Lipocalin 2 induces the epithelial–mesenchymal transition in stressed endometrial epithelial cells: possible correlation with endometriosis development in a mouse model

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Abstract

Lipocalin 2 (LCN2) is an induced stressor that promotes the epithelial–mesenchymal transition (EMT). We previously demonstrated that the development of endometriosis in mice correlates with the secretion of LCN2 in the uterus. Here, we sought to clarify the relationship between LCN2 and EMT in endometrial epithelial cells and to determine whether LCN2 plays a role in endometriosis. Antibodies that functionally inhibit LCN2 slowed the growth of ectopic endometrial tissue in a mouse model of endometriosis, suggesting that LCN2 promotes the formation of endometriotic lesions. Using nutrient deprivation as a stressor, LCN2 expression was induced in cultured primary endometrial epithelial cells. As LCN2 levels increased, the cells transitioned from a round to a spindle-like morphology and dispersed. Immunochemical analyses revealed decreased levels of cytokeratin and increased levels of fibronectin in these endometrial cells, adhesive changes that correlate with induction of cell migration and invasion. *Lcn2* knockdown also indicated that LCN2 promotes EMT and migration of endometrial epithelial cells. Our results suggest that stressful cellular microenvironments cause uterine tissues to secrete LCN2 and that this results in EMT of endometrial epithelial cells, which may correlate with the development of ectopic endometriosis. These findings shed light on the role of LCN2 in the pathology of endometrial disorders.

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Introduction

Endometriosis is an estrogen-dependent disease characterized by the presence of endometrium-like tissue outside of the uterine cavity (Bulun 2009). This disease typically affects organs of the pelvis, such as the ovaries, and may contribute to infertility. In addition, endometriosis is believed to increase the risk of cancer (Melin *et al.* 2011), and links between ovarian endometriosis and certain types of ovarian cancer have been suggested (Somigliana *et al.* 2006). The endometriotic lesions are similar to cancerous tumors in that they require angiogenesis to expand (Taylor *et al.* 2002). However, the pathogenesis of this uterine disorder is poorly understood.

Estrogen induces an epithelial–mesenchymal transition (EMT) during implantation of the ectopic endometrium, which consists of stromal and epithelial cells (Huang *et al.* 2007, Bulun 2009). EMT is a process whereby epithelial cells convert to a mesenchymal phenotype, and EMT may be essential for the migration, invasion, and relocalization of epithelial cells

(Bulun 2009). EMT can be induced by a number of signals, including the acute stress response (Vargha *et al.* 2008). Gene profiling in a rat model of endometriosis identified 71 upregulated and 45 downregulated genes in endometriotic lesions (Konno *et al.* 2007), including an acute-phase protein, lipocalin 2 (LCN2). Thus, LCN2 levels may correlate with EMT and endometriosis onset. Clarifying the molecular mechanisms involved in endometriosis development may provide insights into how endometriotic lesions transition to carcinoma.

LCN2 is an estrogen-dependent, acute-phase protein that in mice has been implicated in multiple processes, including apoptosis, ion transport, inflammation, cell survival, and tumorigenesis (Chu *et al.* 1996, Liu *et al.* 1997, Lee *et al.* 2007, Lin *et al.* 2007). As mentioned earlier, LCN2 is upregulated in the endometriotic lesions (Konno *et al.* 2007, Becker *et al.* 2010). We previously found that LCN2 is upregulated in the endometrial carcinoma cells (RL95-2 cells derived from endometrial epithelia) under oxidative stress and that LCN2-induced secreted cytokines trigger cell proliferation and migration (Lin *et al.* 2011). These findings indicate that

LCN2 is a stress factor that responds to environmental stress by triggering changes in cellular physiology. This stress-linked signaling pathway can be activated under both physiological and pathophysiological conditions *in vivo* (Cannito *et al.* 2010). Oxidative stress regulates EMT (Cannito *et al.* 2010), and LCN2 is associated with breast cancer progression via EMT (Yang *et al.* 2009). In addition, EMT is an important factor in an estrogen-dependent tumorigenesis (Tiezzi *et al.* 2007). On the basis of these data, we hypothesized that the stress- and estrogen-dependent induction of LCN2 triggers EMT within endometrial epithelial cells, which then stimulates the migration of endometrial epithelial cells to ectopic sites and increases the risk of endometriosis.

