Effects of FSTL1 on the proliferation and motility of breast cancer cells and vascular endothelial cells

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Abstract

Background: Treatments that prevent the motility of breast cancer cells and inhibit formation of new capillary vessels are urgently needed. FSTL1 is a secreted protein that has been implicated in maintaining the normal physiological function of the cardiovascular system, in addition to a variety of other biological functions. We investigated the role of FSTL1 in the proliferation and migration of breast cancer and vascular endothelial cells.

Methods: Human umbilical vein endothelial cells and human breast cancer BT-549 cells were used to test the effects of FSTL1 and the N-terminal domain of FSTL1. Immunofluorescence microscopy and 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, transwell invasion, and wound healing assays were conducted.

Results: Different doses of the N-terminal fragment of FSTL1 (FSTL-N) have variable effects on the migration of these cells. However, FSTL1 does not significantly affect tube formation in vitro from vascular endothelial cells. FSTL1-FL and FSTL1-N have modest effects on the invasion of breast cancer and vascular endothelial cells. Interestingly, FSTL1-FL, but not FSTL-N, modulates vascular endothelial cell polarization.

Conclusion: FSTL1 modestly affects the proliferation of breast cancer cells and vascular endothelial cells. Our findings improve our understanding of the functions of FSTL1 in breast cancer development and angiogenesis.

Introduction

Breast cancer is one of the most prevalent cancer types in women worldwide. Because of its high mortality rate and frequent metastasis, new diagnostics and more effective treatments for metastatic breast cancer are needed. Breast cancer metastasis is a complex process comprising multiple steps and mechanisms.¹² Metastasis occurs when unstable cancer cells adapt to and colonize a tissue microenvironment distant from the primary tumor. In order to prevent tumor invasion, it is necessary to inhibit tumor cell migration and angiogenesis, a tightly orchestrated process that requires cell proliferation, cell migration, and tubular morphogenesis.¹³ Both positive and negative regulators of angiogenesis contribute to a variety of physiological and pathogenic mechanisms, including embryonic development and tumor growth.⁶⁷ In the context of cancer, inhibition of tumor angiogenesis is an effective way to prevent tumor invasion.⁸
FSTL1 is a secreted protein that belongs to the follistatin family. It is widely expressed in mammalian tissues and is produced mainly by cells of mesenchymal origin. It has also been detected in medium collected from endothelial cells. However, the precise function of FSTL1 in endothelial cells is not fully understood. FSTL1 appears to play a key regulatory role in maintaining the normal physiological function of the cardiovascular system, where it is highly expressed in the blood vessels of the developing lung. In addition, a recent study showed that FSTL1 expression preserved the viability of endothelial cells subjected to systemic vascular damage. However, little is known about the role of FSTL1 in cancer. In this study, we investigated the role of FSTL1 in the proliferation and migration of breast cancer cells and vascular endothelial cells. Our results provide a deeper understanding of the function and mechanism of FSTL1 in breast cancer development and angiogenesis.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human breast cancer BT-549 cells were purchased from the American Type Culture Collection. HUVECs and BT-549 cells were cultured in RPMI-1640 and Dulbecco’s modified Eagle medium, respectively, containing 10% fetal bovine serum. Cells were maintained in a humidified incubator containing 5% CO2.

Drugs, antibodies, and reagents

Dr. Wen Ning of Nankai University kindly provided purified full-length FSTL1 (FSTL1-FL) and the N-terminal domain of FSTL1 (FSTL1-N). Antibodies against γ-tubulin and α-tubulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate and tetramethylrhodamine-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA), 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) from Songon Biotech (Shanghai, China).

Tube formation

Human umbilical vein endothelial cells were plated in 24-well plates pre-coated with Matrigel (diluted 1:1 with serum-free media). To examine tube formation, images were captured using an Axio Observer A1 fluorescence microscope (Carl Zeiss, Baden-Wuerttemberg, Germany). The degree of tube formation was quantified by measuring the cumulative tube length using IMAGEJ software (NIH, Bethesda, MD, USA), as previously described.

Cell proliferation

Cells were seeded in 96-well plates, and the density of cells was determined by MTT assay, as previously described.

Fluorescence microscopy

For immunofluorescence microscopy, cells were grown on coverslips, fixed with methanol at ~20°C, and blocked with 2% bovine serum albumin in phosphate buffered saline. Cells were incubated with primary antibodies for two hours and then with secondary antibodies for two hours. DAPI was used for DNA staining, and coverslips were mounted in 90% glycerol. Images were captured using an Axio Observer A1 fluorescence microscope (Carl Zeiss).

