Abstract. Endometrial cancer is the most common gynecological cancer. We identified interleukin 11 (IL11) as a critical mediator of endometrial tumourigenesis and demonstrated that IL11 regulates chondroitin sulfate proteoglycan (CSPG4) in human placental trophoblasts. CSPG4 is a cell membrane protein overexpressed in numerous human cancers, although its role in endometrial cancer has not been investigated. We examined CSPG4 expression and localization in primary human type I endometrioid grade (G) 1-3 tumours by qPCR and immunohistochemistry and determined whether IL11 stimulated CSPG4. IL11 upregulated CSPG4 mRNA in HEC1A (G2-derived endometrial epithelial cancer cell line) cells. IL11 administration to BALB/c nude mice enhanced HEC1A xenograft tumour growth and increased CSPG4 protein in tumours. CSPG4 mRNA was unchanged between human G1-3 endometrial cancer and control tissues. CSPG4 protein levels were elevated in the epithelium of G2 and G3 endometrial cancer and in the tumour-associated stroma of G3 tumour tissues compared to proliferative phase or post-menopausal endometrium. CSPG4 knockdown by siRNA reduced HEC1A proliferation and migration in vitro and reduced gene expression of the key epithelial-to-mesenchymal transition (EMT) regulator SNAIL. Our data suggest that CSPG4 inhibition may impair endometrial cancer progression by reducing cancer cell proliferation, migration and potentially EMT.

Introduction

Endometrial cancer occurs in the glandular lining of the uterus and is the most common cancer of the female genital tract and the fourth most common cancer in women (1). Type I ‘endometrioid’ carcinoma accounts for approximately 85% of all cases (2). It is often preceded by endometrial hyperplasia; a proliferative process within the endometrial glands that leads to an increase in the glandular-stromal ratio. This process is commonly associated with unopposed estrogen stimulation and may also be due to specific genetic alterations (2). Type I tumours are staged according to the guidelines of the International Federation of Gynecology and Obstetrics (3). Tumour grade (G1-3) is based histologically on the extent to which the cancer forms glands that display similar morphology to normal endometrium, and also metastatic behavior; the extent to which the cancer invades the uterine corpus and the surrounding peritoneum (2).

Cytokines produced within the tumour microenvironment can promote cancer cell growth, attenuate apoptosis and facilitate invasion and metastasis, making them attractive therapeutic targets. We identified interleukin 11 (IL11) as a critical regulator of placental trophoblast invasion in vitro and in vivo in the uterus during the establishment of pregnancy (4-6) and also endometrial tumourigenesis (7). We demonstrated that targeted blockade of endometrial cancer epithelial cell IL11 signalling reduced primary tumour growth and impaired metastasis in ectopic and orthotopic endometrial tumour xenograft models in vivo. IL11 receptor (R) α inhibition in G2-derived HEC1A cell line xenograft tumours retained a well-differentiated, endometrial epithelial phenotype vs. control, suggesting that it prevented epithelial-to-mesenchymal transition (EMT) (7).

We have previously shown that IL11 upregulates chondroitin sulfate proteoglycan (CSPG4) in primary human first trimester villous trophoblasts (8). CSPG4 silencing promotes trophoblast cell proliferation, but impairs invasion and migration (8). We have reported that IL11 plays opposing roles in trophoblast cell vs. cancer cell invasion in the endometrial lining of the uterus (4,5,9,10), whereby IL11 blocks trophoblast cell invasion, while it facilitates endometrial cancer cell migration/invasion.

CSPG4, also known as nerve glial antigen 2 (NG2), melanoma chondroitin sulfate proteoglycan (MCSP) (11) and N-methylpurine-DNA glycosylase (HMPG) (12) is a single membrane spanning protein (13). CSPG4 is overexpressed in several human cancers, including soft tissue sarcomas (14), mesenchymal tumours (15) and melanomas (16). It is a member of the matrilysin-inducible surface protein component (MISP) family (17) and is strongly expressed in the cancer associated stroma of many human tumours (18).

It is not known whether CSPG4 is upregulated in endometrial cancer (19) or if it plays a role in endometrial cancer progression and metastasis. Here we report that CSPG4 is a new target for endometrial cancer therapy.
melanoma (15) and breast cancer (16). It has established functional roles in promoting cancer cell proliferation, migration and invasion (17-19). These functions are thought to be mediated via CSPG4 interactions with extracellular and intracellular binding proteins (20). However, the expression, regulation and function of CSPG4 in endometrial cancer have not been investigated.