To examine the role of LCN2 in the induction of ectopic endometriosis, here we established a mouse model of endometriosis and used an LCN2-specific antibody to inhibit LCN2 function. We harvested primary endometrial epithelial cells and examined cytokeratin and fibronectin levels to assess EMT under stress-inducing conditions. Through this analysis, we attempted to reveal correlations between LCN2, EMT, and endometriosis. Consequently, we also examined the LCN2 expression in human uterine tissue, including a patient with endometriosis and a patient with a benign tumor of myoma as the normal one. Our results suggest that LCN2 induction during EMT of endometrial epithelial cells promotes endometriosis onset.

Materials and methods

Experimental animals

Outbred female imprinting control region (ICR) mice were bred in the animal center at the National Taiwan University (Taipei, Taiwan) and transferred to Academia Sinica for experimentation. Test animals were housed under controlled light (12 h light:12 h darkness cycle) at a constant temperature ($23 \pm 2^\circ\text{C}$) with water and food (NIH 31) supplied *ad libitum*. All animal procedures were approved by the ethics committee of the animal center at Academia Sinica.

Autotransplantation of mouse endometrial tissue

Autotransplantations were carried out as described by Somigliana *et al.* (1999) and Lin *et al.* (2006) with modifications. Female ICR mice (6 weeks old) were ovariectomized (day 0) and then s.c. injected with estrogen (15 $\mu\text{g}/\text{mouse}$) on days 0 and 3. On day 7, fragments of the right side of the uterus were implanted into the peritoneal surface of the same mouse. Mice were separated into six groups (two control groups, three implanted groups that were killed at 3, 4, and 5 weeks, and one group for antibody treatment), and each group ($n \geq 13$) received s.c. injections of estrogen (15 $\mu\text{g}/\text{mouse}$) twice a week (with a 3–4-day interval) for 3, 4, and 5 weeks after the peritoneal implantation of uterine tissue by suturing into the peritoneum. The antibody-treated group was supplemented with the LCN2 antibody (10 $\mu\text{g}/\text{mouse}$) injected into

peritoneum with the same schedule as for the estrogen supplement until 5 weeks. The mice were then killed at specific time points and LCN2 was analyzed. Finally, eutopic and ectopic endometrial tissues and peritoneal fluid were harvested for analysis. Two groups of sham mice were included: in one group, no estrogen supplement was given after the mice were ovariectomized as a control (NE in Fig. 1); in the other group, mice were estrogen-treated after having been ovariectomized (EE in Fig. 1). Eutopic tissue was from a mouse without endometriosis as a control experiment. Ectopic tissue volume calculation was done following the method of Makino's report (Makino *et al.* 2001): ectopic tissue volume (cm^3) = (large diameter) $\times$ (small diameter) $^2/2$.

Isolation and culture of primary uterine endometrial cells

Mouse endometrial cells were isolated enzymatically as described by Chan *et al.* (1997), with modifications. Uterine tissue was obtained from 3-week-old female ICR mice (3 to 5 mice) and mixed together for use. This avoided confounding effects related to the estrous cycle, which typically begins after 5–6 weeks of age. Isolated cells ($\sim 1.5 \times 10^5$ cells/mouse) were resuspended in Ham's DMEM/F12 culture medium containing 1% charcoal-treated fetal bovine serum (FBS), 1% nonessential amino acids, 100 $\mu\text{U}/\text{ml}$ penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin and the culture medium was changed every 3 days to keep fresh; passage was not necessary. Suspensions were incubated at 37°C in 5% CO_2 in air. No estrogen was added.

Figure 1 Effect of LCN2 on endometriosis development *in vivo*. Using a mouse model of endometriosis in which uterine tissue was implanted in the peritoneum, peritoneal fluid, eutopic endometrial tissues, and ectopic endometrial tissues were harvested after the animals received estrogen treatment for 3, 4, or 5 weeks. (A) Western blot analysis of LCN2 secretion into the peritoneal fluid. (B) QPCR analysis of *Lcn2* mRNA levels in eutopic (gray bars) and ectopic (black bars) endometrial tissues ($n \geq 3$ per group). (C) Endometrial tissues associated with the peritoneal surface in mice treated with anti-LCN2 (a) or not treated (b). Arrows indicate ectopic tissues. (D) Western blotting was used to measure the LCN2 levels in serum for mice with ectopic uterine tissue (EM) or mice that received a sham surgery (without EM) (* $P < 0.05$; $n \geq 13$ per group). EA, estrogen treatment with anti-LCN2 treatment.

