Original Paper

LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a

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Key Words

E-cadherin • miR-200a • FAK • Migration • Tumor cells

Abstract

Background: LEFTY2, a suppressor of cell proliferation, tumor growth, regulator of stemness and embryonic differentiation, is a negative regulator of cancer cell reprogramming. Malignant transformation may lead to migration requiring loss of adhesion and gain of migratory activity. Signaling involved in the orchestration of migration, proliferation and spreading of cells include focal adhesion kinase (FAK) and adhesion molecule E-cadherin. **Aims:** The present study explored whether LEFTY2 influences the proliferation marker MKi67, FAK activity, E-cadherin abundance and migration of Ishikawa human endometrial carcinoma cells. Moreover, the study explored the involvement of microRNA-200a (miR-200a), which is known to regulate cellular adhesion by targeting E-Cadherin. **Methods:** FAK activity was estimated from FAK phosphorylation quantified by Western blotting, migration utilizing a wound healing assay, miR-200a and MKi67 expression levels utilizing qRT-PCR, cell proliferation and apoptosis using BrdU and Annexin V staining, respectively, and E-Cadherin (E-Cad) abundance, using confocal microscopy. **Results:** LEFTY2 (25 ng/ml, 48 hours) treatment was followed by decrease of MKi67 expression, FAK activity and migration. LEFTY2 upregulated miRNA-200a and E-Cad protein level in Ishikawa cells. The effect of LEFTY2 on migration was mimicked by FAK inhibitor PF 573228 (50 µM). Addition of LEFTY2 in the presence of PF-573228 did not result in a further significant decline of migration. **Conclusion:** In conclusion, LEFTY2 down-regulates MKi67 expression and FAK activity, up-regulates miR-200a and E-cadherin, and is thus a powerful negative regulator of endometrial cell proliferation and migration.

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Introduction

LEFTY2, also called endometrial bleeding-associated factor (EBAF), is a recently discovered member of the transforming growth factor (TGF)- β family [1, 2]. Human LEFTY2 is secreted as a 42-kDa precursor susceptible to proteolytic cleavage. LEFTY2 active forms are able to induce mitogen-activated protein kinase activity and to inhibit TGF- β signaling [3].

LEFTY2 expression is low in healthy endometrium, but its expression levels are increased prior to or during menstrual bleeding [4]. Enhanced LEFTY2 expression is associated with infertility [4] and abnormal uterine bleeding [5, 6]. The biological consequence of this over-expression remains to be determined. LEFTY2 also participates in the regulation of stemness and embryonic differentiation [7-19], reprogramming cancer cells [20] and suppression of tumor cells [21-24].

Recent evidence indicates that cancer cells retain many of the same characteristics as stem cells. An in depth understanding of the mechanisms that control stemness may lead to a better understanding of cancer cells. Several recent reports have revealed that embryonic stem (ES) cells are also found in many cancers, suggesting that these shared features may provide clues to designing cancer therapies (1, 2). A clue pointing to this shared feature is Nodal-LEFTY2 signaling. LEFTY is a critical regulator of very early human ES cell fate decisions, as LEFTY negatively modulates Nodal/TGF- β signaling, a key factor in the maintenance of pluripotency in human ES cells. Nodal and its coreceptor Cripto is expressed highly in certain malignant melanoma cells, and their malignant properties can be inhibited by LEFTY [25, 26]. LEFTY2 can inhibit cell proliferation, stimulate apoptosis and thus suppress tumor growth [20, 27-29], which can be quantified from expression of the proliferation marker Ki-67 (MKi67) [30].

A previous study demonstrated that LEFTY2 modulates migration [31]. Signaling contributing to the regulation of migration includes Focal adhesion kinase (FAK) [32-36], a ubiquitously expressed protein tyrosine kinase with an amino acid sequence about 90% homologous between humans, chickens, mice and frogs [37]. Lethality of FAK-deficient embryos at E8.5 suggests that it is critical during mammalian development [38]. FAK is further a powerful regulator of cell stiffness, proliferation and survival [39]. Abnormally high expression of FAK activity is associated with invasion and metastasis of cancer cells as well as tumor angiogenesis [37, 39]. Migration is inhibited by adhesion molecule E-Cadherin [40]. E-Cadherin is up-regulated by miR-200a [41], a powerful regulator of malignant transformation [42].

The present study explored whether LEFTY2 affects FAK activity, miR-200a, E-Cadherin and migration of human endometrial cancer cells.

