Fibulin-3 Has Anti-Tumorigenic Activities in Cutaneous Squamous Cell Carcinoma

Xin Wang1,5, Qing Zhang2,5, Changji Li1,3, Xiaoyan Qu1, Peiwen Yang1, Jinjing Jia1, Liumei Song1, Wenxin Fan1, Huiling Jing1 and Yan Zheng1

Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer. Several previous studies have shown that fibulin-3 participates in the occurrence and development of various tumors; however, its role in cSCC remains unknown. In the present study, we observed that the expression of fibulin-3 was downregulated in cSCC tissues compared with normal skin tissues, which was due to fibulin-3 promoter methylation. In vitro, knockdown of fibulin-3 in cSCC cell lines A431 and SCL-1 cells promoted cell proliferation, protected cells against apoptosis and enhanced migration and invasion abilities. Conversely, overexpression of fibulin-3 inhibited cell proliferation by promoting growth arrest during the G1/S phase transition, induced apoptosis, and reduced migration and invasion abilities. These anticarcinogenic effects of fibulin-3 were associated with inhibition of the AKT signaling pathway. Through a mouse xenograft model, we found that fibulin-3 overexpression inhibited the cSCC tumor growth in vivo. Our results suggest that fibulin-3 has anti-tumorigenic activities in cSCC. Downregulation of fibulin-3 is involved in cSCC development and it may serve as a novel therapeutic target of this disease.


INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a keratinocyte-derived skin malignant tumor. It is the second most common non-melanoma skin cancer worldwide (Brougham and Tan, 2014). cSCC accounts for 15–20% of all cutaneous malignancies, with an increasing incidence rate (Allen and Stolle, 2014). Therefore, there is an urgent need to study novel molecular mechanisms responsible for the development and progression of cSCC.

Fibulin-3 (EFEMP1) is a widely expressed extracellular matrix glycoprotein that is vital to the composition and stability of the extracellular matrix (Marmorstein et al., 2002). It belongs to the fibulin family, and the gene coded for fibulin-3 is EFEMP1 (Giltay et al., 1999). Subsequent studies have shown that fibulin-3 plays an important role in the occurrence and development of a variety of tumors. The methylation of the fibulin-3 promoter decreases fibulin-3 expression in some tumors, which in turn promotes tumor growth and metastasis, and protects tumor cells against apoptosis (Almeida et al., 2014; Kim et al., 2012; Sadr-Nabavi et al., 2009, Tong et al., 2011). It is now considered as a biomarker for poor prognosis in prostate cancer (Almeida et al., 2014), lung cancer (Chen et al., 2014; Kim et al., 2012), hepatocellular carcinoma (Luo et al., 2013), sporadic breast cancer (Sadr-Nabavi et al., 2009), and colorectal cancer (Tong et al., 2011). In other tumors, such as urothelial carcinoma (Han et al., 2017), malignant glioma or glioblastoma (Hu et al., 2009; Nandhu et al., 2017), pancreatic adenocarcinoma (Seeliger et al., 2009) and cervical cancer (Song et al., 2011), fibulin-3 expression was upregulated and the changes of this protein were correlated with tumor cells proliferation, migration, invasion, and poor prognosis of the disease. Thus, the contribution of fibulin-3 in cancer development is context-specific (Albig et al., 2006). To the best of our knowledge, its role in cSCC has not been reported. In the present study, we investigated fibulin-3 expression in cSCC patients. Then we examined the biological function of fibulin-3 by overexpressing and knocking down its expression in cSCC cell lines A431 and SCL-1 cells. Our results indicated that fibulin-3 expression was decreased in cSCC and that downregulation of fibulin-3 contributed to the progression of cSCC by enhancing the AKT signaling pathway.