Immunohistochemistry

Cytokeratin and fibronectin were used as markers of EMT (Xu *et al.* 2009). The endometrial epithelial cells were cultured on coverslips with or without LCN2 for ≥ 24 h. After fixing with methanol, the cells were then incubated in PBS containing 1% BSA and 0.25% (v/v) Triton X-100 (PBST) for 30 min and then incubated with a rabbit antibody against cytokeratin (1:250, ab9377-500; Abcam, Cambridge, UK) in PBST for 2 h at RT. After washing, cells were incubated with Texas Red-conjugated IgG against rabbit (1:500, ab6793; Abcam). After an additional round of washing and blocking with PBST (30 min), samples were incubated with an antibody against fibronectin (1:250, ab2413; Abcam) for 2 h, washed, and then incubated with FITC-conjugated IgG against rabbit (1:500, ab6717; Abcam). 4',6-Diamino-2-phenylindole dihydrochloride (DAPI) staining to label nuclei was also carried out.

For histological studies involving human and mouse tissues, the tissues were fixed overnight in neutral formalin solution (3800598, Leica Surgipath, Singapore), dehydrated using a serial dilution of ethanol (50, 70, 80, 90, and 100% ethanol) and xylene, and embedded in paraffin. The tissue sections (5 μ m) were mounted on coated microscope slides (K8020, Dako, Glostrup, Denmark) and dried at 42 °C for 24 h. The sections were then deparaffinized with xylene and rehydrated using a serial dilution of ethanol and water. The rehydrated sections were incubated with an antibody against mouse LCN2 (1:100, MAB1857; R&D, Minneapolis, MN, USA) or human LCN2 (1:100, MAB1757; R&D) in PBST (contain 3% BSA) at 4 °C overnight. After washing, the sections were incubated in HRP-conjugated IgG against rat in PBST (contain 3% BSA) (1:400, 7077; Cell Signaling, Danvers, MA, USA) at room temperature for 2 h, and the DAB Kit (00-2020; Invitrogen) was used for the substrate reaction (10 min at room temperature). Following the appearance of the brown precipitate, the sections were stained with hematoxylin (HHS16; Sigma) for 1 min, rinsed with water, and mounted in 50% glycerol in PBS. The sections were examined under a light microscope (AH3-RFCA; Olympus).

Cell proliferation assay

The primary endometrial epithelial cells, from 3-week-old female ICR mice, were seeded into 96-well plates at $\sim 1.0 \times 10^4$ cells/well. The cells were incubated in DMEM/F12 medium containing 1 or 10% FBS for 24–120 h. The cells were then harvested and washed with serum-free medium and stained for metabolic activity using the redox dye resazurin and the VisionBlue Quick Cell Proliferation Assay Kit (K303-500; Biolink, San Francisco, CA, USA). Metabolically active cells reduce resazurin, which then becomes highly fluorescent ($\lambda_{\text{ex}} = 545$ nm; $\lambda_{\text{em}} = 595$ nm). The number of proliferating cells was determined using a cell counter (Invitrogen).

Western blotting

The conditioned medium from endometrial epithelial cells cultured in the absence or presence of LCN2 was subjected to 12% SDS-PAGE. The gels were then blotted onto a PVDF

membrane (PL-BSP0161; PALL, Washington, NY, USA). Each membrane was placed into blocking buffer that consisted of 5% skim milk and 0.05% (v/v) Tween-20 (P9416; Sigma) in PBS and then incubated with anti-LCN2 (1:5000, MAB1857; R&D). The membrane was then incubated with HRP-conjugated IgG against rabbit (1:10 000, 7074; Cell Signaling). The protein bands were visualized using an ECL Kit (WBKLS0050; Millipore, Billerica, MA, USA) and X-ray film (Fujifilm, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted from the endometrial epithelial cells using an RNeasy Mini Kit (Qiagen). RNA (500 ng) was reverse transcribed using the MMLV Reverse Transcriptase Kit (M6125H; Epicentre, Madison, WI, USA). Forward and reverse primers for *Lcn2* (forward, CCATCTATGAGCTACAAGAGAA-CAAT and reverse, TCTGATCCAGTAGCGACAGC) and *actin beta* (*Actb*; forward, TGCCTGACATCAAAGAGAAG and reverse, GATGCCACAGGATTCCATA) were used for amplification. To assess mRNA expression levels, quantitative real-time PCR (qPCR) and data analysis were performed using 7300 System SDS Software (Applied Biosystems). Gene expression levels were normalized using *Actb* as the internal standard. The PCR conditions were 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min. Data were standardized by subtracting the signal threshold cycles (C_T) associated with the internal standard (*Actb*) from C_T values associated with the genes of interest.