Transwell invasion

For the invasion assay, chambers were assembled in 24-well plates using 8 μm pore transwell inserts (BD Falcon, Franklin Lakes, NJ, USA). Inserts were coated with Matrigel (diluted 1:4 in serum-free media). HUVECs (1 × 105) were placed in the upper chamber. Invaded cells on the underside of the inserts were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Images were captured using a stereo microscope (Leica, Wetzlar, Germany).

Wound healing

Human umbilical vein endothelial cells treated with the indicated drugs for 48 hours were plated on coverslips and allowed to reach confluence. A 10 μL pipette tip was then used to create a scratch in the monolayer. The cells were washed twice with phosphate-buffered saline to remove the cell debris and cultured in serum-free media for 12 hours. Images of the wound were then captured, and the extent of wound repair was determined using IMAGEJ software.

Cell polarization

Human umbilical vein endothelial cells were plated onto coverslips and allowed to reach confluence. The monolayer was then scratched with a pipette tip to stimulate directed cell migration. Microtubules, centrosomes, and nuclei were stained using α-tubulin and γ-tubulin antibodies and DAPI, respectively. Coverslips were mounted in 90% glycerol and examined under the fluorescence microscope (Carl Zeiss). Border cells that exhibited a centrosome situated between the nucleus and the leading edge were considered as polarized.
Results

Domain organization of full-length FSTL1 and an N-terminal fragment

The FSTL1 protein contains 308 amino acids and has an N-terminal signal peptide of 20 amino acids (Fig 1a). The N-terminal domain of FSTL1 is critical for proper localization of the protein and has been shown to be required for various biological functions. FSTL1 also contains an FST-like domain containing 10 conserved cysteine residues and an extracellular calcium-binding domain that binds two calcium ions through a pair of canonical EF hand motifs. The EF hand is the most common calcium-binding motif found in proteins. Other conserved domains in FSTL1 include a KAZAL-like domain and a Von Willebrand factor type C domain. To characterize the role of FSTL1 in cancer development and angiogenesis, we used both full-length FSTL1 (FSTL1-FL) and the N-terminal fragment of FSTL1 (FSTL1-N) (Fig 1a).

FSTL1 has a modest effect on the proliferation of breast cancer and vascular endothelial cells

Tumor cell proliferation is necessary for tumorigenesis, and vascular endothelial cell proliferation is a key factor influencing tumor-associated angiogenesis. Therefore, we investigated the effects of FSTL1-FL and FSTL1-N on the proliferation of BT-549 breast cancer cells and HUVECs using MTT assays. We observed modest differences in the effects of treatment with low and high doses of FSTL1-FL in HUVECs, but not BT-549 cells (Fig 1b, d). The viability of BT-549 cells was slightly decreased after treatment with high doses of FSTL1-N compared to the vehicle (Fig 1c), but FSTL1-N treatment had no significant effect on HUVEC proliferation (Fig 1e).

Different doses of FSTL1-N have variable effects on breast cancer cell migration

To further explore the role of FSTL1 in cancer, we investigated the effects of FSTL1 on breast cancer cell migration, a critical step in cancer progression. Because FSTL1-N plays a key role in the biological function of FSTL1, we analyzed the effects of FSTL1-N on the migration of BT-549 cells. Wound healing assays revealed that the wounded area fully recovered after 12 hours in control monolayers as a consequence of directed cell migration (Fig 2a). Treatment of BT-549 cells with different doses of FSTL1-N had variable effects on the extent of wound closure (Fig 2b).

FSTL1 does not significantly affect tube formation in vitro from vascular endothelial cells

Angiogenesis plays a pivotal role in cancer progression, and because FSTL1 is a secreted protein, we hypothesized that it might regulate vascular endothelial cell function. To investigate the potential role of FSTL1 in the regulation of angiogenesis, we examined the effects of FSTL1-FL and FSTL1-N on vascular endothelial tube formation in vitro. Three hours after plating the cells, a tubular network of interconnecting branches was observed in the control cells. Measurement of cumulative tube length revealed no significant differences in comparison to FSTL1-FL or FSTL1-N-treated groups (Fig 3), indicating that FSTL1 does not directly affect tubular morphogenesis.
Different doses of FSTL1-N have variable effects on vascular endothelial cell migration

We next sought to examine the effects of FSTL1-N on vascular endothelial cell migration, another critical step in the process of angiogenesis. Results from wound healing assays showed that the wounded area in the control group was fully recovered after 12 hours as a result of directed cell migration (Fig 4a). Similar to the findings in BT-549 cells, treatment of HUVECs with different doses of FSTL1-N had variable effects on the extent of wound closure (Fig 4b).