Materials and Methods

Cell Culture

Ishikawa cells, a well differentiated endometrial carcinoma cell model [43, 44], were cultured in DMEM/F12 without phenol red, containing 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (Invitrogen, Karlsruhe, Germany). Cells were treated as described with recombinant human LEFTY2 (25 ng/ml; 746-LF-025/CF) (R&D Systems, Oxford, UK) and/or FAK inhibitor PF-573228 (50 μ M) (TOCRIS bioscience, Wiesbaden-Nordenstadt, Germany) for 48 hours.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from Ishikawa cultures using Trizol (Invitrogen) based on a phenol-chloroform extraction protocol [45]. Equal amounts of total RNA (2 μ g) were reverse transcribed by using the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) using an oligo dT primer. The resulting first-strand cDNA was diluted and used as template in qRT-PCR experiments. L19 was used as housekeeping gene to normalize for variances in input cDNA. Detection of gene expression was performed with KappaFast

-SYBR Green (Peqlab, Erlangen, Germany) and Quantitative RT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The expression levels of the samples are provided as arbitrary units defined by the $\Delta\Delta C_t$ method. All measurements were performed in triplicate. Melting curve analysis confirmed amplification specificity.

<i>MKi67</i>	forward (5'-3'): CACACTCCACCTGTCCTGAA
	reverse (5'-3'): GACTAGGAGCTGGAGGGCTT
<i>L19</i>	forward (5'-3'): GCAGCCGGCGCAAA
	reverse (5'-3'): GCGGAAGGTACAGCCAAT

miRNAs Quantitative Real-time PCR (miR-qRT-PCR)

Total RNA including miRNAs was extracted from Ishikawa cultures using miRNeasy Kit (Qiagen, Duesseldorf, Germany) as described previously [46]. Equal amounts of total RNA (100ng) were reverse transcribed by using the miRNA universal cDNA synthesis kit II (Exiqon, Vedbaek, Denmark) and PCR reaction was set using miR-200a-LNA™ primers (Exiqon, Vedbaek, Denmark) [46]. Detection of miRNA was performed with KappaFast-SYBR Green (Peqlab, Erlangen, Germany) and miR-qRT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The transcript levels of the samples were expressed as arbitrary units defined by the $\Delta\Delta C_t$ method. All measurements were performed in triplicate. Melting curve analysis confirmed amplification specificity.

BrdU staining

The BrdU staining was performed as recommended by the manufacturers' protocol with slight modification (eBioscience). In brief, Ishikawa cells were treated in 6-well plate as described above. Two hours prior to harvesting the cells BrdU (10μM) was added into the medium. The supernatant was removed and 250 μl of warm trypsin was added to the plate and returned to the incubator. After detachment, the cells were transferred to 15 ml tubes and warm PBS was added up to 10 ml. The tubes were then centrifuged at 300xg for 5 min at room temperature. The supernatant was removed and 200 μl PBS was added to the cell pellet. The resuspended cells were transferred to 96-well plate and centrifuged at 300xg room temperature for 5 min. The supernatant was removed and 200 μl of Fixation buffer (eBioscience) was added and incubated at room temperature for 15 minutes, protected from light. After incubation, the plate was centrifuged at 300xg at room temperature for 5 min then washed twice with staining buffer. 25 μl DNase I was added to 75 μl of staining buffer and incubated at 37 °C for 1 hour, protected from light. The cells were washed twice with the staining buffer. For intracellular staining, 2 μl BrdU antibody was added and mixed gently and incubated at room temperature for 30 minutes in the dark. Then centrifuged at 300xg at room temperature for 5 min and then 200 μl of PBS was added to each sample and transferred to FACS tubes for analysis. All the FACS were analysed by Flowjo software (Treestar, USA).

Annexin V staining

The Annexin V staining was performed as recommended by manufacturer (eBioscience). Cells were washed once with 200 μl of PBS, and once with 1 X binding buffer. Then cells were suspended in 150 μl of 1 X binding buffer and 2μl of fluorochrome-conjugated Annexin V was added. The plate was incubated for 15 minutes at room temperature, protected from the light. Wash cells with 1 X binding buffer and resuspended in 200 μl 1 X binding buffer then the cells were transferred to FACS tubes, and 2 μl of Propidium Iodide (PI; Sigma) was added. The samples were analyzed with flow cytometry within 4 hours. All the FACS were analysed by Flowjo software (Treestar, USA).