RESULTS

Fibulin-3 expression is downregulated in cSCC

First, we investigated fibulin-3 protein expression by immunohistochemistry in cSCC and normal skin samples (Figure 1a–1f). Fibulin-3 was expressed in the extracellular matrix of full-thickness normal skin epidermis. The positive rate for fibulin-3 staining was 73.3% (22 of 30) in normal skin samples. However, fibulin-3 was only weakly expressed in the cytoplasm of, and the matrix around, the well-differentiated cells in cSCC samples. The positive rates for fibulin-3 staining were 33.3% (10 of 30) and 26.9% (7 of 26) in well-differentiated and moderately/poorly differentiated cSCC samples, respectively. The positive rate of fibulin-3 staining in cSCC samples was
Figure 1. Analysis of fibulin-3 expression and promoter methylation in cSCC. Immunohistochemical staining of fibulin-3 in normal skin (n = 30) (a, scale bar = 400 μm; b, scale bar = 200 μm). Well-differentiated cSCC (n = 30) (c, scale bar = 400 μm; d, scale bar = 200 μm). Moderately and poorly differentiated cSCC (n = 26) (e, scale bar = 400 μm; f, scale bar = 200 μm). (g) Semi-quantitative analysis of fibulin-3 staining. wd, well-differentiated; md, moderately differentiated; pd, poorly differentiated. (h) Quantitative real-time reverse transcriptase PCR analysis of fibulin-3 mRNA in primary human epidermal keratinocytes, keratinocyte cell line HaCaT cells, and two cSCC cell lines: A431 and SCL-1 cells. (i) Western blot analysis of fibulin-3 protein expression in primary HEK, HaCaT, A431 and SCL-1 cells. (j) Fibulin-3 mRNA expression in cSCC cells with or without 5-aza-2'-deoxycytidine treatment. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Bar graph was quantification of fibulin-3 mRNA levels. (k) Methylation status of fibulin-3 in cSCC cells. Bar graphs were quantification of fibulin-3 mRNA levels. cSCC, cutaneous squamous cell carcinoma; HEK, human epidermal keratinocyte; IVD, in vitro methylated DNA, positive control; M, primers for methylated DNA; U, primers for unmethylated DNA.
significantly lower than that in normal skin samples (Figure 1g; \( P < 0.001 \)). Then, we examined the mRNA and protein expression of fibulin-3 in cSCC cell lines: A431 and SCL-1 cells. Compared with the primary human epidermal keratinocytes and the keratinocytes cell line HaCaT, A431 and SCL-1 cells had significantly lower levels of fibulin-3 mRNA (Figure 1h) and protein (Figure 1i). Methylation of the promoter has been shown for fibulin-3 in liver and breast cancers (Nomoto et al., 2010; Sadr-Nabavi et al., 2009). To investigate the role of promoter methylation in fibulin-3 expression in cSCC, we treated A431 and SCL-1 cells with 5-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor. As shown in Figure 1j, 5-aza-2'-deoxycytidine restored fibulin-3 expression in both A431 and SCL-1 cells. Moreover, the methylation-specific PCR was performed to detect methylation of the fibulin-3 promoter in A431 and SCL-1 cells. The results indicate that the fibulin-3 promoter was methylated in cSCC cells (Figure 1k).

Fibulin-3 inhibits proliferation and promotes differentiation in cSCC cells
To test the potential effects of fibulin-3 in cSCC, we first overexpressed fibulin-3 using lentivirus vectors (LV5s) in two cSCC cell lines, A431 and SCL-1 (Figure 2a, 2b). As indicated in Supplementary Figure S1 online, fibulin-3 was detected in both the culture medium and cell lysate of fibulin-3-overexpressing cells. Overexpression of fibulin-3 decreased cell proliferation (Figure 2c) and led to growth arrest in the G1/S stage transition (Figure 2d) in both cell
Consistently, the expression of cell cycle regulator CDK2 was also reduced in fibulin-3 overexpressing cells (Figure 2e). Then, we knocked down fibulin-3 with small interfering RNAs in A431 and SCL-1 cells (Figure 3a, 3b). The results showed that depletion of fibulin-3 promoted proliferation, increased the population of S-phase cells, and induced
the expression of CDK2 in both cell lines (Figure 3c–3e). The propidium iodide staining plots were presented in Supplementary Figure S2 online. Taken together, these results indicate that fibulin-3 inhibits cell proliferation via regulating the G1/S progression in cSCC cells. Differentiation is reduced in skin cancer cells. To investigate the role of fibulin-3 on epidermal differentiation, we overexpressed fibulin-3 using LV5s in A431 and SCL-1 cells. Epidermal differentiation was determined by measuring the expression of involucrin, loricrin, and S100A4, three proteins involved in epidermal differentiation. As shown in Figure 3f, fibulin-3 overexpression significantly increased involucrin, loricrin, and S100A4 expression in A431 and SCL-1 cells. Consistently, fibulin-3 knockdown with small interfering RNAs resulted in downregulation of these three proteins in human epidermal keratinocytes and HaCaT cells (Figure 3g). Moreover, fibulin-3 knockdown also increased cell viability in human epidermal keratinocytes and HaCaT cells (Figure 3h). Thus, fibulin-3 promotes epidermal differentiation.

