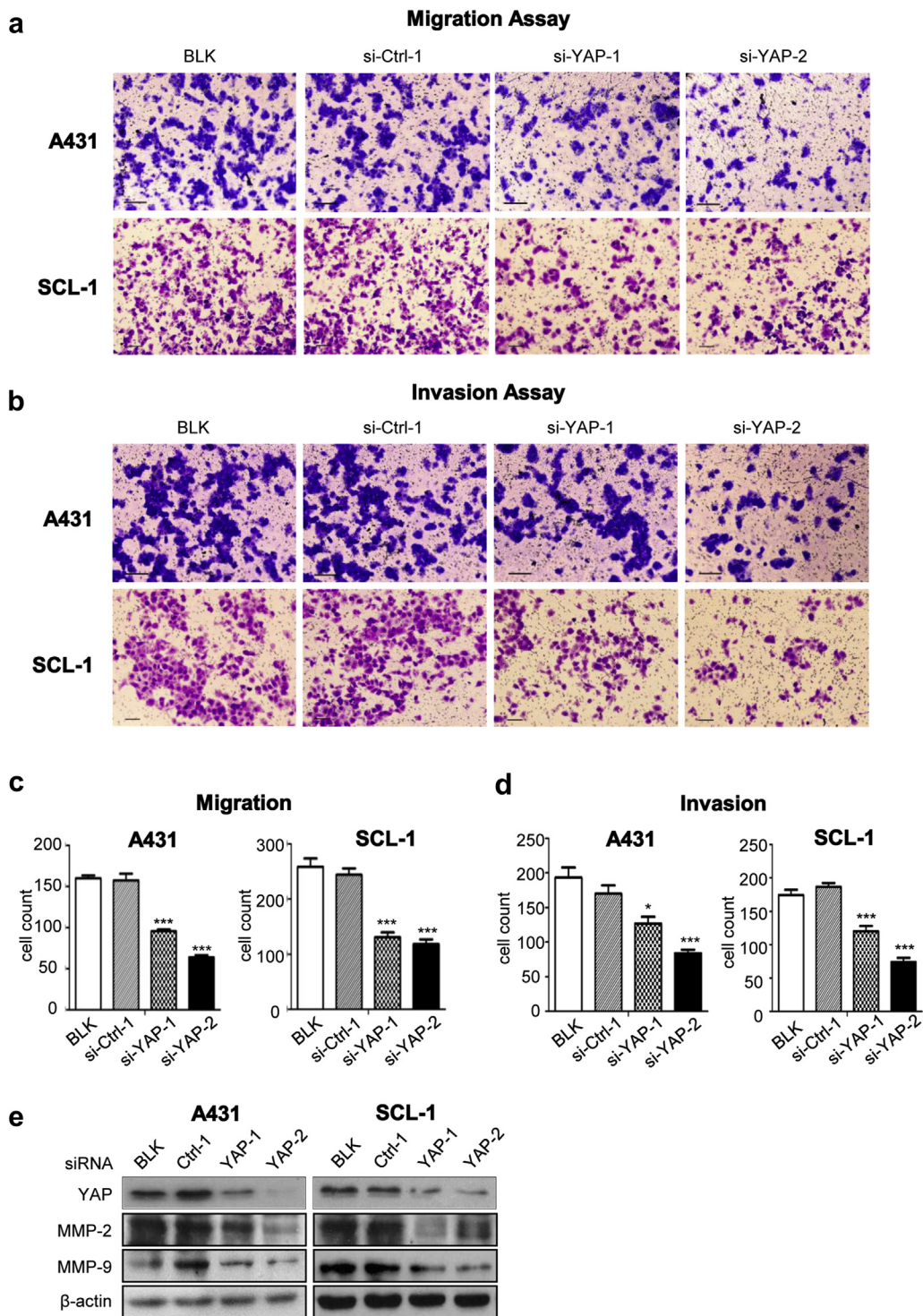
Figure 4. Effects of down-regulation of YAP on migration/invasion ability of cutaneous squamous cell carcinoma cells. Cells were transfected with YAP siRNA for 48 hours, and Transwell assays were performed to measure **(a)** cell migration or **(b)** invasion. Bar length = 100μm. **(c)** Quantification of results from **a**. **(d)** Quantification of results from **c**. **(e)** The protein expressions of MMP-2 and MMP-9 after YAP knockdown were analyzed using Western blot analysis, and β-actin was used as an internal control. Quantitative data were presented as mean ± standard error of the mean (n = 6 for migration assay, n = 5 for invasion assay). *$P < 0.05$, ***$P < 0.001$. BLK, blank transfected group; MMP, matrix metallopeptidase; si, small interfering.

