Down-regulation of Forkhead Box M1 Transcription Factor Leads to the Inhibition of Invasion and Angiogenesis of Pancreatic Cancer Cells

Zhiwei Wang, Sanjeev Banerjee, Dejuan Kong, Yiwei Li, and Fazlul H. Sarkar

Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

Abstract

The Forkhead Box M1 (FoxM1) transcription factor has been shown to play important roles in regulating the expression of genes involved in cell proliferation, differentiation, and transformation. Overexpression of FoxM1 has been found in a variety of aggressive human carcinomas including pancreatic cancer. However, the precise role and the molecular mechanism of action of FoxM1 in pancreatic cancer remain unclear. To elucidate the cellular and molecular function of FoxM1, we tested the consequences of down-regulation and up-regulation of FoxM1 in pancreatic cancer cells, respectively. Using multiple cellular and molecular approaches such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, gene transfection, flow cytometry, real-time reverse transcription-PCR, Western blotting, migration, invasion, and angiogenesis assays, we found that down-regulation of FoxM1 inhibited cell growth, decreased cell migration, and decreased invasion of pancreatic cancer cells. FoxM1 down-regulation also decreased cell population in the S phase. Compared with control, FoxM1 small interfering RNA–transfected cells showed decreased expression of cyclin B, cyclin D1, and Cdk2, whereas p21 and p27 expression was increased. We also found that down-regulation of FoxM1 reduced the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, and vascular endothelial growth factor, resulting in the inhibition of migration, invasion, and angiogenesis. These findings suggest that FoxM1 down-regulation could be a novel approach for the inhibition of pancreatic tumor progression. [Cancer Res 2007;67(17):8293–300]

Introduction

Pancreatic cancer (PC) is one of the most common cancers and is the fourth leading cause of cancer-related death in the United States, with about 32,000 newly diagnosed cases and an approximately equal number of deaths per year (1). This could be due to the fact that no effective methods of early diagnosis are currently available, as well as due to the lack of effective systemic therapies resulting in the high mortality of patients diagnosed with PC. This disappointing outcome strongly suggests that there is a dire need for innovative research that will lead to a dramatic improvement in the survival of patients diagnosed with this deadly disease.

FoxM1 signaling is involved in cell proliferation and apoptosis, which affects the development and function of many organs (2–4). FoxM1 (previously known as HFH-11, MPP2, Win, and Trident) is a member of the Fox transcription factor family (2–6). It has been reported that FoxM1 is a key cell cycle regulator of both the transition from G1 to S phase and the progression to mitosis (2, 3, 7–10). Loss of FoxM1 expression generates mitotic spindle defects, delays cells in mitosis, and induces mitotic catastrophe (9). Moreover, FoxM1 has been shown to regulate transcription of cell cycle genes essential for G1-S and G2-M progression, including Cdc25A, Cdc25B, cyclin B, cyclin D1, p21cip1, and p27kip1 (2, 7, 10–12).

FoxM1 signaling also plays important roles in the cellular developmental pathway including proliferation and apoptosis, and alterations in FoxM1 signaling are associated with tumorigenesis (5, 6, 13). These observations suggest that dysfunction of FoxM1 prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation. It has also been reported that the FoxM1 signaling network is frequently deregulated in human malignancies with up-regulated expression of FoxM1 in lung cancer, glioblastomas, prostate cancer, basal cell carcinomas, hepatocellular carcinoma, and primary breast cancer and PC (2, 5, 6, 9, 13–15). These results suggest that FoxM1 plays important roles in the oncogenesis of several malignancies. Recent data suggest that the FoxM1 gene is up-regulated in PC due to transcriptional regulation by the Sonic Hedgehog pathway (2). There has been some progress toward elucidating the mechanism of action of FoxM1 as well as the consequence of down-regulation of FoxM1; however, the exact mechanism has not yet been fully established. Therefore, we sought to find novel avenues by which FoxM1 could be inactivated, which may represent a promising strategy for the development of novel and selective anticancer therapies for PC. We investigated the consequence of down-regulation of FoxM1 by FoxM1 small interfering RNA (siRNA) on PC cell growth and apoptosis. Moreover, because cell migration and invasion are important processes involved in tumor development and metastasis and because FoxM1 signaling is known to control these processes, we also examined the effect of FoxM1 on the processes of cell migration and invasion of PC cells. We found that down-regulation of FoxM1 inhibits cell growth of PC cell lines. Our data also show that down-regulation of FoxM1 inhibited the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, and vascular endothelial growth factor (VEGF), which could be the mechanism responsible for the inhibition of PC cell migration, invasion, and the activity of conditioned media to inhibit angiogenesis as measured by tube formation of human umbilical vascular endothelial cells (HUVEC).