We hypothesized that CSPG4 expression is overexpressed in human endometrial cancer, as in other epithelial malignancies and IL11 stimulates CSPG4 in endometrial cancer cells. We aimed to determine the expression and localization of CSPG4 in type I human endometrioid endometrial cancer across G1-3 tumours and normal endometrium. We determined the effect of CSPG4 knockdown on HEC1A (endometrial epithelial cancer cell line) cell proliferation and migration using the xCELLigence real-time system, or wound healing migration assays in vitro. Furthermore, we investigated whether IL11 alters CSPG4 levels in human endometrial epithelial cancer cells in vitro and in subcutaneous xenograft tumours in vivo, in mice.

Materials and methods

Cell culture. HEC1A cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA; 2013) and cultured in McCoy's medium with 10% fetal calf serum (FCS). Ishikawa cells were provided by Dr M. Nishida (Tsukuba University, Tochigi, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. Monash Health Translational Precinct Medical Genomics authenticated these cell lines in June 2016.

HEC1A in vitro cytokine treatments. Confluent HEC1A cells were serum starved for 24 h and treated with recombinant human IL11 (10 or 100 ng/ml) or phosphate-buffered saline (PBS) (4). Cell lysates were collected after 6 h of treatment. RNA was extracted and quantitative real-time RT-PCR performed.

Animals. Animal experiments were conducted in female, 5-7 week old, athymic, BALB/c nude mice purchased from the Animal Resources Centre; Western Australia, housed in specific pathogen-free conditions, with food and water available ad libitum and held in a 12-h light and dark cycle. Use of all animals was in accordance with the guidelines of the Monash Medical Centre Animal Ethics Committee under Ethics Approval number MMCB/2012/07.

Subcutaneous tumour inoculation. HEC1A cells were resuspended in serum-free medium at a concentration of 20x10⁶ cells/ml. Both flanks of each animal were inoculated with 100 µl (2x10⁶ cells) (n=3/group). Once palpable, tumours were measured with digital calipers (Hare & Forbes Machineryhouse) and tumour volume calculated using the following formula: (length x width²)/2 (mm³) (7).

Animal treatments and tissue collection. Once subcutaneous tumours measured 80-100 mm², mice were randomized into groups and administered by intraperitoneal injection with recombinant human IL11 (500 µg/mg/kg) (4) or saline vehicle control three times weekly for two weeks. At the completion of the study, tumours were fixed in 4% paraformaldehyde for 24 h and paraffin embedded for immunohistochemistry.

Participants and patient samples. Endometrial cancer tissue biopsies (n=10/grade), or benign endometrium (n=4) were collected from postmenopausal women undergoing total abdominal hysterectomy at the Monash Medical Centre Melbourne, Australia. The Human Ethics Committee approved the research project and informed consent was obtained from each patient. Tumors were graded histologically by a specialist gynecological pathologist according to the guidelines of FIGO, 2009 as previously described (21). Proliferative phase endometrium (n=6) was collected at curettage from women between day 7 and 13 of their menstrual cycle that were scheduled for tubal ligation, as a non-tumour control group. A pathologist declared no obvious endometrial pathology. Women had no steroid treatment or other medication for at least 2 months before tissue collection. Written informed consent was obtained from each patient and the study was approved by the Southern Health Human Research and Ethics committee. Biopsies were fixed in 10% neutral buffered formalin overnight, prior to paraffin embedding.

Immunohistochemistry. Formalin-fixed human endometrial cancer, postmenopausal, or proliferative phase endometrial tissue, or subcutaneous HEC1A xenograft tumour tissue sections (4 µm) were dewaxed in histosol (2x10 min), rehydrated in ethanol and antigen retrieval performed in 0.01 M sodium citrate (pH 6.0) before endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 10 min. Non-specific binding was blocked with 10% normal goat serum and 2% normal human serum, in Tris-buffered saline (TBS) for 30 min. The CSPG4 antibody (#4235; 4 µg/ml; Cell Signaling Technology, Danvers, MA, USA) was applied overnight at 4°C. Equal concentration of negative control isotype rabbit IgG (Dako) was included for every tissue section. Antibody localization was detected by sequential application of biotinylated goat anti-rabbit IgG (7.5 µg/ml; Vector Laboratories, Burlingame, CA, USA) for 30 min followed by Vectastain ABC Elite kit (Vector Laboratories) for 30 min. Peroxidase activity was visualized by the application of diaminobenzidine substrate (DakoCytomation). Tissues were counterstained with Harris hematoxylin (Sigma-Aldrich) and mounted. Staining intensity in the epithelial and stromal compartments were scored from 0, no staining to 3, intense staining by two independent, blinded assessors.