MATERIALS AND METHODS

Patient samples

Patient samples were obtained from the tissue bank of the Department of Dermatology at the Second Affiliated Hospital of Xi'an Jiaotong University. There were a total of 160 samples collected, including 35 well-differentiated cSCCs (22 male and 13 female, age range = 46−91 years), 30 moderately/poorly differentiated cSCCs (16 male and 14 female, age range = 36−91 years), 34 Bowen's disease (22 male and 12 female, age range = 39−89 years), 31 AK (13 male and 18 female, age range = 37−83 years), and 30 normal skin tissues (18 male and 12 female, age range = 43−89 years) obtained from cosmetic surgery. The differentiation stages of all
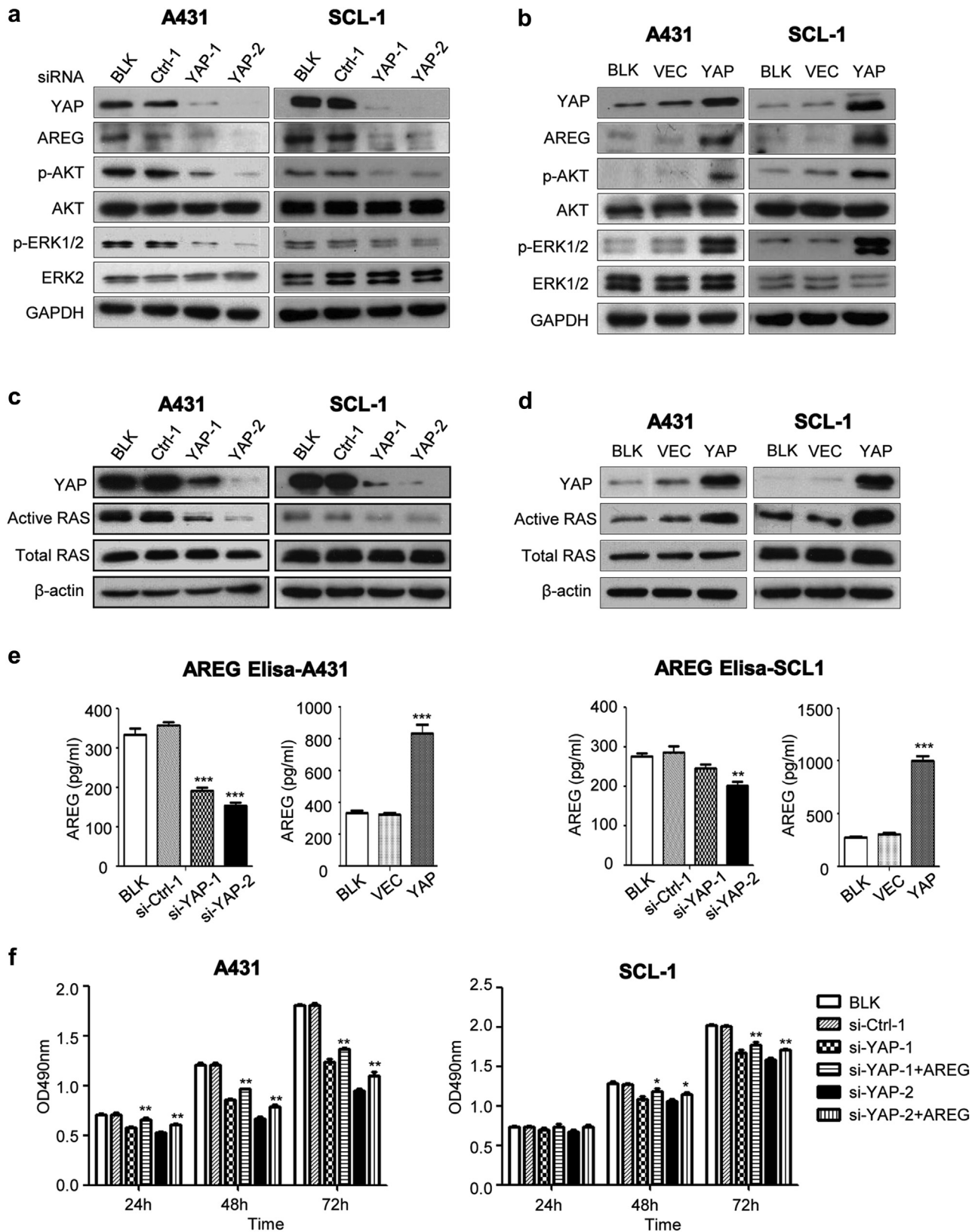
Figure 5. Effects of down-regulation and overexpression of YAP on RAS signaling. The expressions of AREG, AKT, phospho-AKT, ERK1/2 and phospho-ERK1/2 were analyzed by Western blot analysis in A431 and SCL-1 cells transfected with either **(a)** YAP-siRNA or **(b)** YAP-plasmid. GAPDH was used as a loading control. RAS pull-down assays on cutaneous squamous cell carcinoma cells transfected with **(c)** YAP-siRNA or **(d)** YAP-plasmids. **(e)** ELISA assays measuring the concentrations of AREG in conditioned medium (24 hours) of A431 or SCL-1 