Cell migration assay

The wound-healing assay is used to assess directional cell migration *in vitro*, and it mimics cell migration during wound healing *in vivo* (Rodriguez *et al.* 2005). The primary endometrial epithelial cells, from 3-week-old female ICR mice, were cultured in 24-well plates until confluent, and then a pipette tip was used to generate a scratch. The detached cells were removed and a fresh medium was added. The simulated wound was observed over the next 12 h as it closed. In addition, the Transwell migration assay was used to quantify the number of migrating cells. For this assay, endometrial epithelial cells were cultured in 1% FBS/DMEM/F12 for 48 h, and then the resultant LCN2-containing conditioned medium was collected. Additional primary endometrial epithelial cells, from 3-week-old female ICR mice, were grown to logarithmic phase, harvested, and resuspended ($\sim 2 \times 10^5$ cells/ml) in the conditioned medium. Cell suspensions (400 μ l) were placed into upper compartments of 24-well plates containing Transwell inserts with 8- μ m pores (Transwell Costar 3422; Corning, Tewksbury, MA, USA). The lower compartments were filled with 100 μ l DMEM/F12 containing 1% FBS, and the chambers were incubated at 37 °C for 24 h. The Transwell insert membranes were fixed with methanol and stained with 0.5% crystal violet (C0775; Sigma) for 20 min, and then rinsed twice with water. The cells on the upper surface of the filters were removed by cotton swab. The crystal violet dye retained on the filters was then extracted with 150 μ l of 33% acetic acid, and the absorbance of this solution was measured at 570 nm using an ELISA reader (Lee *et al.* 2012).

Cell invasion assay

The tissue culture plates (24-well plates) containing Transwell inserts with 8- μ m pores were coated with 10 μ l Growth Factor Reduced Matrigel (BD 354230; BD, Franklin Lakes, NJ, USA). The cells were cultured at 37 °C in 5% CO₂. Murine endometrial epithelial cells ($\sim 1 \times 10^5$) were transfected with *pKLO.1/Lcn2* siRNA (see below) or a mock vector and placed on the top of the Matrigel: 10% FBS DMEM/F12 culture medium (200 μ l) was then added to the lower chamber, and the cells were incubated overnight to allow adherence of the cells to the Matrigel. After various incubation periods, the Matrigel and cells were removed, and the upper side of each membrane was cleaned with a cotton swab. The filters were stained with 0.5% crystal violet (C3886; Sigma) and mounted on the glass microscope slides. The total number of cells that invaded the underside of the filter was counted using a light microscope at 100 $\times$ magnification.

Lcn2 siRNA transfection

The vectors were obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The uterine tissue-derived endometrial epithelial cells were transfected with five siRNA-containing vectors (pLKO.1-TRCN0000055328 to pLKO.1-TRCN0000055332, which we denote RNAi28 to RNAi32) using an Amaxa Basic Nucleofector Kit for Primary Mammalian Epithelial Cells (VPI-1005; Lonza, Walkersville, MD, USA). After transfection, the levels of *Lcn2* expression were then measured using qPCR. These levels were confirmed using western blotting. Representative *pKLO.1/Lcn2* siRNA-transfected cells were used for the cell migration and invasion assays.

