Antitumor effect of focal adhesion kinase inhibitor PF562271 against human osteosarcoma *in vitro* and *in vivo*

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Focal adhesion kinase (FAK) overexpression is related to invasive and metastatic properties in different kinds of cancers. Target therapy by inhibiting FAK has achieved promising effect in some cancer treatments, but its effect in human osteosarcoma has not been well studied. In the present study, we analyzed the antitumor efficacy of PF562271, an FAK inhibitor, against osteosarcoma *in vitro* and *in vivo*. Phosphorylated FAK (Y397) was highly expressed in primary human osteosarcoma tumor samples and was associated with osteosarcoma prognosis and lung metastasis. PF562271 greatly suppressed proliferation and colony formation in human osteosarcoma cell lines. In addition, treatment of osteosarcoma cell lines with PF562271 induced apoptosis and downregulated the activity of the protein kinase B/mammalian target of rapamycin pathway. PF562271 also impaired the tube formation ability of endothelial cells *in vitro*. Finally, oral treatment with PF562271 in mice dramatically reduced tumor volume, weight, and angiogenesis of osteosarcoma xenografts *in vivo*. These results indicate that FAK inhibitor PF562271 can potentially be effectively used for the treatment of osteosarcoma.

Osteosarcoma (OS) is the most common primary malignant bone tumor and leads to a large number of cancer-related deaths in children and young adults, mainly due to the development of lung metastases.[1–3] The use of neo-adjuvant chemotherapy significantly increased 5-year survival of localized OS patients from approximately 20–60%.[1] However, there have been only minimal improvements in the prognosis of osteosarcoma patients in the last two decades.[4] Thus, a novel strategy that efficiently inhibits metastasis is demanded.

Focal adhesion kinase (FAK), a non-receptor cytoplasmic protein tyrosine kinase, is a key regulator of signals from the ECM mediated by integrin and growth factor receptors.[5] Focal adhesion kinase has been implicated in the regulation of a variety of cellular signaling pathways that control cell proliferation, cell cycle progression, migration, apoptosis, and cell survival.[6] Expression of FAK is reported to be upregulated in many tumor types including colon,[7] breast,[8] prostate,[9] thyroid,[10] liver,[11] and esophageal,[12] and elevated expression of FAK correlates with poor survival rates. In OS, it has been reported that phosphorylated (p-)FAK (Y397) highly expressed in human OS samples and the FAK expression profile were independent indicators of both overall and metastasis-free survival. Small interfering RNA-based knockdown of FAK not only dramatically reduced the migration and invasion of MG63 and 143B cells, but also had a distinct effect on OS cell proliferation and apoptosis.[13] These results collectively suggest that FAK could be a potential target for the treatment of OS.

Several small molecule inhibitors of FAK have been described lately. Most notably, PF562271 is a potent small molecule inhibitor of FAK. PF562271 is reported to be efficacious in inhibiting the proliferation of tumors in both xenograft and transgenic mouse models.[14,15] The mechanism by which PF562271 affects OS cells is unknown. In this research, we tested the effect of PF562271in several OS cell lines and investigated how FAK signaling is involved in the progression of OS.

Materials and Methods

Tissue microarrays and immunohistochemical analyses. The OS tissue microarray was provided by the Department of Pathology of Ruijin Hospital (Shanghai, China). Two representative cores of each paraffin-embedded tissue sample (1 mm in diameter) were selected for immunohistochemical staining, which was carried out on 5-μm-thick paraffin-embedded sections. All experimental protocols for OS samples were approved by the ethics committee of Shanghai Ruijin Hospital.

Osteosarcoma cell lines. Six OS cell lines (143B, MG63, SAOS2, MG63.2, HOS, and U2OS) were purchased from ATCC (Manassas, VA, USA). The WELL5 cell line was established by our group.[16] All cells were cultured in DMEM (Invitrogen Carlsbad, CA, USA), supplemented with 10% FBS (Invitrogen), penicillin (100 U/mL) and streptomycin (100 mg/mL; Invitrogen), at 37°C in a 5% CO_2 incubator.

Small molecule inhibitors. PF562271, a tyrosine kinase inhibitor of FAK, was ordered from Selleck Chemicals (Houston, TX, USA). PF562271 was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a 10-mM concentration, stored at −20°C, and diluted to an appropriate final concentration in culture media before use.

Cell proliferation assay. The cellular viability of OS cells was determined by the Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) cell proliferation assay. Briefly, 5000 cells/well were planted in 96-well plates and rendered quiescent by incubation in serum-free medium at 37°C for 24 h. Osteosarcoma cells were treated with PF562271 at different concentrations (0, 0.5, 1, 2.5, and 5 μM) and incubated for 72 h. Subsequently, 10 μL CCK-8 solution was added to each well. Then cells were incubated at 37°C for 3 h and the absorbance (A450) was finally read at 450 nm using a microplate reader. All experiments were carried out three times. The cell growth inhibitory rate was calculated as: inhibitory rate (%) = (1 − A450 [treated]/A450 [blank]) × 100%.

Inhibitory concentration curve calculation. GraphPad Prism 5.0 software (Aspire Software International, Washington, DC, USA) was used to graph cell viability data. Raw data were matched to non-linear regression dynamic fitting curves for dose inhibition. Statistical analyses were done using this program and IC_{50} values were calculated using three- or four-parameter variable slope log inhibitor response curves.

Apoptosis. Apoptosis assay was undertaken with a PE Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) to identify apoptotic nuclei. One hundred millimeter plates were seeded with 143B, WELL5, U2OS, and MG63 cells at a concentration of 1×10^6 cells in normal growth media. After overnight incubation, plates were treated with one of several doses of PF562271 or the DMSO control. After another 24 h of incubation, cells were collected with trypsin and, following centrifugation, were washed with 1× PBS. Again, cells were centrifuged, PBS was removed, and cells were resuspended in binding buffer and filtered through BD mesh tubes. Annexin V and propidium iodide (PI) were added to the solution, vortexed, and incubated for 15 min in the dark. Binding buffer was added and the cells were analyzed by flow cytometry within 1 h.