cells. **(f)** MTT assays of control or YAP-depleted cutaneous squamous cell carcinoma cells in the presence or absence of AREG. Quantitative data are presented as mean ± standard error of the mean (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. AREG, amphiregulin; BLK, blank transfected group; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, hours; MMT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; si, small interfering; VEK, vector transfected group.
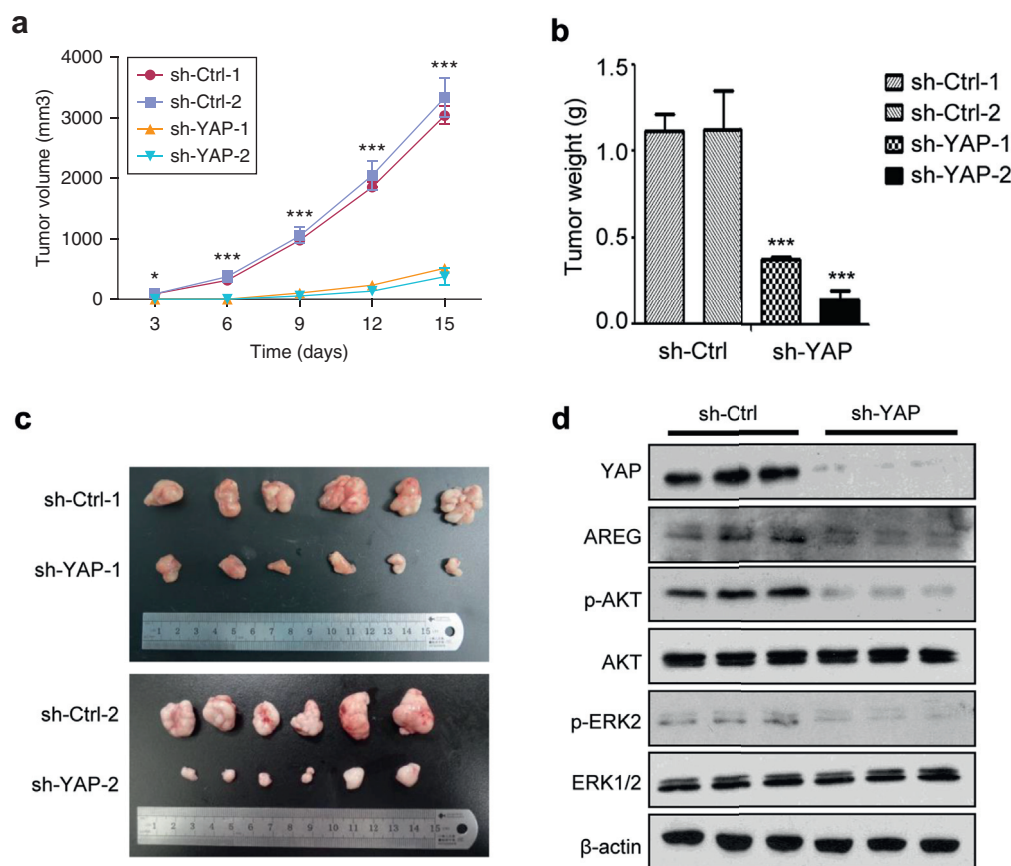
Figure 6. YAP depletion inhibits the growth of cutaneous squamous cell carcinoma tumor xenograft in vivo. (a) The growth curve of the sh-Ctrl or sh-YAP containing tumor xenograft in the nude mouse model. (b) Comparison of the average weight (n = 6) among the sh-Ctrl-1, sh-Ctrl-2, sh-YAP-1, and sh-YAP-2 xenografts at the end point. (c) Comparison of the sizes of tumors from sh-Ctrl or sh-YAP xenografts at the end of the experiment (day 15). (d) Western blot analysis of YAP, AREG, p-AKT, AKT, p-ERK and ERK expressions in sh-Ctrl or sh-YAP xenografts. β-Actin is used as an internal control. The graph shows the mean ± standard error of the mean (n = 6). * $P < 0.05$, ***$P < 0.001$. AREG, amphiregulin; ERK, extracellular signal-regulated kinase.