Statistical analysis

One-way ANOVA with a *post hoc* Dunnett's test was performed using InStat, version 3.00 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

Relationship between LCN2 and ectopic endometriosis

To determine whether LCN2 levels correlate with the adhesiveness of endometrial epithelial cells *in vivo*, we established an animal model of endometriosis, in which the uterine tissue was ectopically implanted into the peritoneum. Control mice were subjected to a sham surgery, i.e. without the implantation of uterine tissue. After estrogen was administered to mice with implanted endometrial tissue, LCN2 protein levels increased significantly in the peritoneal fluid. This effect was seen 5 weeks after implantation (Fig. 1A). Trace amounts of LCN2 were present in the peritoneal fluid of control animals with or without estrogen treatment (Fig. 1A). The peritoneum was washed with 1 ml PBS to obtain the peritoneal fluid. The implanted endometrial tissue grew over time, as the proportion of mice with large

Table 1 Ectopic implantation of endometrial tissue in female ICR mice.

Ectopic tissue sizes	EM group (%)			EA group (%) (n=20)
	3 weeks (n=40)	4 weeks (n=14)	5 weeks (n=16)	
Large (≥ 8 mm ³)	60	86	100	20
Medium (≥ 1 mm ³ to < 8 mm ³)	20	14	0	40
Small (< 1 mm ³)	20	0	0	30
None	0	0	0	10
Total (%)	100	100	100	100

EM, ectopic implantation without LCN2 antibody treatment; EA, ectopic implantation with LCN2 antibody treatment.

endometrial lesions within the peritoneum increased from 60% after 3 weeks to 100% after 5 weeks (Table 1), coincident with increases in LCN2 levels (Fig. 1A).

Mice with implanted endometrial tissue that received i.p. injections of anti-LCN2 (10 μ g for 3 days) exhibited slower growth of endometrial tissue. Substantially fewer of these mice developed lesions – indeed only 20% had large endometrial lesions after 5 weeks (Table 1), an effect that could be related to a reduction in *Lcn2* mRNA level (Fig. 1B). Estrogen treatment also elevated *Lcn2* mRNA levels within eutopic endometrial tissue, and anti-LCN2 blocked this effect (Fig. 1B, gray bars). The amount of ectopic endometrial tissue in the mouse abdominal cavity was also reduced upon treatment with anti-LCN2 (Fig. 1C). Furthermore, LCN2 levels within mouse serum were elevated by endometriosis (Fig. 1D). Together, these data suggested that LCN2 levels increased during endometriosis development and that LCN2 may promote ectopic endometrial implantation. Eutopic endometriosis, which is a stress-like condition, also seemed to be induced by LCN2. Thus, LCN2 secreted from the uterine epithelia may trigger the movement of endometrial epithelia into the peritoneum where it forms an ectopic tissue.

We subjected ectopic endometrial tissue to immunohistochemistry (IHC) and found that LCN2 localized to these cells (Fig. 2A and D). We also visualized LCN2 within human endometrium, both normal and ectopic. LCN2 was essentially absent from normal endometrium (Fig. 2B and E) but was expressed at high levels by ectopic endometriotic lesions (Fig. 2C and F). This suggested that LCN2 levels correlate with endometriosis.

EMT of primary endometrial epithelial cells cultured in nutrient-deprived medium

We previously showed that LCN2 mediates autocrine signaling within the endometrial epithelium (Huang *et al.* 1999, Lee *et al.* 2012). As LCN2 is a stress protein (Lin *et al.* 2007), we used a 1% FBS culture medium, which causes nutrient-deprivation stress, to induce LCN2 production. We then characterized physiological changes to primary endometrial epithelial cells. Between 48 and 120 h of nutrient deprivation, cellular

Figure 2 LCN2 expression in uterine tissue. IHC staining for LCN2 (brown precipitate) in mouse ectopic uterine tissue (A and D), human eutopic uterine tissue (B and E), and human ectopic uterine tissue (C and F). Right panels represent boxed regions in left panels. Nuclei are stained blue with hematoxylin. The arrows indicate glandular epithelium (GE) cells. The archived samples were from Mackay Memorial Hospital. Normal group, the endometrium collected from a benign tumor of myoma patient. Experimental group, the endometrium collected from endometriosis patients. The benign tumor tissue has been defined as a normal tissue.

morphology went from rounded to spindle-like, and the cells dispersed (Fig. 3A). These morphological and spatial changes suggest cell migration. Bright-field microscopy showed an increase in cell expansion after long incubation periods (Fig. 3A). Nutrient deprivation also decreased levels of cytokeratin (an epithelial marker) and increased levels of fibronectin (a mesenchymal marker). These changes in cytokeratin and fibronectin levels were seen with IHC (Fig. 3A) and western blotting (Fig. 3B). Multiple bands within the western blot represent different isoforms of cytokeratin and fibronectin. IHC and western blotting data both showed the absence of fibronectin at 48 h of nutrient deprivation (Fig. 3A,b) but high levels at 96 h (Fig. 3A,d). The fibronectin increase coincided with the cell expansion shown in Fig. 3A and indicated the increase in cell adhesion.