Western blotting

Whole-cell protein extracts were obtained by direct lysis in Laemmli buffer heated to 95 °C. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes [47]. The membranes were incubated overnight at 4 °C with anti-phospho-FAK (Tyr397) antibody (#8556), anti-total FAK antibody (A-17;#13309) or anti GAPDH antibody (1:1000; #2118, Cell Signaling, Leiden, The Netherlands), and then with secondary anti-rabbit HRP-conjugated antibody (1:2000; Cell Signaling) for 1 hour at room temperature. Following incubation with HRP-conjugated secondary antibodies the chemiluminescence was

visualized using the Novex ECL⁺ kit (Novex® ECL, Invitrogen). Densitometry was performed on scanned immunoblot images using the ImageJ gel analysis tool.

Confocal microscopy

Cells were treated and grown in sterile 4-well tissue culture chambers (Sarstedt, Germany). They were washed with ice-cold PBS once and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were washed 3x with ice-cold PBS, and permeabilised with 1x permeabilization buffer (eBioscience) for 15 minutes at room temperature. E-Cadherin primary antibody (#3195; Cell Signaling) [diluted in permeabilization buffer (1:100)] was added and kept in a humidified chamber at 4°C overnight. The following day, cells were washed with ice-cold PBS (x4) and incubated with the secondary antibody goat anti-rabbit IgG-FITC (#sc-2012; Santa Cruz Biotechnology) (1:200) for 1 hour at room temperature. The chambers were then washed three times with ice-cold PBS. Then DRAQ5™ (nuclear stain; eBioscience) was added. To mount Ishikawa cells CC/Mount (Sigma) was added and the cover slip was placed. Confocal microscopy was performed with a confocal laser-scanning microscope (LSM 5 Exciter, Carl Zeiss, Germany) with a C-Apochromat 63/1.3 NA DIC water immersion objective. (Carl Zeiss, Germany)

Migration assays

Ishikawa cells were counted and seeded in sterile 6 well plates and grown to confluence. Each well was scratched with a sterile 10 µl pipette tip, washed with PBS, and placed into fresh medium and treated as described. The sites at which wounds were to be measured were marked on the undersurface of wells to ensure that measurements were made at the same place. Wound width was measured by phase-contrast microscopy (Nikon Diaphot 300, Amsterdam, The Netherlands) immediately and after 48 h. Data were acquired with Bresser Mikrocarn (Bresser GmbH, Rhede, Germany) camera using MikroCamLab software and analysed with ImageJ software. Wound closure was calculated and expressed as a percentage of the initial wound width as described previously [48]. The data shown represent the means ± SEM of 3 measurements from 3 or more independent experiments.

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test and only results with *P* < 0.05 were considered statistically significant.

Results

The present study explored effects of LEFTY2 on proliferation and migration of human endometrial cancer (Ishikawa) cells. To this end, confluent cultures of Ishikawa cells were treated with 25 ng/ml of recombinant LEFTY2 for 48 hours. As illustrated in Fig. 1A, LEFTY2 significantly decreased the transcript levels of the proliferation marker MKi67. To investigate the effect of LEFTY2 during cell proliferation on Ishikawa cells BrdU assay was implemented. As shown in Fig. 1B, the proliferative ability of cells was significantly decreased (34%) after a 48h treatment with LEFTY2.

In order to test whether LEFTY2 affects migration, a wound-healing assay was performed. To this end, confluent Ishikawa cells were treated by mechanical wounding and subsequently treated with or without LEFTY2 (25ng/ml) for 48 hours. Wound widths were measured immediately (0 h) and after 48 h, and the percentage of wound closure was determined (Fig. 2A, B). Wound closure in the LEFTY2 treated cells was significantly reduced compared to the controls. Therefore, a significant decrease in cell reconstitution/migration was observed when recombinant LEFTY2 was added.

A further series of experiments explored whether the effect of LEFTY2 on migration was paralleled by an influence on the activity of focal adhesion kinase (FAK). To this end, confluent cultures of Ishikawa cells were treated with 25 ng/ml of recombinant LEFTY2 for 48 hours and total protein lysates harvested for Western blot analysis. FAK activity was quantified from FAK-phosphorylation (Tyr397), utilizing a specific antibody. Total FAK levels

Fig. 1. Effect of LEFTY2 on proliferation marker MKi67 in Ishikawa cells. A. Arithmetic means $\pm$ SEM ($n = 4$, arbitrary units) of MKi67 transcript levels. B. Original FACS plot of BrdU staining (% of proliferative cells). C. Fold Change ($\pm$ SEM) of cell proliferation ($n = 4$). * ($P < 0.05$), ** ($P < 0.01$) indicates statistically significant difference, Student's *t*-test.