RNA preparation and quantitative real-time RT-PCR. RNA from human endometrial cancer or benign endometrium whole tissue was obtained from the Victorian Cancer Biobank (Project #13018). Total RNA was isolated from endometrial epithelial cancer cell lines, or subcutaneous HEC1A xenograft tumour tissue using TRI reagent (Sigma-Aldrich). Genomic DNA was digested using the DNA-free kit (Ambion) according to the manufacturer's instructions. To test the RNA yield, purity and concentration, 2 µl was analyzed using the NanoDrop spectrophotometer (Thermo Fisher Scientific) at an absorbance ratio of A260/280 nm. cDNA was synthesized from total RNA (250 ng) using SuperScript III reverse transcriptase.
Results

CSPG4 mRNA and protein are stimulated by IL11 in HEC1A endometrial epithelial cancer cells and xenograft tumours in mice. To investigate the stimulation of CSPG4 in human endometrial epithelial cancer cells, HEC1A cells were treated with IL11 in vitro. There was a significant 3-fold increase in CSPG4 gene expression following treatment with IL11 (100 ng/ml) compared to control or IL11 at (10 ng/ml) after 6 h (P<0.05, n=3/gp) (Fig. 1A). To confirm this effect in vivo, subcutaneous HEC1A xenograft tumours were established in mice, and mice were administered with saline vehicle control, or recombinant IL11 (500 µg/mg/kg). IL11 significantly increased subcutaneous tumour growth vs. saline control (P<0.01, n=3/gp) (Fig. 1B). Following IL11 treatment, immunohistochemical staining of tumour tissue identified that CSPG4 predominantly localized to the cell membrane and to a lesser extent to the cytoplasm increased in IL11-treated tumours vs. control (Fig. 1C). Semi-quantification of the immunostaining intensity confirmed a significant increase in CSPG4 staining intensity supplied with the instrument (ACEA) and exported for statistical analysis.

Wound healing migration assay. HEC1A cells were seeded at 100,000 cells/well in a 12-well plate and grown to subconfluence. Cells were transfected with siRNA or control as above, before wounding by using vacuum suction through a protein electrophoresis pipette tip (Bio-Rad Laboratories). On the day of wounding, designated time 0 (0 h), cell wounds were photographed using a Motic AE31 inverted microscope and camera and Motic Images Plus 2.0 software (Motic microscopy; Motic). To assess differences in wound repair, the area of each wound was manually outlined and they are quantitated using ImageJ (v10.2 NIH) software, at 0 and at 48 h. The 48-h time-point was chosen since a clear wound boundary was distinguishable, as the wound was not fully repaired. Data was expressed as percent repair at 48 h vs. the 0 h time-point. For each experiment, wounds were performed and assessed in triplicate and repeated in three separate experiments.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism (GraphPad Software 6.0) and data assessed by the Student’s t-test for two groups. Multiple groups were compared using one-way ANOVA, with the Tukey’s post-hoc test. Results of P<0.05 were considered statistically significant.

Table I. Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5'-3')</th>
<th>Reverse primer sequence (5'-3')</th>
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<tbody>
<tr>
<td>CSPG4</td>
<td>AGCTAGCCAGGACTGATGGA</td>
<td>CAGCCTAACCTGTCCAAAG</td>
</tr>
<tr>
<td>ECAD</td>
<td>ACACCATCTGTCGCCACTTT</td>
<td>CAGGTCTCCCTCTTGGCTCTG</td>
</tr>
<tr>
<td>TWIST</td>
<td>GGAATCCGAGGCTTACGAG</td>
<td>TGGAGGACCTGGTATAGGAA</td>
</tr>
<tr>
<td>SNAIL</td>
<td>AGATGATCATGCGGACCCCCAC</td>
<td>CCTCATTTGTTGCGAGGAGA</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>GGCCGCGGACCTTATACAC</td>
<td>TAGACACTTGAGCTCGGCA</td>
</tr>
<tr>
<td>18s</td>
<td>GATCCATTGGAGGCAAGTCTT</td>
<td>CCAAGATCCAACACTACGAGCTT</td>
</tr>
</tbody>
</table>

Small interfering RNA (siRNA) transfections. HEC1A cells were cultured to 70% confluence and transfected with commercially generated and validated ON-TARGETplus SMARTpool siRNA (Dharmacon, Lafayette, CO, USA) targeting CSPG4 (CSPG4 siRNA) or no specific sequence as a scrambled siRNA (Dharmacon, Lafayette, CO, USA) targeting CSPG4 specifically and validated ON-TARGETplus SMARTpool. Delivery was performed using the Lipofectamine RNAiMAX (Invitrogen-Life Technologies) according to the manufacturer’s instructions. Cells were transfected for 72 h prior to RNA collection to test for transfection efficiency or prior beginning functional experiments (8).

xCELLigence real-time cell proliferation assay. Experiments were carried out using the RTCA DP xCELLigence instrument (Roche), which was placed in a humidified incubator maintained at 37°C with 95% air/5% CO2. Cells were seeded in E-plate 96 at 10,000 cells/well in 5% FCS DMEM medium and the plate was monitored once every hour for a total of 48 h (22). Data was calculated using RTCA Software 1.2, as the endogenous control for normalization.