Western blot analysis. Whole-cell lysates were prepared using pre-chilled RIPA (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholate). The cell lysates were centrifuged at 12 000 g for 20 min at 4°C and the supernatants were collected for protein concentration determination. The total proteins were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane (Millipore, Boston, MA, USA). The membrane was incubated with blocking buffer and then incubated overnight with appropriate primary antibodies at 4°C. The primary antibodies used were anti-p-FAK (Y397; 1:1000), anti-FAK (1:1000), anti-p-AKT (S473) (1:1000), anti-AKT (1:1000), anti-mammalian target of rapamycin (mTOR) (1:1000), anti-p-mTOR (S2448; 1:1000), anti-poly(ADP-ribose) polymerase (PARP) (1:1000) (all Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH (1:500; Santa Cruz, CA, USA). Membranes were then washed three times in TBST solution for 15 min each time, and then incubated with secondary antibodies. The membranes were visualized using LI-COR infrared imaging system (LI-COR Biosciences, Shanghai, China) following the manufacturer's guidance.

Endothelial tube formation assay. Briefly, each well of pre-chilled 48-well culture plates was coated with a thin layer of Matrigel (BD Biosciences), which was left to polymerize at 37°C for 1 h. Human umbilical vein endothelial cells (4×10^4 cells/well) treated with one of several doses of PF562271 or the DMSO control were added to the polymerized Matrigel. After 4 h of incubation at 37°C with 5% CO_2, tube forming ability was evaluated by counting the number of tubes and their length in five random fields using Image-Pro Plus software (Rockville, MD, USA) according to Mirshahi's method.[17]

Colony formation assay. Three 6-well plates were seeded with MG63, U2OS, and WELL5 cells at a concentration of 600 cells per well in 2 mL media. 143B cells were plated at 400 cells and 2 mL media per well. After overnight incubation (37°C, 5% CO_2), each well was treated with a different PF562271 concentration and left to incubate. After 14 days, the media was aspirated from each well and then the cells were incubated in 1% crystal violet for 30 min to stain them. The colony number in each well was counted in duplicate plates.

Cell death assay. The TUNEL assay was carried out on tumor tissue as a measurement of in situ apoptosis. Previously sectioned and fixed tumor samples were processed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Negative controls were obtained for each sample by omission of incubation with the TUNEL reaction mixture. Sections were viewed under light microscopy and photographs obtained.

Mice xenograft models. Four-week-old male BALB/C nude mice were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). All experiments were carried out in accordance with the official recommendations of the Chinese animal community. Mice was injected s.c. into the right flank with 143B cells (200 μL, 2×10^6 cells/mL). Tumor volume was measured periodically using calipers to monitor tumor growth (volume = 0.5 × length × width²). When the average tumor volume in all animals reached approximately 100 mm³, twice-daily treatment was administered by oral gavage with either PF562271 (50 mg/kg) (in a volume of 10 mL/kg) or vehicle control (30% PEG, 0.5% Tween 80, and 5% propanediol in sterile water; $n = 6$ per condition). When tumor volumes exceeded 2000 mm³, the mice were killed and tumors were removed and weighed.

Statistical analyses. One- or two-way ANOVA with Tukey *post hoc* tests were used to compare groups. Statistical analyses were carried out using GraphPad Prism 5.0 (Aspire Software International).
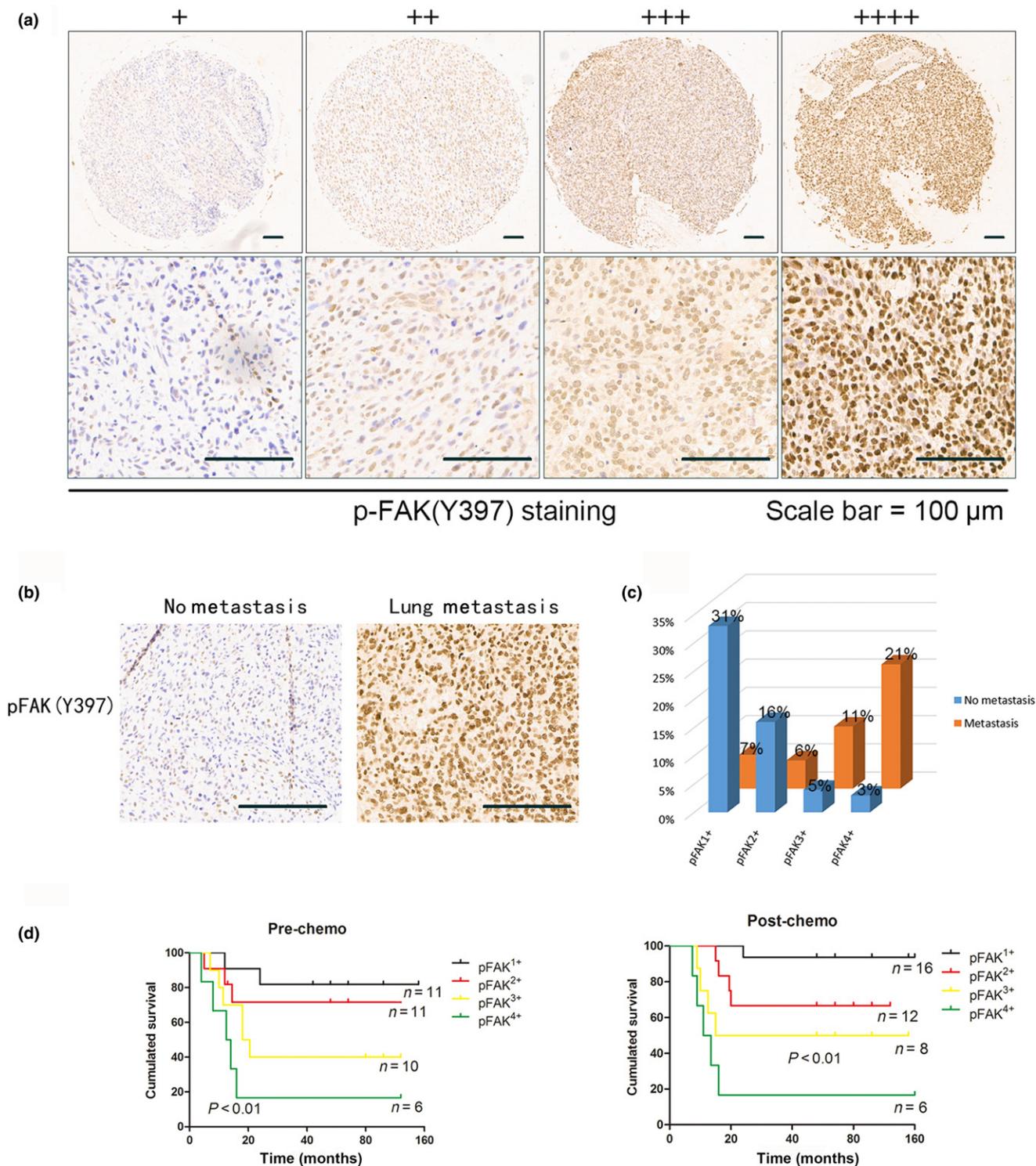
Fig. 1. Phosphorylated focal adhesion kinase (p-FAK) (Y397) expression is associated with osteosarcoma prognosis and lung metastasis. (a) p-FAK (Y397) staining patterns representative of four grades of human primary osteosarcoma samples (1+ to 4+). (b) Representative images of p-FAK (Y397) expression in osteosarcoma with or without lung metastasis (scale bar = 100 μm). (c) Correlation between p-FAK (Y397) staining intensity and occurrence of lung metastasis in osteosarcoma patients. (d) Overall survival of patients with discrete p-FAK (Y397) staining intensities in osteosarcoma cases, as measured before (left panel) or after (right panel) the first round of chemotherapy. *P*-values: compared with the p-FAK (Y397)1+ subgroup.