LCN2 expression in primary endometrial epithelial cells

To investigate cell proliferation and *Lcn2* expression under stress conditions, primary endometrial epithelial cells were cultured in stress-inducing, nutrient-deprived medium (1% FBS/DMEM/F12) or in nutrient-complete medium (10% FBS/DMEM/F12). The primary endometrial epithelial cells cultured in nutrient-complete medium remained viable for 72 h, but viability declined after 96 h (Fig. 4A, gray bars). Interestingly, primary

endometrial epithelial cells grown in nutrient-deprived medium showed a ~ 1.8 -fold increase in cell number after 72 h (Fig. 4A, black bars). *Lcn2* mRNA levels were significantly elevated in the stressed cells, reaching a maximum at 72 h (Fig. 4B). By contrast, only low levels of LCN2 were detected in nonstressed cells (Fig. 4B). Western blotting was used to measure both intracellular (cell lysate) and extracellular (conditioned medium, i.e. the secreted form) levels of LCN2 in the stressed cells. Intracellular and extracellular LCN2 levels increased over time, reaching a maximum at 72 h (Fig. 4C). LCN2 secretion was detected after 24 h of culture (Fig. 4C), suggesting that, if LCN2 mediates an autocrine signal, cells should respond to LCN2 after ~ 24 h of stress.

Figure 3 Expression of EMT markers in the endometrial epithelial cells cultured under stress conditions. (A) Nutrient deprivation affected cell morphology (bright field microscopy in a, c and e) and expression of the epithelial marker cytokeratin (red) and the mesenchymal marker fibronectin (green) (b, d and f) in cultured cells, indicating EMT. Culture durations of 48, 96, and 120 h are shown. (B) Western blot analysis of cytokeratin and fibronectin levels in cell lysates. Upper panel represents the western blot. Lower panel represents densitometric analysis of the western blot (cytokeratin, gray bars; fibronectin, black bars). GAPDH served as the internal control.

Figure 4 LCN2 expression in primary endometrial epithelial cells. Primary endometrial epithelial cells ($\sim 2.5 \times 10^4$) were harvested from the uterine tissue of 3-week-old mice and cultured in the medium containing 1 or 10% FBS for different periods. (A) Cells were harvested and the number of live cells was counted. Fold change relative to the initial number of cells is shown. Black bars, cells cultured in 1% FBS; gray bars, cells cultured in 10% FBS. $**P < 0.01$; $***P < 0.005$; $n \geq 3$ per group. (B) *Lcn2* mRNA levels in endometrial epithelial cells cultured in 1 or 10% FBS. (C) Intracellular and extracellular levels of LCN2 in endometrial epithelial cells cultured in 1% FBS. GAPDH served as the internal control.

To determine the mechanism by which LCN2 induces EMT in primary endometrial epithelial cells, we cultured cells in nutrient-deprived medium and added anti-LCN2 (Fig. 5). The cells cultured in nutrient-complete medium served as the control. Anti-LCN2 inhibited the stress-induced increase in fibronectin but did not affect cytokeratin levels, which remained elevated over the culture period (Fig. 5A). IHC verified that anti-LCN2 suppressed stress-induced increases in fibronectin (Fig. 5B). These results indicated that LCN2 induced EMT (i.e. elevated levels of the mesenchymal marker

fibronectin) in primary endometrial epithelial cells cultured under stress conditions.

Because EMT can initiate epithelial cell migration (Bulun 2009), we investigated the relationship between LCN2 level and the migration of primary endometrial epithelial cells cultured in nutrient-deprived medium. We used the supernatant harvested from the cells cultured for 48 h in nutrient-deprived medium (LCN2-containing medium as shown in Fig. 4C) as conditioned medium in cell migration assays. Wound-healing assays showed that anti-LCN2 slowed cell migration during the 12-h incubation period (Fig. 6A). Transwell analysis showed that anti-LCN2 reduced cell migration in a dose-dependent manner (Fig. 6B). These results indicated that LCN2 is involved in the migration of primary endometrial endothelial cells under stress conditions.