Materials and Methods

Cell culture and experimental reagents. Human PC cell lines AsPC-1, BxPC-3, COLO-357, HPAC, L3.6pl, MIAPaCa, and PANC-1 were used in this...
study. BxPC-3, HPAC, and PANC-1 [American Type Culture Collection (ATCC)] were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. AsPC-1, COLO-357, L3.6pl, and MIA PaCa cells were generously provided by Dr. Paul Chiao (M.D. Anderson Cancer Center, Houston, TX) and grown as a monolayer cell culture in DMEM containing 4.5 mg/mL d-glucose and 1-glutamine supplemented with 10% fetal bovine serum. HUVECs (ATCC) were cultured in F12K medium (ATCC) supplemented with 10% fetal bovine serum, 0.1 mg/mL heparin sulfate, 0.05 mg/mL endothelial cell growth factor supplement (BD Biosciences), 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured in a 5% CO2-humidified atmosphere at 37°C.

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris (pH, 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 μL/mL protease inhibitor cocktail, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system. Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane for Western blotting as described earlier (18).

Real-time reverse transcription-PCR analysis for gene expression studies. The total RNA from transfected cells was isolated by TRIZol (Invitrogen) and purified by RNeasy Mini Kit and RNease-free DNase Set (Qiagen) according to the manufacturer’s protocols. About 1 μg of total RNA from each sample was subjected to first-strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems) in a total volume of 50 μL, including 6.25 units MultiScribe reverse transcriptase and 25 pmol random hexamers. RT reaction was done at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. The primers used in the PCR reaction are FoxM1 forward primer (5′-AACCGCTACTTGACATTGG-3′) and reverse primer (5′-GCAGTGGCTCTCATCTTC-3′); Cdc25A forward primer (5′-ACACGAGACTAGCTCCATCAG-3′) and reverse primer (5′-GCCACGCCTCCTACCTACGC-3′); Survivin forward primer (5′-GCTTGTTGGTGAATCTTAC-3′) and reverse primer (5′-GATGTGGATGTCTCGGCTTC-3′); p21 forward primer (5′-GCCGAGCCAGCTTCTACCACAG-3′) and reverse primer (5′-GCCATGTACTCCGACCTAC-3′); P27 forward primer (5′-CGCTGGCCAGCTTCTACCACAG-3′) and reverse primer (5′-ACAAAGGAAACACAC-3′); E2F1 forward primer (5′-CAAGATGGCAAGAGCACATC-3′) and reverse primer (5′-CTGCTTCGTCCTCTCTC-3′); FoxM1 protein levels were measured by FoxM1 cDNA plasmid. A and B, FoxM1 mRNA and protein levels were measured by real-time RT-PCR and Western blotting, respectively. C, FoxM1 mRNA levels were down-regulated by FoxM1 siRNA. Columns, mean of three independent experiments; bars, SD. D, FoxM1 protein levels were down-regulated by siRNA and up-regulated by FoxM1 cDNA plasmid in three PC cells, respectively. *, P < 0.05 relative to control.
Real-time PCR amplifications were done as described earlier (18).

**MMP-2 assay.** The culture medium of the FoxM1 siRNA- or control siRNA-transfected cells grown in six-well plates was collected. After collection, the medium was spun at 800 g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for later MMP-2 assay or assayed immediately using commercially available ELISA kits (R&D Systems, Inc.).