Results

Phosphorylated FAK (Y397) expression is associated with OS prognosis and lung metastasis. To determine the expression of p-FAK (Y397) in OS, immunohistochemistry was carried out in a tissue microarray comprising 109 cases of OS samples. The p-FAK (Y397) staining intensities were arbitrarily determined as 1+, 2+, 3+, or 4+ (Fig. 1a). Figure 1(b) shows that p-FAK (Y397) expression was higher in tumor tissues with
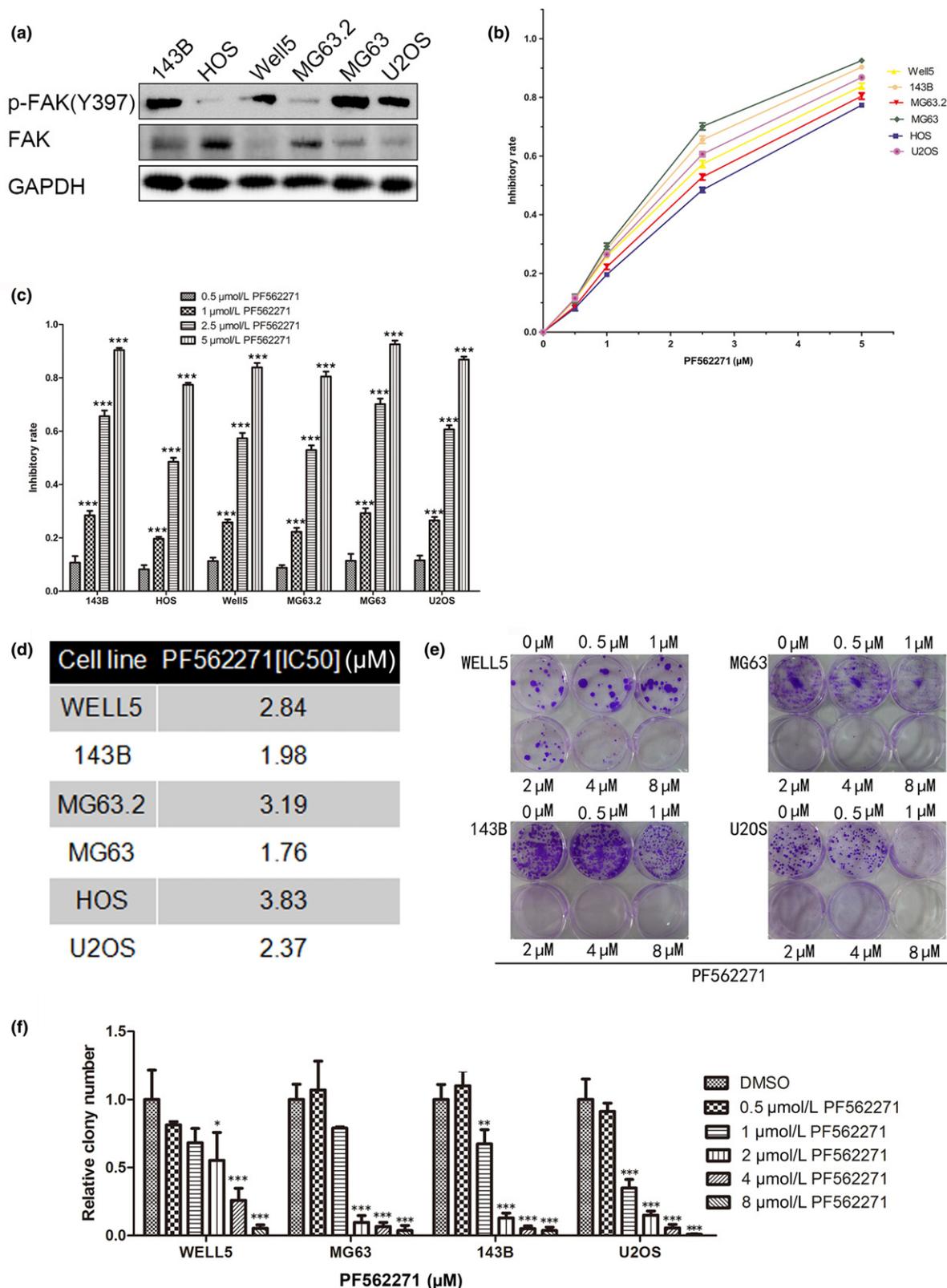
Fig. 2. PF562271 inhibits cell viability and colony formation in osteosarcoma cells. (a) Phosphorylated focal adhesion kinase (p-FAK) (Y397) and total FAK expression in osteosarcoma cell lines (143B, HOS, WELL5, MG63.2, MG63, and U2OS). (b, c) Osteosarcoma cell lines were treated with control (PF562271 0 μM) and PF562271 at a variety of concentrations (0.5, 1, 2.5, and 5 μM) for 72 h. CCK-8 assay showed that PF562271 inhibited the growth of osteosarcoma cells and the inhibitory effects were dose-dependent. Shown are the mean of three replicates ± SD. (d) IC_{50} values were calculated using GraphPad Prism from each PF562271 dose–response curve. (e) Effects of PF562271 treatment on colony formation in osteosarcoma cell lines WELL5, MG63, 143B, and U2OS relative to DMSO. (f) Colony counts from the colony formation assay show that colony growth is inhibited by PF562271 in a dose-dependent manner. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Mean relative colony number (±SD) of three replicates is shown.