FSTL1-FL and FSTL1-N have modest effects on the invasion of breast cancer and vascular endothelial cells

To further explore the potential role of FSTL1 in cell motility, we conducted transwell invasion assays in the presence and absence of FSTL1-FL and FSTL1-N. As shown in Figure 5a, treatment with FSTL1-FL or FSTL1-N had modest effects on the invasion of HUVECs and BT-549 cells through the porous membrane. We also quantified the effects of FSTL1-FL and FSTL1-N on the invasion of HUVECs or BT-549 cells by staining cells...
(Fig 5b,c) or counting cell numbers (Fig 5d,e), confirming that FSTL1-FL and FSTL1-N had modest effects on cell invasion.

**FSTL1-FL, but not FSTL-N, modulates vascular endothelial cell polarization**

Polarization is a critical step in cell migration that involves the rearrangement of microtubules and reorientation of the centrosome toward the leading edge of cells.3,20,21 Therefore, we performed polarization assays to characterize any potential involvement of FSTL1 in HUVEC motility. In the control group, cells at the wound margin demonstrated a typical polarized orientation, with the centrosome positioned between the nucleus and the leading edge (Fig 6a). This polarized morphology was modestly affected in cells treated with FSTL1-FL, but not FSTL1-N (Fig 6b). These data indicate that FSTL1-FL, but not FSTL-N, modulates vascular endothelial cell polarization.

**Discussion**

An increasing number of studies have indicated that the secreted protein FSTL1 plays key roles in cardiovascular diseases and might be involved in the pathogenesis of cancer.22–25 However, it is not clear whether FSTL1 is involved in breast cancer development and progression. Excessive proliferation plays a critical role in tumor growth; therefore, in this study we tested the effects of FSTL1 on proliferation of BT-549 breast cancer cells. We also tested the influence of FSTL1 on proliferation of HUVECs. Our results demonstrate that FSTL1 modestly affects the proliferation of these cell types.

Tumor cell migration is an important function associated with tumor invasion and metastasis.26 Similarly, endothelial cell migration is required for neovascularization.3,27,28 Because the N-terminal domain of FSTL1 plays a central role in the regulation of its cellular functions, we examined the effects of FSTL-N on cell migration. We found that different doses of FSTL-N had variable effects on the migration of breast cancer and vascular endothelial cells.

Angiogenesis plays a critical role in tumor development by supplying solid tumors with necessary nutrients.4 Therefore, we explored additional roles of FSTL1 in key processes associated with angiogenesis, including tube formation and polarization of endothelial cells. Cell polarization is the earliest step in cell migration, in which cells rearrange their cytoskeletons to prepare for migration.3,20 Using HUVECs, we have shown that FSTL1 does not significantly affect tube formation in vitro from vascular endothelial cells and that FSTL1-FL, but not FSTL-N, modulates vascular endothelial cell polarization.

Our results indicate that FSTL1 plays a modest role in cell motility and does not significantly affect angiogenesis in vitro. However, it is still possible that FSTL1 could play an indirect role in these physiological processes together with other regulatory factors. Additional studies are needed to explore the impact of this protein on cell proliferation and motility to better understand the role of FSTL1 in breast cancer development and progression.
Figure 5 Effects of full-length FSTL1 (FSTL1-FL) and its N-terminal fragment (FSTL1-N) on the invasion of human umbilical vein endothelial cells (HUVECs) and BT-549 cells. (a) Equal numbers of HUVECs and BT-549 cells treated with the vehicle, FSTL1-FL, or FSTL1-N were plated in transwell inserts. After 20 hours, cells that invaded to the underside of the inserts were stained with crystal violet and photographed. (b, c) Measurement of the absorbance produced by crystal violet of the stained (b) HUVECs and (c) BT-549 cells. (d, e) Quantification of the number of invaded (d) HUVECs and (e) BT-549 cells. OD, optical density.

Figure 6 Effects of full-length FSTL1 (FSTL1-FL) and its N-terminal fragment (FSTL1-N) on the polarization of human umbilical vein endothelial cells (HUVECs). (a) Immunofluorescence microscopy images of control, FSTL1-FL-treated, and FSTL1-N-treated HUVECs at the margins of wounded monolayers. Staining of α-tubulin (green), γ-tubulin (red), and DNA (blue) was used to visualize cell polarization. (b) Quantification of the percentage of polarized cells from experiments performed as in panel a.
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Disclosure

No authors report any conflict of interest.

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