samples were determined by two experienced pathologists. Written informed consent for tissue procurement was obtained from all patients before study initiation, and ethics approval was obtained from the Institutional Ethics Committee of Xi'an Jiaotong University.

Immunohistochemistry

Immunohistochemical staining was performed using a standard immunoperoxidase staining procedure (Pinheiro et al., 2008). The staining results were evaluated under microscope by two independent pathologists and quantified based on the following scoring system. A positive rate score was first assigned according to the percentage of positive cells ($\leq 5\% = 0$, $6-25\% = 1$, $26-50\% = 2$, $51-75\% = 3$, $>75\% = 4$). A second score of staining intensity was then assigned (colorless = 0, light yellow = 1, yellowish brown = 2, chocolate brown = 3). The overall score for each microscopic field was calculated by the product of the two scores. The average score of five fields was taken as the final score of YAP expression for each slide.

Cell culture

The human cSCC cell line A431 was obtained from ATCC (USA) through an authorized local ATCC dealer (Xiangf Biotechnology, Shanghai, China). SCL-1 (Boukamp et al., 1982) and HSC-1 (Kondo and Aso, 1981) cells were from Beijing Beinachuanglian Biotechnology Research Institute (Beijing, China). The cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were routinely cultured in a humidified incubator at 37°C and 5% CO_2. Lysates of primary human epidermal keratinocytes were kindly provided by Dr. Andrew Aplin at Thomas Jefferson University, Philadelphia, PA, USA. These keratinocytes were isolated from neonatal foreskin tissues.

Transient transfection of YAP siRNA and plasmid

The siRNA oligonucleotides were synthesized by Shanghai Gene-Pharma (Shanghai, China), and their sequences are shown in Supplementary Table S1 online. YAP expression plasmid was obtained from Addgene (Cambridge, MA) (pcDNA Flag YAP, Plasmid #18881). Cells were transfected with siRNAs or plasmids according to the recommended procedures for Lipofectamine2000 Transfection Reagent (Invitrogen, Carlsbad, CA).

Lentivirus transduction

Lentiviruses of control nontargeting shRNAs (sh-Ctrl-1 and sh-Ctrl-2) and YAP targeting shRNAs (sh-YAP-1 and sh-YAP-2) were from KeyGEN Biotech (Nanjing, China). The shRNAs share the same core sequences of their siRNAs counterparts listed in Supplementary Table S1. Transduction was carried out by adding 10 μl of virus suspension (titer 1 × 10^9 transducing units/ml) to cells cultured in l ml complete culture medium containing 5 μg/ml Polybrene (YEA-SEN Biotechnology Co. Ltd., Shanghai, China). Forty-eight hours after transfection, cells were selected with puromycin (5 μg/ml) containing medium for 2 weeks.

Quantitative Real-Time PCR

Total RNA was extracted from the cells using the Trizol reagent (Invitrogen, Carlsbad, CA) and then reverse transcribed into complementary DNA. The PCR primers were synthesized by Sangon Biotech (Shanghai, China). Primer sequences are shown in Supplementary Table S2 online. Reactions were performed using SYBR premix EX Taq I (Takara, Japan) and analyzed by the 7500 real-

time PCR system (Applied Biosystems, Foster City, CA). Relative mRNA levels were calculated using the comparative Ct (ΔCt) method. Experiments were repeated three times, and three technical replicates were included for each data point.