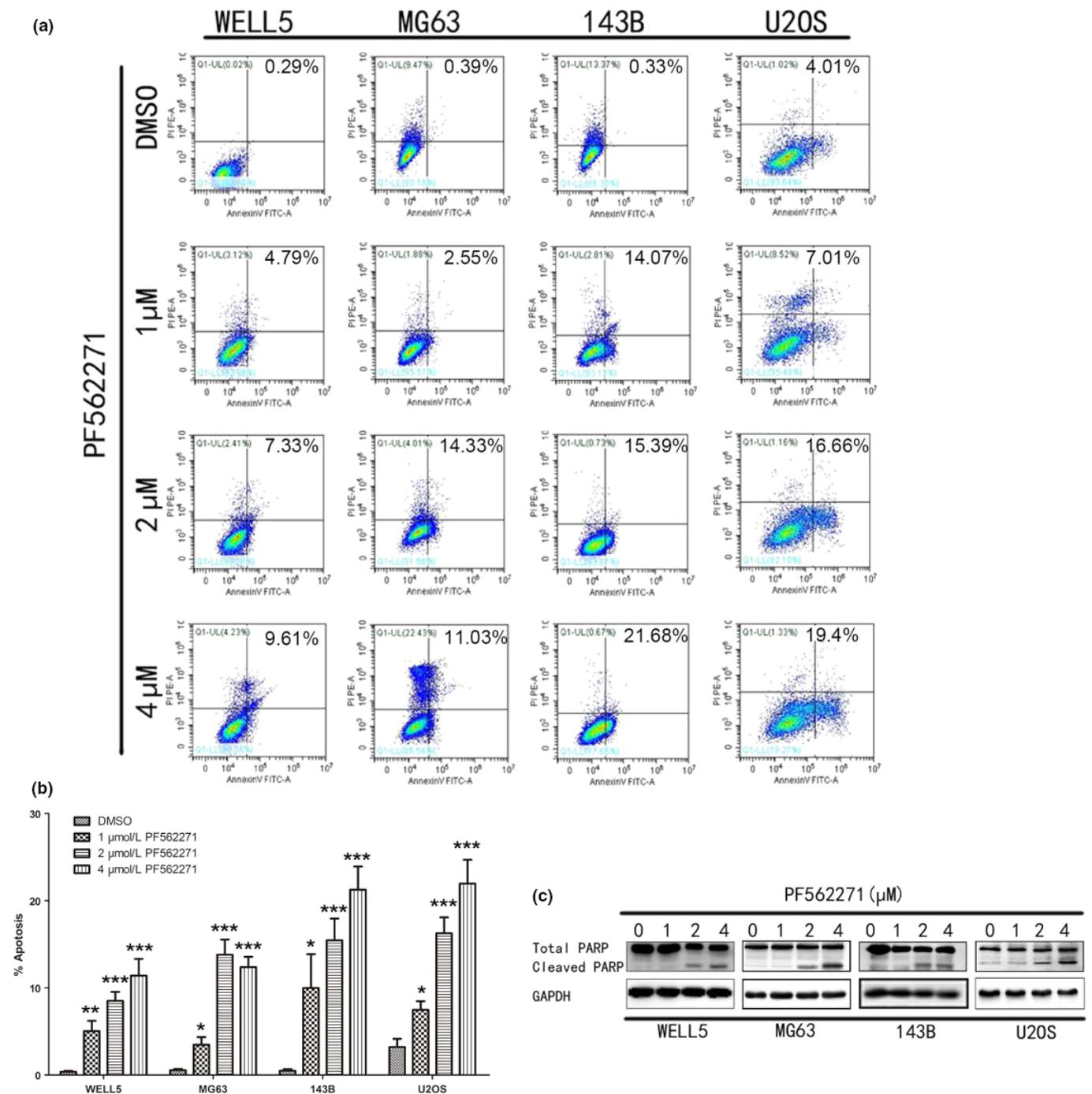
Fig. 3. PF562271 induced apoptosis in osteosarcoma cells. (a) WELL5, MG63, 143B, and U2OS cells treated with PF562271 for 48 h at different concentrations (1, 2.5, and 5 μM) relative to DMSO was analyzed by flow cytometry after staining with annexin V-FITC/propidium iodide. (b) Effects of PF562271 treatment on apoptosis were concentration-dependent in osteosarcoma cell lines relative to DMSO. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Shown are the mean percentages of cells undergoing apoptosis ($\pm$SD) of three replicates. (c) Detection of total poly(ADP-ribose) polymerase (PARP) and cleaved PARP following 48 h of treatment with PF562271 in osteosarcoma cells by Western blotting. GAPDH was included as the loading control.

metastasis than without metastasis and Figure 1(c) further indicated that p-FAK (Y397)[3+–4+] samples were more highly distributed within tumor samples with metastasis than within tumor samples without metastasis. Remarkably, p-FAK (Y397) staining intensities as measured before or after the first round of chemotherapy were correlated inversely with the overall survival probabilities of patients (Fig. 1d). Strikingly, >80% of

p-FAK(Y397)[1+] cases, but no p-FAK(Y397)[2+–4+] cases, survived more than 10 years after diagnosis.