**MMP-9 activity assay.** The FoxM1 siRNA- or cDNA-transfected cells were seeded in six-well plates and incubated at 37°C. After 24 h, the complete medium was removed, and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 24 h. MMP-9 activity in the medium was detected by using Fluorokine E Human MMP-9 activity assay kit (R&D Systems) according to the manufacturer's protocol.

**VEGF assay.** The FoxM1 siRNA- or cDNA-transfected cells were seeded in six-well plates (1.0 × 10^5 cells per well) and incubated at 37°C. After 24 h, the cell culture supernatant was harvested, and cell count was done after trypsinization. After collection, the medium was spun at 800 × g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for later VEGF assay or assayed immediately using commercially available ELISA kits (R&D Systems).

**Cell migration and invasion assay.** Cell migration was assessed using 24-well inserts (BD Biosciences) with 8-μm pores according to the manufacturer's protocol. The invasive activity of the FoxM1 or control siRNA-transfected cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences). Briefly, transfected PC cells (5 × 10^4) with serum-free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 24 h of incubation, the cells in the upper chamber were removed, and the cells that had invaded through Matrigel matrix membrane were stained with 4 μg/mL calcein AM in Hanks' buffered saline at 37°C for 1 h. The fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

**Matrigel in vitro HUVECs tube formation assay.** The FoxM1 siRNA- or cDNA-transfected cells were cultured in serum-free RPMI 1640 for 24 h. The conditioned media were collected, centrifuged, and transferred to fresh tubes and stored at −20°C. Growth factor–reduced Matrigel (125 μL.) was then incubated at 37°C for 30 min to allow the Matrigel to polymerize. HUVECs were trypsinized and seeded (5 × 10^4 cells per well) in each well with 250 μL of conditioned medium from FoxM1 siRNA or control siRNA-transfected BxPC-3 or HPAC cells. The chamber was incubated for 6 h. Each well was photographed using an inverted microscope with digital camera. The assessment of vessel number and length of vessel perimeter in each of the entire field was calculated using the Scion Image analysis program.1

**Densitometric and statistical analysis.** The cell growth inhibition after transfection was statistically evaluated using GraphPad StatMate software (GraphPad Software, Inc.). Comparisons were made between control and transfection. P < 0.05 was used to indicate statistical significance.

**Results**

**Down-regulation of FoxM1 expression by siRNA inhibited cell growth.** The baseline expression of FoxM1 was determined in a panel of human PC cell lines that included AsPC-1, BxPC-3, COLO-357, HPAC, L3.6pl, MIAPaCa, and PANC-1. The results showed that FoxM1 was frequently but differentially expressed in different human PC cell lines (Fig. 1A). We also examined the relative mRNA levels of FoxM1 in three PC cell lines such as BxPC-3, HPAC, and PANC-1 by real-time reverse transcription-PCR (RT-PCR). All three

1 Scion Image was downloaded from NIH Web site http://www.scioncorp.com.
cell lines expressed high levels of FoxM1 in both mRNA and protein level (Fig. 1A and B). To determine whether FoxM1 could be an effective therapeutic target for PC, the effect of FoxM1 siRNA on cell growth of the PC cells was examined. The efficacy of FoxM1 siRNA for knockdown of FoxM1 mRNA and protein was confirmed by real-time RT-PCR and Western blotting. We observed that both FoxM1 mRNA and protein levels were barely detectable in FoxM1 siRNA-transfected cells compared with siRNA control-transfected cells (Fig. 1C and D). The cell viability was determined by MTT, and the effect of FoxM1 siRNA on the growth of cancer cells is shown in Fig. 2. We found that the down-regulation of FoxM1 expression caused cell growth inhibition in all three PC cell lines.

**Overexpression of FoxM1 by cDNA transfection promoted cell growth.** PC cells AsPC-1, PANC-1, and Colo-357 were transfected with human FoxM1 or empty vector alone. The reason for choosing these three PC cell lines was due to the fact that these cell lines showed the lowest expression of FoxM1. The proteins were measured using Western blotting. The results showed that FoxM1 protein level was increased by FoxM1 cDNA transfection (Fig. 1D). FoxM1 cDNA-transfected cells showed a significant promotion of cell growth compared with empty vector–transfected control cells (Fig. 2).