PCR and gel electrophoresis. RNA and cDNA were prepared as above. PCR reactions were performed using PCR express machine (Thermo Fisher Scientific) and GoTaq Master Mix (Promega) according to the manufacturer’s instructions. cDNA was analyzed for CSPG4 and 18s (Table I) using reaction conditions of denaturation at 95°C for 3 min, followed by 30 cycles of: denaturation, 95°C for 30 sec; annealing, 60°C for 30 sec; extension, 72°C for 1 min; with a final extension at 72°C for 10 min. PCR products were run on 1.5% agarose gel with 1000 bp DNA ladder (Invitrogen). Real-time RT-PCR analyses were performed on the ABI 7500HT fast block real-time PCR system (Applied Biosystems) in triplicate (final reaction volume, 10 µl) in Optical 384-well microplates (Applied Biosystems). For each sample, 25 ng of cDNA was added to a PCR mix made with the 2X FastStart SYBR-Green Master Mix containing ROX passive reference dye (Applied Biosystems) and 10 nM primers. The primer sequence details are listed in Table I. A template-free negative control in the presence of primers and RNase-free water and, RNase-free water only negative controls were added for each run. The PCR protocol was as follows: 95°C for 10 min and 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. Relative expression levels were calculated by the comparative cycle threshold method (ΔΔCt) as outlined in the manufacturer’s user manual, with 18s ribosomal RNA serving as the endogenous control for normalization.

Experiments were performed and assessed in triplicate and repeated in three separate experiments.
in the cell membrane following IL11 treatment compared to control (P<0.05, n=3/group) (Fig. 1D).

CSPG4 protein levels are elevated in type I endometrioid cancer with increasing tumour grade. CSPG4 protein was immunolocalized in human G1-3 endometrial tumour or proliferative phase or post-menopausal endometrial tissue. CSPG4 predominantly localized to the epithelial compartment of G1-3 endometrial tumours (Fig. 2A) and staining intensity levels were significantly elevated in the epithelium of G2 and G3 tumour tissue compared to proliferative phase or post-menopausal endometrium (P<0.05, P<0.01; n=6-10/group) (Fig. 2B). Very minimal staining was evident in the stroma of proliferative phase or postmenopausal endometrium (Fig. 2A). Staining intensity levels were significantly elevated in the stromal compartment in G3 malignant tumour tissue vs. non-malignant tissue (P<0.05, P<0.01; n=6-10/group) (Fig. 2C).

CSPG4 mRNA expression in primary human endometrial cancer and cancer cell lines. Quantitative real-time RT-PCR was performed to measure CSPG4 mRNA levels in whole tissue from G1-3 endometrial tumours vs. benign endometrium. There were no differences in gene expression across all endometrial cancer grades compared to the benign group (n=10/group) (Fig. 3A). To conduct functional studies, we determined CSPG4 gene expression in human endometrial epithelial cancer cell lines. G2-derived HEC1A cells expressed CSPG4, although G1-derived Ishikawa cells did not (Fig. 3B).

CSPG4 siRNA knockdown decreases HEC1A cell proliferation and migration. To determine the functional role of CSPG4 in endometrial epithelial cancer cell proliferation and migration, we transiently silenced CSPG4 gene expression using siRNA in HEC1A cells. This resulted in significant knockdown of CSPG4 at the transcript level in siRNA-treated
HEC1A cells compared to scrambled (scr) control (P<0.001; n=4/group) (Fig. 4A). We examined HEC1A cell proliferation using the xCELLigence real-time system. After 72 h, there was a significant decrease in proliferation in response to CSPG4 siRNA treatment vs. scr control (P<0.05; n=4/gp) (Fig. 4B). Cell migration was assessed by performing a wound healing assay on CSPG4 siRNA treated, or scr control HEC1A cells (Fig. 4C). Analysis of the area of repair between 0 and 48 h following wounding demonstrated a significant 16±3% reduction in migration of CSPG4 siRNA treated HEC1A cells compared to control (Fig. 4D).