PF562271 inhibited cell growth and colony formation of OS *in vitro*. We undertook an immunoblot analysis to confirm the basal expression of p-FAK (Y397) in human OS cell lines 143B, HOS, WELL5, MG63.2, MG63, and U2OS. As shown in Figure 2(a), p-FAK (Y397) was highly expressed in 143B,
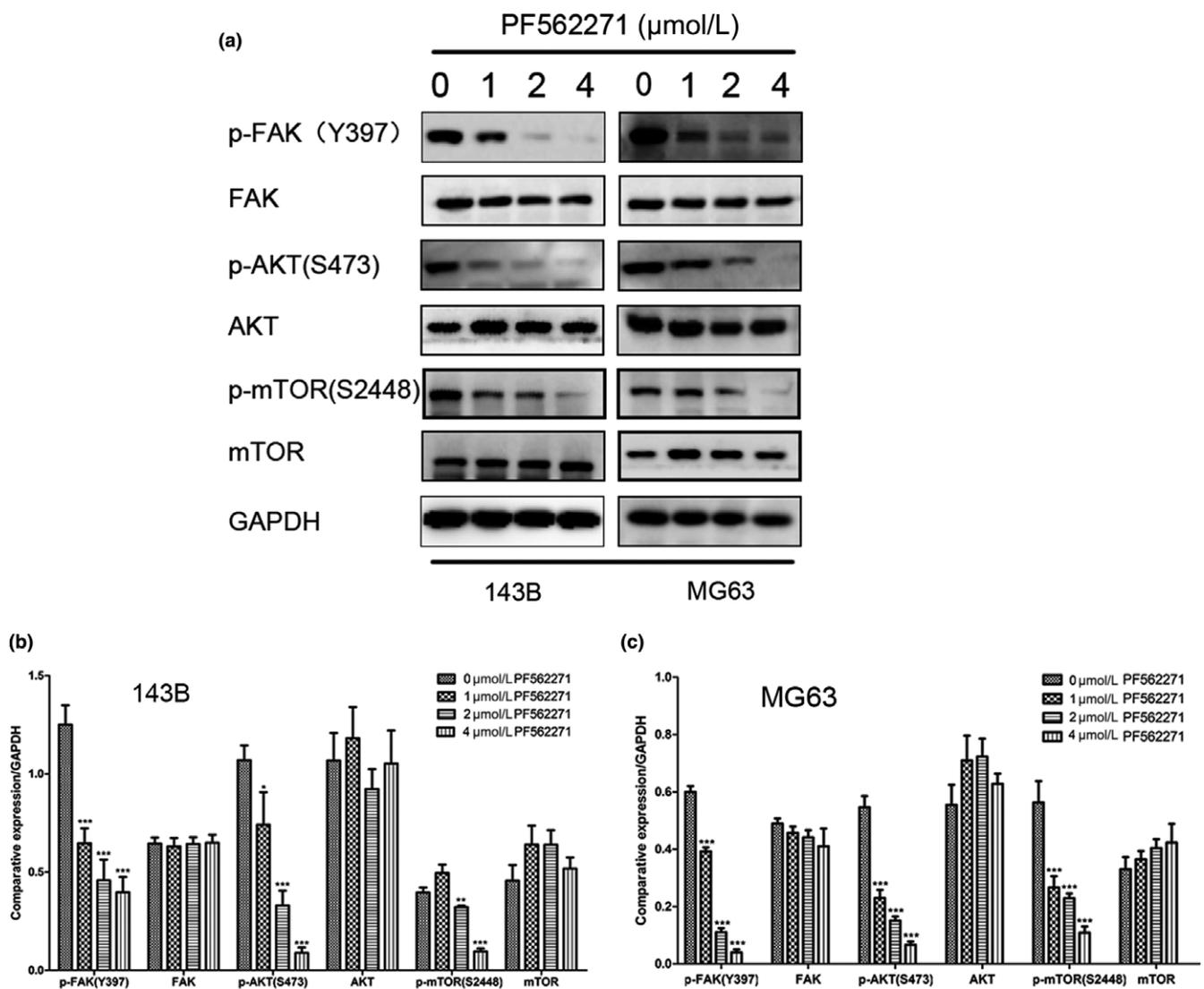
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Fig. 4. Focal adhesion kinase (FAK) inhibition with PF562271 downregulated the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway. (a) Protein levels measured by Western blot analysis for AKT/mTOR pathway proteins in 143B and MG63 osteosarcoma cells treated with PF562271 for 24 h. GAPDH was included as the loading control. (b, c) Semiquantitative histograms of Western blot results.

WELL5, MG63, and U2OS cells. Its expression was lower in HOS and MG63.2 cells. To determine the inhibitory effect on cell growth by PF562271 and to optimize its concentration for further experiments, the CCK-8 assay was completed using a range of PF562271 concentrations on all cell lines (143B, WELL5, MG-63, U2OS, MG63.2, and HOS). PF562271 caused dose-dependent cell growth inhibition across all OS cell lines (Fig. 2b,c). The IC_{50} values after 72 h of treatment were calculated on the basis of these results and are shown in Figure 2(d). All OS cell lines were sensitive to PF562271 treatment, with IC_{50} values from 1.76 to 3.83 μM. 143B and MG63 cells seemed to be the most sensitive to PF562271 (IC_{50} = 1.98 μM for 143B; 1.76 μM for MG63). Then four OS cell lines (143B, WELL5, MG-63, and U2OS) that had high expression levels of p-FAK (Y397) were treated with PF562271 for colony formation assays. PF562271 treatment decreased colony formation in a dose-dependent manner in four OS cell lines, corresponding to the IC_{50} values of each cell line (Fig. 2e,f).

PF562271 induced apoptosis in OS cell lines. Next four OS cell lines (143B, WELL5, MG-63, and U2OS) that had high

expression levels of p-FAK (Y397) were treated with PF562271 and apoptosis assay was carried out using annexin V-FITC/PI staining. Apoptosis was significantly increased in a dose-dependent manner across all cell lines tested (Fig. 3a,b). To confirm this finding, we measured PARP cleavage by Western blot analysis in OS cells treated with PF562271 for 48 h. There was a concentration-dependent increase in PARP cleavage that correlated with our flow cytometric findings (Fig. 3c).