Fig. 2. Effect of LEFTY2 treatment on migration of Ishikawa cells. A. Original photographs illustrating migration of human endometrial cancer Ishikawa cells in the absence (left panels) and presence (right panels) of LEFTY2 (25ng/ml) in a wound-healing assay at 0 hours (upper panels) and 48 hours (lower panels) after wounding. B. Arithmetic means $\pm$ SEM ($n = 10$) of the percentage of wound closure determined by image analysis in the absence and presence of LEFTY2 (25ng/ml). ** ($P < 0.01$) indicates statistically significant difference, Student's *t*-test.

Fig. 3. Effect of LEFTY2 on focal adhesion kinase (FAK) phosphorylation in Ishikawa cells. A. Representative original Western blots showing phosphorylated-FAK (Tyr397), total FAK and GAPDH protein abundance in human endometrial cancer Ishikawa cells after 48 hours culture in the absence or presence of LEFTY2 (25ng/ml). B. Arithmetic means $\pm$ SEM ($n = 4$, arbitrary units) of phosphorylated-FAK/total FAK protein ratio normalized to GAPDH in Ishikawa cells after 48 hours culture in the absence or presence of LEFTY2 (25ng/ml). ** ($P < 0.01$) indicates statistically significant difference using Student's *t*-test.

remained unchanged. As illustrated in Fig. 3A, B, treatment of Ishikawa cells with LEFTY2 was followed by a significant decrease of FAK phosphorylation pointing to a decrease of FAK activity.

Fig. 4. Effect of FAK inhibition on migration, proliferation and apoptosis of Ishikawa cells. A. Representative original Western blots showing phosphorylated-FAK (Tyr397), total FAK and GAPDH protein abundance in human endometrial cancer Ishikawa cells after 48 hours culture in the absence or presence of LEFTY2 (25ng/ml) $\pm$ FAK inhibitor PF 573228 (50 μ M). B. Original photographs illustrating migration of human endometrial cancer Ishikawa cells in the absence and presence of LEFTY2 (25ng/ml), FAK inhibitor PF-573228 alone (50 μ M) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μ M) at 0 hours (upper panels) and 48 hours (lower panels) after wounding. C. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of wound closure determined by image analysis in the absence and presence of LEFTY2 (25ng/ml), FAK inhibitor PF 573228 (50 μ M) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μ M). **($P < 0.01$), ***($P < 0.001$) indicates statistically significant difference, Student's *t*-test. D. Human endometrial cancer Ishikawa cells in the absence and presence of LEFTY2 (25ng/ml), FAK inhibitor PF-573228 alone (50 μ M) and LEFTY2 (25ng/ml) together with FAK inhibitor PF-573228 (50 μ M) for 48 hours. Expression levels of MKi67 were measured using qRT-PCR. * $P < 0.05$ indicate statistically significant difference, Student's *t*-test. E. In parallel experiments cells were subjected to Propidium Iodide (PI) and Annexin-V staining. Original FACS plots shown.

In additional experiments Ishikawa cells were treated for 48 hours with LEFTY2 (25 ng/ml) either with or without FAK inhibitor PF 573228 or left untreated (control). Cell lysates were subjected to Western blot analysis to assess levels of total FAK and phosphorylated FAK (Tyr397). As shown in Fig. 4A, there was a significant reduction in phospho-FAK in cells treated with LEFTY2 with or without inhibitor PF 573228, compared to the control. No change was observed in total FAK levels.

A next series of experiments determined whether inhibition of FAK phosphorylation was paralleled by inhibition of migration. To this end, a wound-healing assay was performed on Ishikawa cells treated with LEFTY2 (25 ng/ml) with or without PF-573228 or control cells. As shown in Fig. 4B and C, the percentage of wound closure was significantly decreased by a 48 hours treatment with FAK inhibitor PF 573228, which thus fully mimicked the effect of LEFTY2 on migration. The additional presence of LEFTY2 did not lead to a further significant decrease of migratory activity, indicating that LEFTY2 was effective in large part