CSPG4 knockdown reduces SNAIL mRNA expression in HEC1A cells. From assessment of key regulatory transcription factors involved in EMT, CSPG4 knockdown resulted in significantly reduced SNAIL gene expression compared to control in HEC1A cells (P<0.05) (Fig. 5A), although TWIST, ECAD and ICAM-1 mRNA levels were unchanged (Fig. 5B-D).

Discussion

This study is the first to identify and determine a functional role for CSPG4 in endometrial cancer. The findings established that CSPG4 protein levels are elevated in G2 and G3 endometrial tumour epithelium and G3 tumour stroma compared to normal proliferative phase or post-menopausal endometrium. IL11, a crucial mediator of endometrial tumourigenesis and EMT (7), upregulated CSPG4 mRNA and protein in G2-derived HEC1A endometrial epithelial cancer cells in vitro and xenograft tumours in vivo. Functionally, CSPG4 knockdown impaired HEC1A cell proliferation and migration in vitro, and led to the downregulation of a central EMT transcription factor, SNAIL, suggesting that CSPG4 may promote endometrial cancer progression in women.

These data are supported by previous studies in other tumour types, where CSPG4 is overexpressed in human breast carcinoma (12,16), glioblastoma (19) and melanoma (11,17). Interestingly, CSPG4 gene expression levels were unchanged between endometrial tumour G1-3 and benign endometrial tissue. This could be attributed to the heterogeneity of whole endometrial tumour tissue; therefore, immuno localization studies were performed to determine which cell types express CSPG4 protein. Since it is typically a cell surface proteoglycan (14), the localization pattern of CSPG4 as a membrane protein on the tumour epithelial cell surface was in line with reports in other tumour cell types.

Recently, we identified CSPG4 in the human placenta, showing that it localized to invasive and proliferative trophoblast subtypes of the placenta and the maternal uterine decidua (8). Furthermore, it was revealed that knockdown of CSPG4 stimulated HTR8/SVneo trophoblast cell line proliferation, but decreased migration. The data from this study highlights similar roles for CSPG4 in human trophoblast and endometrial cancer cell migration, but opposing regulation of proliferation, suggesting that the functional roles of CSPG4 in regulating cell cycle/growth in the uterine environment are cell-dependent.

The present study is the first to localize CSPG4 in the non-pregnant, cycling human endometrium. In normal proliferative phase endometrium, CSPG4 predominantly localized to the glandular epithelium. This localization pattern, together with its well-established role in promoting cell-cell adhesion in other cell types, such as breast cancer cells (23), suggest that CSPG4 could enhance non-malignant endometrial epithelial...
CSPG4 knockdown reduced HEC1A cell migration mesenchymal markers such as vimentin (24). We found that cases, loss of epithelial markers such as E-cadherin and gain of induction transcription, eventually causing degradation of the environment to promote EMT (24). facilitate tumour progression by acting on the local tumour environment to promote EMT (24).

Central to EMT is the activation of TWIST and SNAIL induced transcription, eventually causing degradation of the basement membrane by induction of matrix metalloproteinases, loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin (24). We found that CSPG4 knockdown reduced HEC1A cell migration in vitro. Assessment of pathways involved in EMT showed that CSPG4 silencing significantly downregulated SNAIL gene expression. These findings suggest that CSPG4 inhibition can prevent cancer cell migration, potentially attributed to inhibition of EMT. In line with these findings, we previously demonstrated that IL11 induces SNAIL in HEC1A xenograft tumours in vivo (7). Here we reported that IL11 also upregulates CSPG4, highlighting a new mechanism by which IL11 may promote endometrial tumourigenesis.

Preclinical monoclonal antibody treatment studies have shown promising results in breast cancer models (23,25). Further investigation of the role of CSPG4 in endometrial cancer pre-clinical cancer models in vivo animal models, such as orthotopic xenograft tumour models (7), could support also our findings and determine the potential of targeting CSPG4 for clinical translation as a new treatment option for women with endometrial cancer.

In summary, in the present study we have demonstrated that CSPG4 is a cell surface proteoglycan regulated by IL11 in human endometrial epithelial cancer cells which is upregulated in the epithelial compartment in human type I G2 and G3 endometrioid endometrial cancer. Functionally, loss of CSPG4 gene expression in endometrial epithelial cancer cells reduced proliferation and migration of HEC1A cells in vitro, potentially mediated by suppression of the EMT factor, SNAIL, suggesting that elevations of CSPG4 expression/function could be pro-tumourigenic in women.

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References