Western blot analysis

Protein expression levels were analyzed by standard Western blotting protocols. The following antibodies were used in this study: YAP (#4912), AKT (pan) (#4691), ERK 1/2 (#9102), phospho-AKT (#2965), phospho-ERK 1/2 (#4376), cleaved caspase-3 (#9664), Bcl-2 (#2870), and BAX (#2872); antibodies were from Cell Signaling Technology (Danvers, MA, USA). Cyclin A (sc-596), cyclin B1 (sc-595), cyclin D1 (sc-246), cyclin E (sc-247), p53 (sc-126), MMP-9 (sc-10737), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724), and β-actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). CDK1 (cdc2) (19532-1-AP) and MMP-2 (10373-2-AP) antibodies were purchased from Proteintech (Chicago, IL, USA). Puma (ab33906) and Noxa (ab140129) antibodies were from Abcam (Cambridge, UK). AREG antibody (AF262) was from R&D Systems (Minneapolis, MN, USA).

Cell proliferation assay

Cell proliferation was assessed using the MTT assay. Cells were seeded at a density of 5×10^3 cells per well in 96-well plates and transfected with siRNA or plasmids the next day in the presence or absence of recombinant AREG (R&D Systems). After that, relative cell numbers were measured every 24 hours by incubating cells with 0.5 mg/ml MTT followed by optical density (OD) reading. Mean readings from three independent experiments were plotted for each time point. Five technical replicates were performed for each data point.

PI cell cycle analysis

Cells were harvested, fixed with 70% ethanol, and stored overnight at −20°C. For the analysis, PI staining solution (50 μg/mL PI and 100 μg/mL ribonuclease A) was added to the cells, which were then incubated for 30 minutes in the dark at 37°C. The cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA USA). Modifit version 3.3 software (Verity Software House, USA) was used to analyze the results. Three independent experiments were performed.

Annexin V/PI staining

Plasmid-transfected cells were treated with 5 μg/ml of 5-Fu for 24 hours before harvest. The siRNA-transfected cells were directly harvested 48 hours after transfection. For the analysis, cells were stained with the annexin V-fluorescein isothiocyanate/PI Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China), following the manufacturer's instructions. Stained cells were analyzed by flow cytometry (FACSCalibur from BD Biosciences). Three independent experiments were performed.

Transwell migration and invasion assay

For the migration assay, 1×10^5 siRNA⁻ or plasmid-transfected cells (48 hours posttransfection) were added to each Transwell chamber (8-μm pore size, EMD Millipore) seated in a 24-well plate and 500 μl of culture medium with 10% fetal bovine serum was added to the lower chamber. After culturing for 24 hours, the residual cells on the top surface of the membrane were gently wiped out with a cotton swab. The cells that migrated to the bottom surface of the membrane were stained with staining solution (0.1% crystal violet). The average number of migrated cells was obtained from countings of five microscopic fields (up, down, left, right, and middle). Invasion assay was performed similarly, except that the Transwell chamber membrane was precoated with Matrigel (BD Biosciences) and the culture time was extended to 36 hours. Migration and invasion assays were repeated six and five times, respectively, and three technical replicates were performed for each data point.

RAS pull-down assay

Active RAS-guanosine triphosphate was pulled down from the whole cell lysates by using the Active RAS detection kit (Cell Signaling Technology) following the manufacturer's instructions and was detected by Western blot analysis.

AREG ELISA

AREG protein levels in conditioned medium were measured by a Human Amphiregulin Quantikine ELISA Kit from R&D Systems following the manufacturer's instructions. Three independent experiments were performed, and three technical replicates were included for each data point.

Animal studies

Female BALB/c/nu nude mice (4−5 weeks old) were purchased from Beijing Vital River Company (Beijing, China) (License number: SCXK (Jing) 2012-0001). Six animals were individually inoculated subcutaneously on their left and right flanks with 5×10^6 sh-Ctrl-1 A431 and sh-YAP-1 A431 cells, respectively. The other six animals were similarly inoculated with sh-Ctrl-2 A431 and sh-YAP-2 A431 cells. Tumor sizes were measured every 3 days with a digital microcaliper, and tumor volumes were calculated using the following formula: tumor volume = length × width × width/2. After 15 days, the mice were killed and the tumors were excised for weight measurement, Western blot analysis and histological analysis. All animal protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Statistical analysis

All data are presented as the mean ± standard error of the mean. The SPSS statistical package (SPSS, Chicago, IL, USA) was used for data analysis. The Pearson chi-square test and Wilcoxon signed rank test were used for immunohistochemistry analysis. The Student *t* test was used for comparisons between two groups, and one-way analysis of variance was used for experiments with more than two groups. $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.02.005.

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