Fibulin-3 promotes apoptosis in cSCC cells

In order to detect the role of fibulin-3 in cell apoptosis, we measured apoptosis by Annexin V/7-aminoactinomycin D staining and flow cytometry. Nontransfected or negative control LV5-transfected cells showed a low basal apoptosis rate of about 3% in both A431 and SCL-1 cells. Overexpression of fibulin-3 increased the apoptosis rate to 12–14% (Figure 4a, 4b). The increased apoptosis was associated with a lower expression of Bcl-2 and a higher expression of p53, indicative of the activation of the apoptosis pathway (Figure 4c). The basal apoptosis levels were low in A431 and SCL-1 cells; therefore, to evaluate the effects of fibulin-3 depletion on apoptosis, we used 5-fluorouracil to induce cell apoptosis. The results showed that 5-fluorouracil increased the apoptosis rate to around 30% in blank and negative control transfected cells. Fibulin-3 knockdown decreased 5-fluorouracil–induced apoptosis to 14–19% in both A431 and SCL-1 cells (Figure 4d, 4e), which was associated with increased Bcl-2 expression and reduced p53 expression (Figure 4f). However, both A431 and SCL-1 cells have p53 mutations (Popp et al., 2002; Somers et al., 1992). To further define the role of the p53 pathway in fibulin-3’s effect on cell proliferation and survival, we analyzed p53 mediators p21 and Noxa expression in fibulin-3 overexpressing or knockdown A431 and SCL-1 cells. As shown in Figure 4c and 4f, the expression of p21 was increased in fibulin-3 overexpressing cells and decreased in fibulin-3 knockdown cells. But fibulin-3 overexpression or knockdown had no effects on Noxa expression in A431 and SCL-1 cells. Taken together, these results suggest that downregulation of fibulin-3 protects cSCC cells against apoptosis; however, the role of the p53 pathway in apoptosis warrants further investigation.

Fibulin-3 decreases the migration and invasion ability of cSCC cells

To explore the impact of fibulin-3 on the migration and invasion ability of cSCC cells, we performed in vitro Transwell assays. Overexpression of fibulin-3 significantly reduced the migration and invasion ability of both A431 and SCL-1 cells (Figure 5a–5d). Conversely, knockdown of fibulin-3 increased their migration and invasion ability (Figure 5e–5h). Therefore, fibulin-3 decreases the migration/invasion ability of cSCC cells.