**Down-regulation of FoxM1 decreased cell population in the S phase.** To further investigate the growth-inhibitory effect of FoxM1 knockdown in PC cells, we did cell cycle analysis by propidium iodide staining and flow cytometry. Depletion of FoxM1 levels in BxPC-3, HPAC, and PANC-1 cells caused a significant accumulation of cells in the G0-G1 phase and a marked decrease in the S phase compared with control siRNA-transfected cells (Fig. 3). However, as shown in Fig. 3, the percentage of FoxM1 cDNA-transfected cells in the S phase was increased compared with that of control vector–transfected cells.

To further characterize the G0-G1 arrest, we examined the level of expression of several known G0-G1 cell cycle regulatory factors. Consistent with cell cycle arrest, the expression of cyclin D1 and Cdk2 at both the mRNA and protein levels was found to be decreased, whereas p21 and p27 expression was increased (Fig. 4), suggesting the mechanistic roles of these molecules during FoxM1-induced cell cycle progression and cell cycle arrest by FoxM1 siRNA. To confirm our data, we also found that the expression of FoxM1 target genes involved in cell proliferation and survival, such as E2F-1, survivin, cyclin B, and cdc25A, was down-regulated in FoxM1 siRNA-transfected cells (Fig. 4).

**Down-regulation of FoxM1 decreased MMP-2 and MMP-9 gene transcription and their activities.** It has been reported that MMP-9 expression is elevated in the liver of FoxM1B transgenic mice (19). We therefore investigated whether MMP-9 and MMP-2 were down-regulated by FoxM1 siRNA in PC cell lines. To explore whether FoxM1 siRNA transfection could decrease the expression of MMP-2 and MMP-9, real-time RT-PCR and Western blotting were conducted. We found that both MMP-2 and MMP-9 mRNA and protein levels were dramatically decreased in the FoxM1 siRNA-transfected cells (Fig. 5A and B). Next, we examined whether the down-regulation of FoxM1 could lead to a decrease in MMP-2 and MMP-9 activity in PC cells. There was a marked decrease in the activity of MMP-2 and MMP-9 in FoxM1 siRNA-transfected cells (Fig. 5C and D). However, overexpression of FoxM1 by cDNA transfection led to an increase in MMP-2 and MMP-9 expression and activity in Colo-357 and PANC-1 cells (data not shown).

**FoxM1 siRNA decreased VEGF activity.** FoxM1 has been shown to regulate VEGF signaling in various cell types (20). To further explore whether FoxM1 siRNA reduced VEGF activity, we examined the levels of VEGF activity secreted in the culture medium. We found that FoxM1 could lead to a decrease in the levels of VEGF secreted in the culture medium (Fig. 5E). However, there was a marked increase in the activity of VEGF in FoxM1 cDNA-transfected Colo-357 and PANC-1 cells (data not shown).

**FoxM1 siRNA reduced uPAR gene transcription and translation.** The urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) are able to regulate the MMP-9 activity in PC (21).
To further investigate whether FoxM1 siRNA has any effect on reducing the level of uPAR, real-time RT-PCR and Western blotting were done to detect the expression of uPAR. We found that both uPAR mRNA and protein levels were dramatically reduced in FoxM1 siRNA-transfected cells (Fig. 5A and B).

Down-regulation of FoxM1 decreased PC cell migration and invasion. MMP-2, MMP-9, VEGF, and uPAR are thought to be critically involved in the processes of tumor cell migration, invasion, and metastasis. Because FoxM1 siRNA inhibited the expression and activity of MMP-2, MMP-9, VEGF, and uPAR, we tested the effects of FoxM1 down-regulation on cancer cell migration and invasion. We found that down-regulation of FoxM1 decreased PC cell migration. Moreover, as illustrated in Fig. 6B, FoxM1 siRNA-transfected cells showed a low level of penetration through the Matrigel-coated membrane compared with the control cells. The value of fluorescence from the invaded PC cells was decreased about 3- to 4-fold compared with that of control cells (Fig. 6B). However, FoxM1 cDNA-transfected Colo-357 cells showed a marked increase in cell migration and invasion (data not shown).