PF562271 downregulated activity of AKT/mTOR pathway. Recent research has reported the aberrant activation of mTOR in OS.[18] Focal adhesion kinase is reported to regulate the activity of the AKT pathway, suggesting a possible mechanistic link in OS.[19] Therefore, we chose 143B and MG63 cell lines, which were most sensitive to PF562271 treatment, to examine the expression of the AKT/mTOR pathway after PF562271 treatment for 24 h. PF562271 downregulated FAK (Y397), AKT (S473), and mTOR (S2448) phosphorylation at concentrations that impaired cell growth and colony formation (Fig. 4).

PF562271 has an anti-angiogenic effect *in vitro*. To clarify whether PF562271 has an anti-angiogenic effect, we first
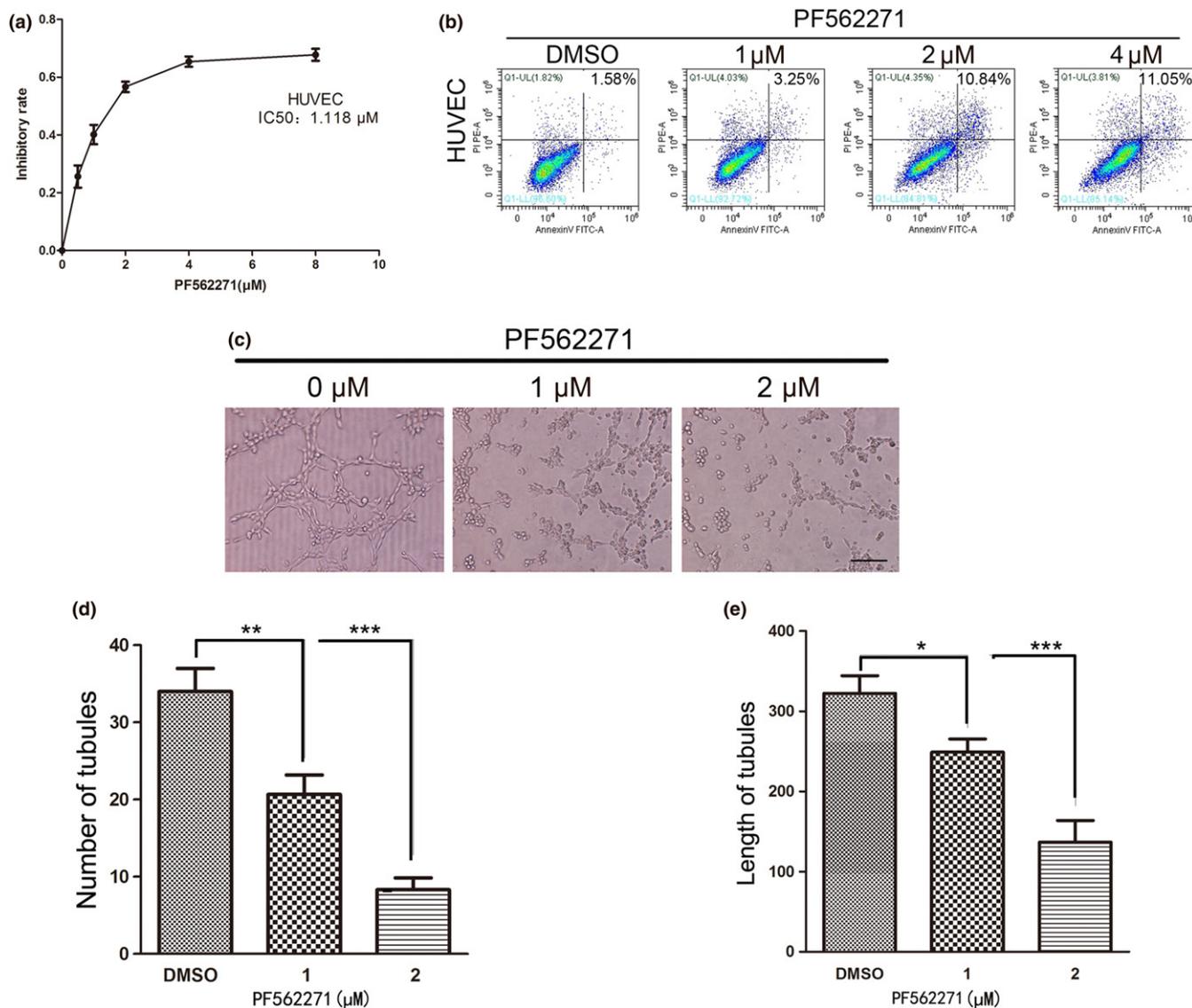
Fig. 5. PF562271 inhibited proliferation, induced apoptosis, and impaired tube-forming ability in HUVECs. (a) Treatment with PF562271 inhibited the growth of HUVECs in a dose-dependent manner, with an IC_{50} of 1.118 μM after exposure for 24 h. (b) PF562271-treated cells showed dose-dependent increases in apoptosis detected by staining with annexin V-FITC/propidium iodide in HUVECs. (c) Treatment with PF562271 at different concentrations (1 and 2 μM) could inhibit the tube-forming ability in HUVECs relative to DMSO. Scale bar = 50 μm. (d, e) Mean numbers of tubules (±SD) (d) and mean tubular lengths (±SD) (e) of three replicates. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

determined that PF562271 caused a dose-dependent cell growth inhibition of normal HUVECs; the IC_{50} value after 24 h of treatment was 1.118 μM (Fig. 5a). To examine the effect of PF562271 on apoptosis, annexin V-FITC/PI staining was carried out. Apoptosis of HUVECs significantly increased in a dose-dependent manner (Fig. 5b). Next, we investigated the tubular formation ability of HUVECs treated with PF562271. PF562271 significantly inhibited the tube number and length of HUVECs in a dose-dependent fashion (Fig. 5c–e).