Fibulin-3 reduces cSCC cell growth by inhibiting the AKT signaling pathway

A recent study (Hwang et al., 2010) has shown that fibulin-3 suppresses nasopharyngeal carcinoma cell migration and invasion by diminishing the activity of phospho-AKT. To
Figure 4. Effects of fibulin-3 overexpression or knockdown on cutaneous squamous cell carcinoma cell apoptosis. (a) Flow cytometry analysis of apoptosis in A431 and SCL-1 cells at 48 hours after being transfected with fibulin-3 overexpressed or control lentivirus vectors. (b) Quantification of the results from (a). (c) Western blot analysis on indicated proteins at 48 hours after transfection. Bar graph was quantification for the Western blotting result. (d) Flow cytometry analysis of apoptosis in A431 and SCL-1 cells after transfected with fibulin-3 or control siRNA and treatment with 5 mg/ml of 5-fluorouracil (5-FU). (e) Quantification of the results from (d). (f) Western blot analysis on indicated proteins at 48 hours after transfection. β-actin was used as an internal control. Bar graph was quantification for the Western blotting result. n = 3 per group, mean ± standard error of the mean, **P < 0.01, ***P < 0.001. BLK, blank transfected group; cSCC, cutaneous squamous cell carcinoma; Ctrl, control; FBL3, fibulin-3; LV5, lentivirus 5; NC, negative control; si, small interfering.
To clarify the molecular mechanisms of fibulin-3’s effects on cSCC, we analyzed AKT, a critical signaling pathway involved in the regulation of cell proliferation, survival, and migration. Fibulin-3 overexpression blocked the AKT pathway as demonstrated by the reduction in the level of phospho-AKT, but not in total AKT (Figure 5i). On the
contrary, knockdown of Fibulin-3 activated the AKT pathway by promoting AKT phosphorylation (Figure 5j). Fibulin-3 contains a signal peptide, five tandem arrays of calcium binding EGF domains preceded by a modified calcium binding EGF domain. To investigate the role of the EGF receptor in fibulin-3-associated AKT activation, we treated fibulin-3 knockdown cSCC cells with AG-1478, a specific inhibitor of EGF receptor kinase. As shown in Figure 5k, AG-1478 reduced phosphorylation of AKT in fibulin-3 knockdown cells, suggesting the EGF receptor is upstream of AKT in cSCC cells. Moreover, the phosphorylation of PDK1 was decreased in fibulin-3 overexpressing cells (Supplementary Figure S3 online), as suggested by the phosphorylation of AKT at T308. On the other hand, the ERK signaling pathway was not affected by the overexpression of fibulin-3 (Supplementary Figure S3). These results suggest that downregulation of fibulin-3 contributes to the growth of cSCC cells by enhancing the AKT signaling pathway.

Overexpression of fibulin-3 constrains cSCC tumor growth in vivo

We used a mouse xenograft model to investigate the impact of fibulin-3 on cSCC tumor growth in vivo. A431 and SCL-1 cells that stably express negative control (A431 LV5-NC and SCL-1 LV5-NC) or overexpressed fibulin-3 (A431 LV5-FBL3 and SCL-1 LV5-FBL3) were subcutaneously implanted into the right flank of nude mice. Tumor growth was monitored over time. As shown in Figure 6a–6d, fibulin-3 overexpression considerably inhibited tumor growth in vivo. Immunohistochemistry showed that Ki67 and Bcl-2 levels were decreased in fibulin-3 overexpressing tumor tissues (Figure 6e), suggesting an anti-proliferation and pro-apoptosis role of fibulin-3 in vivo. The overexpression efficiency of fibulin-3 was confirmed by Western blot and immunohistochemistry analyses at the end point of the experiment (Figure 6e, 6f). Fibulin-3 knockdown, on the other hand, promoted tumor growth in vivo (Supplementary Figure S4a–Sf online).

DISCUSSION

In recent years, an increasing number of studies have implicated the role of fibulin-3 in occurrence and progression of various tumors. However, to our knowledge, whether it has an effect on the development of cSCC has not been reported. In the present study, we provided evidence that fibulin-3 was downregulated in cSCC and then found that fibulin-3 inhibited cSCC cell proliferation, survival, and migration via blocking the AKT signaling pathway. Lastly, we used a mouse xenograft model to clarify that fibulin-3 overexpression inhibited cSCC tumor growth in vivo.

Fibulin-3 regulates the growth of various tumors in a context-specific manner. According to our data, fibulin-3 was downregulated in cSCC due to its promoter methylation. Fibulin-3 suppressed cSCC cell growth by blocking G1/S phase transition, reduced the expression of CDK2, a G1/S regulator, and promoted epidermal differentiation, whereas depletion of fibulin-3 reversed this regulation pattern. In pancreatic cancer, however, an opposite effect of fibulin-3 has been observed. Seeliger et al. (2009) found that overexpression of fibulin-3 promoted cell cycle progression in human pancreatic adenocarcinoma cells. Cell cycle shifted from the G0–G1 phase toward the S phase and mitosis in fibulin-3 overexpressed pancreatic adenocarcinoma cells. The specific mechanism of molecular regulation of fibulin-3 in different cancers is not clear and apparently warrants further investigation. Weng et al. (2001) revealed that the tumor suppressor PTEN in breast cancer cells causes G1/S
arrest mediated by the PI3K/AKT pathway. Fibulin-3 blocked the signaling pathways involved in cell growth in lung cancer and gliomas (Hu et al., 2011; Kim et al., 2012). In our current study, we also found fibulin-3 blocked the AKT signaling pathway through an EGF receptor-dependent mechanism in cSCC. Thus, fibulin-3 may play a mitogenic role indirectly by blocking the AKT signaling pathway in cSCC.