Reduced tube formation of HUVECs induced by conditioned media from FoxM1 siRNA-transfected cells. Inhibition of FoxM1 reduced tumor cell proliferation and angiogenesis in hepatocellular carcinomas (22). FoxM1 has been reported to promote both angiogenesis and metastasis in certain tumor models (22). Because FoxM1 siRNA inhibited VEGF expression, we tested whether conditioned media from FoxM1 siRNA-transfected cells could reduce the tube formation, an indirect measure of angiogenesis. We did the tube formation assay in growth factor–reduced Matrigel in vitro. As shown in Fig. 6C, conditioned media from FoxM1 siRNA-transfected BxPC-3 and HPAC cells were able to significantly reduce the tube formation of HUVECs in 6 h incubation compared with the medium from control siRNA-transfected cells. However, conditioned media from FoxM1 cDNA-transfected Colo-357 and PANC-1 cells increased the tube formation of HUVECs in 6 h incubation compared with the medium from control cDNA-transfected cells (data not shown).

Discussion

FoxM1 signaling plays important roles in maintaining the balance between cell proliferation, differentiation, and apoptosis (2, 5–7). The FoxM1 gene is abnormally activated in many human malignancies (5–7, 9, 15). It was found that FoxM1 gene is up-regulated in PC due to the transcriptional regulation by the Sonic Hedgehog pathway (2). We have previously reported that docetaxel (taxotere) alone or in combination with estramustine down-regulated the expression of FoxM1 in prostate cancer leading to cell growth inhibition and induction of apoptosis (23, 24). We also found that 3,3'-diindolylmethane (DIM) reduced the expression of FoxM1 in breast cancer cells (25). However, very little or no information is available regarding the consequence of FoxM1 down-regulation in PC. Thus, in the present study, we investigated the role of FoxM1 in cell proliferation in PC cell lines. In our study, down-regulation of FoxM1 elicited a dramatic effect on growth inhibition of PC cells as shown by MTT assay. Our results provide in vitro evidence in support of the role of FoxM1 as an oncogene in PC cells because FoxM1 is known to induce oncogenesis, and its down-regulation causes inhibition of cell growth.
Because down-regulation of FoxM1 by siRNA reduced cell growth, we postulated whether cell growth inhibition was due to cell cycle arrest in any specific phase of the cell cycle. Indeed, we found that FoxM1 down-regulation increased cell population in the G0-G1 phase and decreased cell progression into the DNA replication phase (S phase). Diminished G1-S progression and growth rate was associated with increased expression of the CdkI protein p21CIP and p27KIP1, which is known to have negative effects on cell cycle machinery by binding to various cyclin-Cdk complexes and inhibiting their activities (26–29). We also observed a marked reduction in cyclin B, cyclin D1, and Cdk2 expression in FoxM1 siRNA-transfected cells. In our study, the decrease in cyclin D1, cyclin B, and Cdk2 and the increased expression of CdkI proteins, including p21CIP and p27KIP1, were strongly correlated with the altered cell cycle distribution phenotype and growth suppression. These results suggest that FoxM1 affects the PC cell cycle by regulating the expression levels of some cyclins (cyclin D1 and cyclin B) and CDK inhibitors (p21CIP and p27KIP1).