Oral administration of PF562271 suppresses s.c. tumor growth *in vivo*. To analyze the antitumor effect of PF562271 *in vivo*, we established OS xenograft tumors derived from 143B cells in nude mice. When the average tumor volume in all animals reached approximately 100 mm^3, twice-daily treatment was given by oral gavage with PF562271 (50 mg/kg) or vehicle control. Animals were killed when tumor volumes exceeded

2000 mm^3. The tumors were excised, weighed, and examined histologically. As shown in Figure 6, both tumor weight and volume were significantly decreased in mice that had PF562271 treatment compared with mice treated with vehicle only. No severe side-effects occurred during the treatment. As show in Figure 7(a,b), expression of p-FAK (Y397) protein was significantly suppressed in tumor sections of mice treated with PF562271 compared to control mice. There was no statistical significance in terms of total FAK expression. Immunohistochemical staining analysis and TUNEL assay further showed a significant reduction in CD31-positive endothelial cells and an increase in TUNEL-positive cancer cells in tumor sections of mice treated with PF562271 (Fig. 7c,d). These results suggested that PF562271 could suppress tumor growth by the regulation of tumor cell survival and angiogenesis.
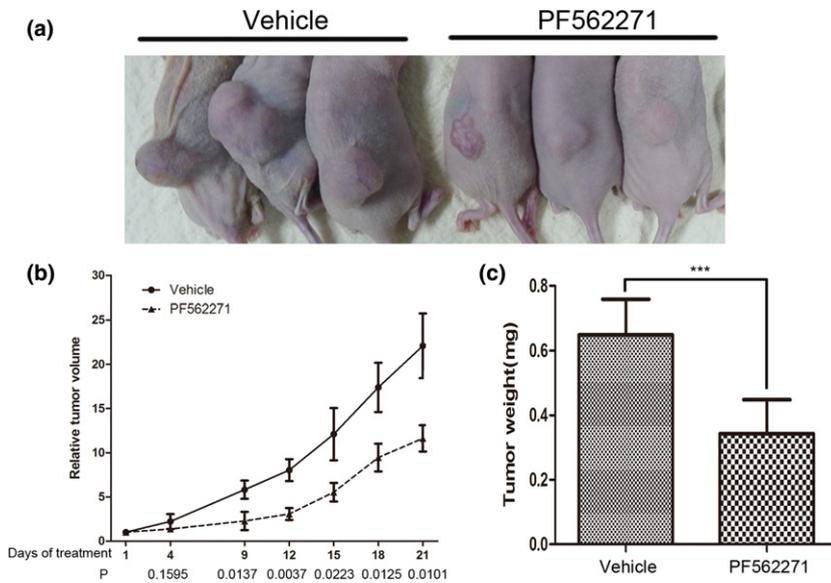
Fig. 6. Effect of PF562271 on osteosarcoma xenografts. (a) Macroscopic appearance of 143B tumors in BALB/c nude mice at 21 days after treatment ($n = 6$/group). Antitumor effect of PF562271 against established flank 143B xenografts tumors in nude mice. On day 21, all of the mice were killed and tumors were removed and weighed. (b) PF562271 treatment significantly reduced tumor volume after s.c. injection of 143B cells in human tumor xenograft models of osteosarcoma. Relative tumor volumes were calculated by dividing the tumor volume measurement for each animal by the corresponding day 0 measurement for that animal. *P*-values calculated by Student's *t*-test for each time point. Shown are the mean relative tumor volumes ($\pm$SD). (c) Mean tumor weight ($\pm$SD). ***$P < 0.001$.
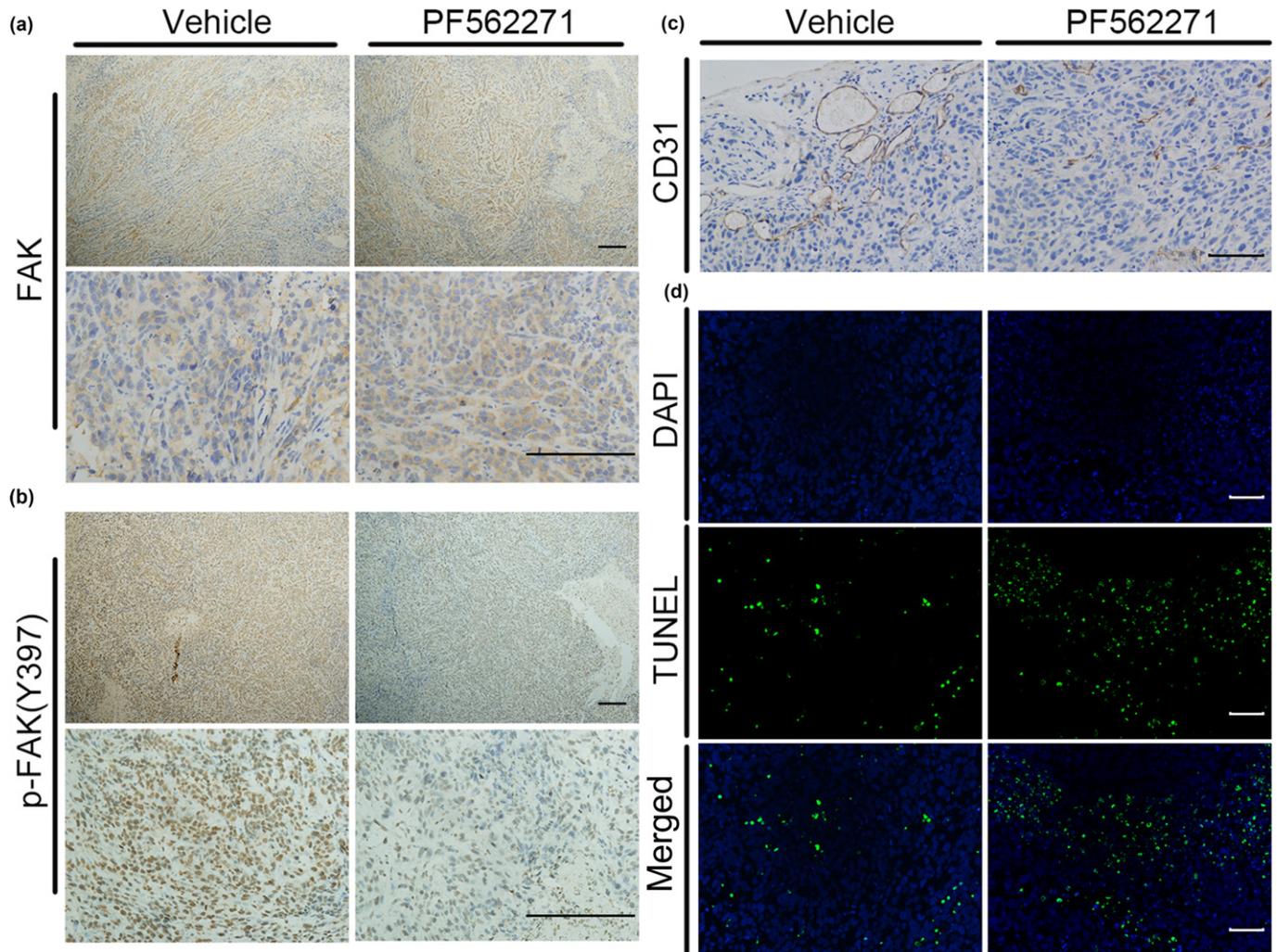