Here, we also found that fibulin-3 could promote cSCC cells apoptosis. Consistent with our finding, Almeida et al. (2014) showed that depletion of fibulin-3 protected tumor cells against apoptosis in prostate cancer. However, Seeliger et al. (2009), found that overexpressed fibulin-3 reduced cell apoptosis in human pancreatic adenocarcinoma cells. In the current study, we also found that fibulin-3 overexpression promoted apoptosis, as fibulin-3 knockdown decreased 5-fluorouracil-induced apoptosis in cSCC cells. It has been confirmed in many studies that by regulating the PI3K/AKT pathway, cells can resist the apoptosis induced by various factors, such as TNF-α and hypoxia (Alvarez-Tejado et al., 2001; Osawa et al., 2001). Accordingly, we have reason to assume that fibulin-3 may promote cSCC cell apoptosis by blocking the AKT pathway.

Lastly, we studied the influence of fibulin-3 on cSCC cell migration and invasion. We found that overexpression of fibulin-3 significantly inhibited cell migration and invasion, while knockdown fibulin-3 achieved an opposite result. Because of the low basal expression of endogenous fibulin-3, the result from the knockdown experiment was not as prominent as that from the overexpression experiment. Several previous studies have proved that fibulin-3 inhibited some tumor cell migration and invasion by reducing AKT phosphorylation and blocking the PI3K/AKT pathway.

Figure 6. Fibulin-3 overexpression inhibits cutaneous squamous cell carcinoma tumor growth in mice. (a) The representative picture of nude mice implanted with either LV5-NC or LV5-FBL3 transfected cutaneous squamous cell carcinoma cells. (b) The growth curves of the tumor xenograft in nude mice. Comparison of the average weight (c) and sizes (d) of the tumors at the end of the experiment (day 15). (e) Immunohistochemical staining of Ki67, Bcl-2 and fibulin-3 in xenograft tumors. Bar length = 200 μm. (f) Western blot analysis of fibulin-3, p-AKT, AKT expressions in xenografts. β-actin was used as an internal control. n = 6 per group, mean ± standard error of the mean, *P < 0.05, **P < 0.01, ***P < 0.001.
(Hwang et al., 2010); however, in some other tumors, fibulin-3 could promote the level of phospho-AKT (Camaj et al., 2009). Given the excess of molecular and cellular irregularities in cancer cells, such divergences may be associated with different membrane receptors and/or downstream signaling pathways in different types of cancers (Hu et al., 2011).

In summary, our results suggest that fibulin-3 has antitumorigenic activities in cSCC. Downregulation of fibulin-3 is involved in the development of cSCC and it may serve as a new biomarker of cSCC.

MATERIALS AND METHODS

Patient samples
A total of 56 cSCC samples, including 30 well-differentiated cSCCs (21 male and 9 female, aged 38–90 years) and 26 moderately/poorly differentiated cSCCs (15 male and 11 female, aged 48–92 years), along with complete clinical and pathological data were obtained from the tissue bank of the Department of Dermatology at The Second Affiliated Hospital of Xi’an Jiaotong University. Thirty normal skin tissues (14 male and 16 female, aged 28–85 years) obtained from cosmetic surgery were used as normal controls. The study protocol was approved by the Institutional Ethics Committee of Xi’an Jiaotong University, and written informed consent was obtained from all patients and control subjects before sample collection.

Immunohistochemistry
Formalin-fixed and paraffin-embedded sample sections were used for immunohistochemical staining as described previously (Pinheiro et al., 2008). The immunohistochemistry results were evaluated by two independent scientists blinded to the corresponding clinical-pathological data. The following antibodies were used in this study: anti-fibulin-3 antibody (sc-33722; Santa Cruz Biotechnology, Dallas, TX), anti–Bcl-2 antibody (sc-7382; Santa Cruz Biotechnology), and anti-Ki67 antibody (#12202; Cell Signaling Technology, Danvers, MA).