Fig. 5. Effect of LEFTY2 treatment on miR-200a and E-Cadherin in Ishikawa cells. A. Human endometrial cancer Ishikawa cells in the absence and presence of LEFTY2 (25ng/ml), FAK inhibitor PF-573228 alone (50 μ M) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μ M) for 48 hours. Expression levels of miR-200a/5S rRNA were measured using qRT-PCR. B. Confocal Images of E-Cadherin (E-Cad) with and without LEFTY2 treatment in the presence or absence of FAK inhibitor PF573228 (50 μ M). Green (E-Cad), Red (Draq5; nucleus). * $P < 0.05$, ** $P < 0.01$ indicate statistically significant difference, Student's *t*-test.

by inhibiting FAK. To investigate if the effect of LEFTY2 on migration was dependent upon cell proliferation we performed qPCR for MKi67. As illustrated in Fig. 4D and E, treatment with LEFTY2 again decreased MKi67 transcript levels. However treatment with the FAK inhibitor slightly decreased MKi67 levels, though not reaching statistical significance. FAK inhibitor PF 573228 treated cells showed a slight increase in apoptotic cells.

We further explored the possibility that LEFTY2 treatment can influence the expression of E-Cadherin and of miR-200a. Ishikawa cells either remained untreated or were treated with LEFTY2 (25ng/ml) with or without the FAK inhibitor PF-573228 for a total of 48 hours. As showed in Fig. 5A, miR-200a was significantly upregulated in LEFTY2, PF-573228 and LEFTY2+ PF 573228 treated cells compared to the control. In parallel cultures, confocal microscopy revealed that LEFTY2 with or without PF-573228 treatment further increased E-Cadherin protein abundance (Fig. 5B).

Discussion

The present study uncovers a powerful inhibitory effect of LEFTY2 on the activity of FAK. FAK is a component of focal adhesions considered as a point of convergence of integrin and growth factor mechanical signaling [39, 49]. FAK is important for maintaining cell rigidity (stiffness) through promoting a static and highly aligned contractile cytoskeleton [50, 51]. It is a powerful regulator of actin polymerization and distribution of microfilaments [52] by activating downstream molecules such as: PI3-K [53-55], PAK1 [56, 57], Nhe1 [58, 59], rho kinase [38, 60, 61] or Src [62]. In FAK-deficient cells, reduced cytoskeletal stability is attributed to a compensatory increase of rhoA-kinase and ROCK activity [38, 60, 63]. Further, FAK directly interacts with multiple cell surface receptors and signaling proteins [39, 60, 62, 64] thus mediating a variety of physiological responses. Therefore, by regulating the activity of FAK, LEFTY2 could contribute to regulation of other physiological processes beyond regulating actin polymerization and cell stiffness [65].

As a regulator of focal adhesion dynamics, FAK is a critical mediator of signaling events between cells and their extracellular matrix. Up-regulation of FAK was seen in both endometrial hyperplasia and carcinoma, implying that FAK may play an important role in epithelial-mesenchymal transition (EMT) [66-71] and migration [32-36] during endometrial carcinogenesis [72]. According to our results both, LEFTY2 and PF 573228, decrease phosphorylation of FAK, an observation pointing to inactivation of FAK [73]. Deletion or inhibition of FAK impairs migration of fibroblasts [74]. Thus, treatment with LEFTY2 or PF-573228 decreases migration of Ishikawa cells. LEFTY2 could counteract migration by down-regulating microRNA miR-200a, a powerful stimulator of E-Cadherin expression [75, 76].

By supporting adhesion LEFTY2 counteracts detachment of cells from adjacent cells, thus suppressing migration and metastasis of tumor cells. LEFTY2 may further influence migration by down-regulation of the Na⁺/H⁺ exchanger [77], a carrier participating in the machinery of cell migration [78-84]. The molecular link between signaling and migration was recently described in colon cancer cells [85]. LEFTY2 may further be effective by down-regulation of FAK/phosphatidylinositol-3-kinase/Akt signaling, which is followed by actin reorganization controlling the migratory potential of tumor cells [65, 85]. By whatever mechanism, LEFTY2 does not only counteract tumor growth but as well as the migration of tumor cells and thus presumably tumor metastasis. Clearly, further investigations are required to fully uncover the pleiotropic effects of this interesting mediator on tumor cells.

In conclusion, LEFTY2 treatment of human endometrial carcinoma cells down-regulates the activity of the FAK and up-regulates E-Cadherin. LEFTY2 is thus a powerful inhibitor of Ishikawa cell migration.

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Disclosure Statement

The authors state that they have no conflict of interest.

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