Fig. 7. PF562271 reduces microvessel density and induces apoptosis in 143B-derived osteosarcoma xenograft tumors. (a–c) Representative microscopic images of immunohistochemical analysis of focal adhesion kinase (FAK) (a), phosphorylated FAK (p-FAK) (Y397) (b), and CD31 (c) expression in paraffin sections of tumor tissue of 143B xenograft mice treated with vehicle control or PF562271. Scale bar = 100 μm. (d) Representative microscopic images of nuclear DAPI staining (upper panels), TUNEL immunofluorescent staining (middle panels), and merged images (lower panels) in paraffin sections of tumor tissue of 143B xenograft mice treated with vehicle control or PF562271. Scale bar = 100 μm.

Discussion

Previous studies have revealed that FAK is a key molecule for cell proliferation, migration, and invasion during cancer progression.[20–23] Focal adhesion kinase has also been shown to be upregulated in many cancers, including OS.[13] Ren *et al.* reported that p-FAK (Y397) was highly expressed in 37% of primary human OS tissues (42/113) and greater p-FAK (Y397) overexpression was associated with more aggressive OS and poor prognosis. Thus, FAK inhibition is a viable therapeutic strategy, especially in more aggressive phenotypes.[13] In this study, we further indicated that p-FAK (Y397) was highly expressed in primary human OS tumor samples and was associated with OS prognosis and lung metastasis. Inhibition of FAK by PF562271 may be a promising approach for treatment of human OS.

Several studies have indicated that FAK-mediated signaling plays a critical role in the regulation of cancer cell survival.[24] Anoikis is a form of programmed cell death that occurs when a cell detaches from its ECM, in which FAK activity is lost and, therefore, results in cell apoptosis.[25] In contrast, FAK is often highly expressed in cancer cells and it helps them to resist anoikis or survive and grow in the absence of anchorage to the ECM. Indeed, in these cancer cells, the increased FAK/Src complex confers the activation of both PI3K/AKT and MEK/ERK1/2 signal transductions, thereby enhancing the abilities of cancer cell survival and growth in a cell-detached condition.[26] In the present study, we showed that inhibition of FAK by PF562271 significantly inhibited the proliferation and colony formation in OS cells and induced apoptosis *in vitro* and *in vivo*. Ren *et al.* and Wang *et al.*[13,27] showed that inhibition of FAK using FAK-directed shRNA induced apoptosis in several OS cell lines. This was consistent with our studies. Taken together, it is clear that FAK has an important role in cell survival and promotes OS progression.

Target therapy in OS is still in its infancy.[28,29] However, studies have shown that aberrant activation of mTOR has been detected in OS and activation of mTOR is associated with OS cell growth, proliferation, and metastasis.[18,30] Inhibiting this pathway might be a promising approach for treating OS. In addition, using a combination of inhibitors that target of multiple nodes in a critical cancer pathway was suggested to improve response and prevent the development of resistance.[31] Studies showed that the AKT/mTOR pathway has a role in resistance to sorafenib in high-grade OS[32] and a recent phase II study showed good responses in a subset of patients with inoperable high-grade OS when combining sorafenib with the mTOR inhibitor everolimus.[33] Therefore, treatment of OS cells with PF562271 inhibits the activity of the AKT/mTOR pathway, suggesting that the inhibition of FAK is not only a potential method for OS treatment but also may be a promising candidate for combination therapy either with sorafenib or AKT/mTOR-targeted treatment.

Hypervascularization is an important pathophysiological feature of OS; therefore, anti-angiogenic therapy could be beneficial for OS treatment.[34,35] Several studies have supported the importance of FAK expression and activity in endothelial cells during vascular development and tumor angiogenesis. *In vitro* primary endothelial cells from mice with FAK knockout[36–38] or FAK knockdown[39,40] showed defects in survival, proliferation, sprouting, migration, and tubulogenesis. Furthermore, FAK inhibition reduces tumor angiogenesis in animal models of human colon, ovarian, and hepatocellular carcinoma, supporting a stimulating role of FAK activity in angiogenesis. In our data, the proliferation of HUVECs was inhibited in a dose-dependent manner with the inhibition of FAK by PF562271 treatment for 24 h, and the IC_{50} was 1.118 μM. In addition, PF562271 could induce apoptosis in HUVECs and inhibit its tubular formation ability in a tubular formation assay. Furthermore, neovascularization was suppressed by oral treatment with PF562271 in OS xenograft mice. These data suggested that the inhibition of FAK by PF562271 might suppress angiogenesis and endothelial cell survival in human OS.

In summary, our results show that FAK should be considered as a target for the treatment of OS. Our findings clearly show that PF562271 inhibits OS cell proliferation and survival as well as angiogenesis *in vitro* and *in vivo*. These results strongly suggest that the use of PF562271 alone or in combination with other agents might be an attractive approach for OS treatment.

Acknowledgments

Disclosure Statement

The authors have no conflict of interest.

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