Cell culture
A431 cells and primary human epidermal keratinocytes were obtained from ATCC (Manassas, VA) in 2017. SCL-1 (Boukamp et al., 1982) and HaCaT cells were purchased from Beijing Beinaichuanglian Biotechnology Research Institute (Beijing, China) in 2017. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in a humidified incubator at 37°C and 5% CO2. The A431 and SCL-1 cells were pretreated with 1 μM AG1478 (Sigma-Aldrich, St Louis, MO) for 1 hour. 5-Aza-2′-deoxycytidine (5 μmol/l; Sigma-Aldrich) was used for 6 days to demethylate the cells.

DNA extraction, bisulfite modification, and methylation-specific PCR
Genomic DNA was extracted from human epidermal keratinocytes, HaCaT, A431, and SCL-1 cells using QIAamp DNA mini kit (Qiagen, Valencia, CA). Genomic DNA (400 ng) from each sample was modified by sodium bisulfite using EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA). Methylation-specific PCR was done using 2 μl bisulfite-modified DNA and 2 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) in a 20-μl final volume. Primers of methylated and unmethylated fibulin-3 were listed in Supplementary Table S1 online. PCR products were analyzed by electrophoresis on 2% agarose gels.

Fibulin-3 small interfering RNA transient transfection and lentivirus transduction
The small interfering RNA oligonucleotides of negative control and fibulin-3 were synthesized by Shanghai GenePharma Company (Shanghai, China) and their sequences are shown in Supplementary Table S2 online. Cells were transfected with small interfering RNAs according to the manufacturer’s instructions (Invitrogen). Lentiviruses of negative control, fibulin-3 overexpression (LV5), and fibulin-3 knockdown (LV3) were obtained from Shanghai GenePharma Company. Transduction was performed according to the manufacturer’s instructions.

Quantitative real-time PCR
Total RNA was extracted from the samples according to the manufacturer’s instructions (Invitrogen). cDNA was generated and then amplified with the SYBR Premix Ex TaqTM Kit (Takara Bio, Kusatsu, Shiga, Japan) using the ABI StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR primers for human fibulin-3 (NM_001039348.3), mouse fibulin-3Q2 (XM_011243695.1), human glyceraldehyde-3-phosphate dehydrogenase (NM_002046.7), and mouse glyceraldehyde-3-phosphate dehydrogenase (XM_017321385.1) are shown in Supplementary Table S3 online (Sangon Biotech, Shanghai, China).

Western blot
Western blot analysis was used to measure protein expression levels as we described previously (Jia et al., 2016). The antibodies against fibulin-3 (sc-33722), p53 (sc-126), cyclin A (sc-596), CDK2 (sc-6248), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology. AKT (pan) (#4691), phospho-AKT (Thr 308) (#2965), and Bcl-2 (#2870) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Involucrin (P07476), Loricrin (P23490), S100A4 (P26447), EGF receptor (P00533), PDK1 (O15530), AKT-S473 (P31749), and p21 (P38936) antibodies were obtained from Abways Technology (Shanghai, China).

Cell viability assay
The CCK8 assay was used to measure cell proliferation. The assay was performed according to the manufacturer’s instructions.

Cell cycle analysis
The cell cycle was analyzed as we described previously (Jia et al., 2016). Briefly, the cells were stained with propidium iodide solution and analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). At least three independent experiments were performed.

Apoptosis assay
Cell apoptosis was assessed using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. At least three independent experiments were performed.

Transwell migration and invasion assay
The migration and invasion assays were performed as we described previously (Jia et al., 2016). The assays were repeated four times and three technical replicates were performed for each data point.

Animal studies
Experiments were performed on female BALB/c nu nude mice (4–5 weeks old; Beijing Vital River Company, license number: SCXK [Jing] 2012-0001; Beijing, China). The mice were inoculated subcutaneously on their right flank with 5 × 106 cSCC cells. Tumor size was measured every 3 days for 15 days. Then, the mice were...
sacrificed and the tumors were harvested for various measurements. The animal protocol was approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University.