Because FoxM1 has been reported to promote both angiogenesis and metastasis in certain tumor models and inhibition of FoxM1 reduced tumor cell proliferation and angiogenesis in hepatocellular carcinomas (22), MMP-9 expression was also elevated in the liver of FoxM1B transgenic mice (19). It is known that MMPs are critically involved in the processes of tumor cell invasion and metastasis, and that MMP-9 and MMP-2 are directly associated with angiogenesis and metastatic processes (30–33). Both MMP-9 and MMP-2 have been implicated in metastasis because of their role in the degradation of the basement membrane collagen (30). Here, we showed that the down-regulation of FoxM1 inhibited MMP-9 and MMP-2 expression. We also found that the down-regulation of FoxM1 inhibited the activities of MMP-2 and MMP-9 in the culture medium of PC cells. However, overexpression of FoxM1 increased the expression and activities of MMP-2 and MMP-9. Thus, these results suggest that the down-regulation of FoxM1 could potentiate antitumor and antimetastatic activities, partly through the down-regulation of the expression of MMPs.

Another important molecule involved in tumor cell invasion and metastasis is VEGF. Many studies have documented that VEGF is a critical mediator of angiogenesis and regulates most of the steps in the angiogenic cascade, including proliferation, migration, and tube formation of endothelial cells (34). Investigations by other laboratories have shown that VEGF promotes migration and invasion of PC cells (35). The results of these investigations also suggested a trend toward an association between the expression of VEGF and distant metastasis (35, 36). It has been reported that FoxM1 regulates VEGF signaling in various cell types (20). In this study, we found a significant reduction of VEGF secretion in the culture medium of PC cells by FoxM1 down-regulation. We also found a marked increase in the activity of VEGF in FoxM1 cDNA-transfected cells. There are several other genes that also play important roles in tumor cell invasion and metastasis. It is well accepted that uPA and its receptor (uPAR) are important genes in the processes of tumor cell invasion and metastasis (37). uPA systems, including uPA and uPAR, play important roles in many aspects of the angiogenic process, including extracellular cascade of proteolysis, basement membrane degradation, cell migration, and invasion. It has been reported that cancer cells with higher levels of uPA or uPAR will tend to invade surrounding tissues and

![Figure 5](image-url)
subsequently migrate to blood vessels, thereby developing cancer cell metastasis (38). Indeed, in the present study, we found that the down-regulation of FoxM1 inhibited the expression of uPAR. Our results suggest that the down-regulation of FoxM1 could potentiate the antimetastasis activities partly through the down-regulation of VEGF and uPAR expression.

Because we observed that the down-regulation of FoxM1 inhibited the expression of uPAR, our results suggest that the down-regulation of FoxM1 could potentiate the antimetastasis activities partly through the down-regulation of VEGF and uPAR expression. However, further in-depth studies are needed to ascertain the precise molecular regulation of FoxM1 and NF-κB and their cross-talks in elucidating the role of FoxM1 in cell growth, invasion, and angiogenesis of PC cells in animal models and in human PC.

In summary, we presented experimental evidence that strongly supports the role of FoxM1 down-regulation as antitumor and antimetastatic mechanisms in PC. The down-regulation of FoxM1 induced G0-G1 phase cell cycle arrest with reduced levels of cyclin D1 expression and increased p21CIP and p27KIP1 expression. From these results, we conclude that the down-regulation of FoxM1 could potentially be an effective therapeutic approach for the inactivation of MMP-2, MMP-9, and VEGF, which is likely to result in the inhibition of cell growth, migration, invasion, angiogenesis, and metastasis of PC.

Figure 6. FoxM1 siRNA decreased PC cell migration and invasion and reduced the HUVECs tube formation. The experiments were repeated thrice. *, P < 0.05 relative to control. A, top, migration assay showing that FoxM1 siRNA decreased PC cell migration. Bottom, value of fluorescence from the migrated cells. B, top, invasion assay showing that FoxM1 siRNA-transfected cells resulted in lower penetration through the Matrigel-coated membrane, compared with control cells. Bottom, value of fluorescence from the invaded cells. The value indicated the comparative amount of invaded cells. C, top, Conditioned media from FoxM1 siRNA-transfected BxPC-3 and HPAC cells were able to significantly reduce the tube formation of HUVECs in 6 h incubation compared with the medium from control. Bottom, image analysis of tubule/capillary length was carried out using software image analysis program Scion Image. Quantification of cumulative tube length of endothelial cells.

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