Statistical analysis
Results were expressed as the mean ± standard error of the mean. Student t test or one-way analysis of variance was used to analyze the differences between groups by SPSS software (Chicago, IL). P < 0.05 represents a significant difference.

Data availability statement
The data sets generated during and analyzed during the current study are available in the Dryad repository: https://doi.org/10.5061/dryad.q564pc4.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We would like to thank Yi Lv and his colleague from National Local Joint Engineering Research Center for Precision Surgery and Regenerative Medicine, Shaanxi Province for Physical Examination, and the National Natural Science Foundation of China (81573055, 81371732), and was partially supported by Funds of Shaanxi Province (2015JCTCL03-10) and The Second Hospital of Xi’an Jiaotong University.

AUTHOR CONTRIBUTIONS
Conceptualization: QZ, YZ; Data curation: XW; Formal analysis: XQ, LS; Funding acquisition: YZ; Investigation: XW; Methodology: CL, JJ; Project administration: XW; Resources: PY; Supervision: YZ; Validation: HJ; Visualization: WF; Writing - original draft: XW; Writing - review & editing: QZ.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.01.022.

REFERENCES
Supplementary Figure S1. Expression and release of FBL3 in cutaneous squamous cell carcinoma cells. Western blot analysis of FBL3 in the culture medium or cell lysate of A431 and SCL-1 cells with non-treated (blank), transfected with FBL3 overexpressed or control lentivirus vectors for 48 hours. β-actin was used as an internal control. Bar graph was quantification for the Western blotting result. n = 3 per group, mean ± standard error of the mean, *P < 0.05, **P < 0.01, ***P < 0.001. BLK, blank transfected group; FBL3, fibulin-3; LV5, lentivirus 5; NC, negative control.
Supplementary Figure S2. The propidium iodide staining plots for the cell cycle analysis. Cell cycle profiles were analyzed by flow cytometry at 48 hours after FBL3 overexpression or control lentivirus vector transfection in (a) A431 cells or (b) SCL-1 cells. Cell cycle profiles were analyzed by flow cytometry at 48 hours after FBL3 siRNA or control siRNA transfection in (c) A431 cells or (d) SCL-1 cells. BLK, blank transfected group; Ctrl, control; FBL3, fibulin-3; h, hours; LV5, lentivirus 5; NC, negative control; siRNA, small interfering RNA.
Supplementary Figure S3. The effect of FBL3 overexpression on p-ERK and p-PDK1 expression in A431 and SCL-1 cells. Western blot analysis of p-ERK, total ERK and p-PDK-1 in FBL3 overexpression A431 and SCL-1 cells. β-actin was used as an internal control. Bar graph was quantification for the Western blotting result. n = 3 per group, mean ± standard error of the mean, *P < 0.05. BLK, blank transfected group; Ctrl, control; FBL3, fibulin-3; LV5, lentivirus 5; NC, negative control.
Supplementary Figure S4. FBL3 knockdown promotes cutaneous squamous cell carcinoma tumor growth in mice. (a) The representative picture of nude mice implanted with either LV3-NC or LV3-FBL3-KD transfected cSCC cells. (b) The growth curves of the tumor xenograft in nude mice. Comparison of the average weight (c) and sizes (d) of the tumors at the end of the experiment (day 15). (e) Immunohistochemical staining of Ki67, Bcl-2 and FBL3 in xenograft tumors. Scale bar = 200 μm. (f) Western blot analysis of FBL3, p-AKT, AKT expressions in xenografts. β-actin was used as an internal control. n = 5 per group, mean ± standard error of the mean. *P < 0.05, **P < 0.01. FBL3, fibulin-3; KD, knockdown LV3; NC, negative control.
### Supplementary Table S1. The primers for methylation-specific PCR used in this study

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M, methylated sequence; U, unmethylated sequence.

### Supplementary Table S2. The sequences of small interfering RNAs used in this study

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### Supplementary Table S3. The sequences of primers for reverse transcriptase PCR used in this study

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GAPDH, glyceraldehyde-3-phosphate dehydrogenase.