To further investigate the effects of LCN2 on cell migration, we performed cell migration and invasion assays after siRNA transfected to *Lcn2* was used to knockdown *Lcn2* expression in the primary endometrial epithelial cells. Decreased *Lcn2* expression in response to siRNA treatment was confirmed with qPCR and western blotting (data not shown). Cell migration and invasion assays showed that *Lcn2* knockdown significantly reduced cell migration and invasion under conditions of stress (Fig. 6C). These data indicated that LCN2 is important for the migration and invasion of endometrial epithelial cells under stress conditions.

Discussion

Endometriosis is a disorder associated with both estrogen and cellular oxidative stress (Augoulea *et al.* 2009, Bulun 2009). Cellular stress promotes endometriosis by inducing the migration and implantation of endometrial cells in the peritoneum (Alpay *et al.* 2006). Our current study showed that LCN2 was induced in the endometrial epithelial cells by stressful conditions (*in vitro*), or by estrogen (*in vivo*). Our study assumes that estrogen-dependent LCN2 affects endometrial physiology. We used a mouse model of endometriosis to demonstrate that an antibody specific to LCN2 inhibited the development of ectopic endometriosis. IHC data (Fig. 2) showed expression of LCN2 in mammalian uterine tissue during endometriosis formation. Our findings are consistent with a previous report that showed LCN2 upregulation in a rat model of endometriosis (Konno *et al.* 2007).

Based on our evidence, we propose that LCN2 induces EMT in endometrial epithelial cells under nutrient-deprived conditions (a stress condition) and promotes the development of endometriosis. Cellular stress may initiate endometriosis by affecting the immune system, thereby allowing tissue from the uterine lining to grow in an abnormal location (Augoulea *et al.* 2009). We have previously shown that LCN2 increases intracellular oxidative stress and triggers the secretion of cytokines

Figure 5 Antibodies that functionally inhibit LCN2 to block stress-induced EMT in endometrial epithelial cells. (A) Western blot analysis of EMT markers in endometrial epithelial cells cultured in 1% FBS with (+Ab) or without (-Ab) anti-LCN2 (0.02 μM) for 48–120 h. Cells cultured in 10% FBS without anti-LCN2 served as the control (Con). GAPDH served as the internal control. (B) IHC staining for cytokeratin (red) and fibronectin (green) in endometrial epithelial cells cultured for 72, 96, or 120 h in nutrient-deprived conditions with (+) or without (-) anti-LCN2. Nuclei are labeled blue with DAPI.

from nutrient-deprived cells (Lin *et al.* 2011). Cytokines likely prevent endometrial cell apoptosis, thereby facilitating cell migration. Our data indicate that cytokines such as GRO, MCP1, IL6, and IL8 could be upregulated in the endometrial cells in response to LCN2 and that these factors could induce cell migration. MCP1, IL8, and IL6 are important for endometriosis formation (Ulukus *et al.* 2009, McKinnon *et al.* 2012), so reducing the levels of these factors might inhibit endometriosis.

This type of change to the cellular microenvironment may explain why anti-LCN2 reduced endometrial growth within the peritoneum. In this study, we found that LCN2 altered the cellular microenvironment and increased cell proliferation after nutrient deprivation (Fig. 4A). LCN2 also triggered cell migration and invasion (Fig. 6), which may have contributed to the implantation of ectopic endometrial tissues. Changes in the expression of EMT markers were associated with changes in LCN2 levels in

Figure 6 Endometrial epithelial cell migration assays. (A) Wound-healing assay: endometrial epithelial cells ($\sim 5 \times 10^4$) were cultured until nearly confluent, and then a pipette tip was used to create a wound. LCN2-containing conditioned medium that included (+) or did not include (-) anti-LCN2 (0.02 μM) was then added, and the simulated wound was allowed to heal for 12 h. Green lines indicate wound edges. (B) Transwell assay: endometrial epithelial cells ($\sim 1 \times 10^5$) in 1% FBS were added to Transwell units. LCN2-containing conditioned medium that included different concentrations of anti-LCN2 was then added and the cells were allowed to migrate for 24 h. Cells in the membrane insert were stained (lower panel) and counted (upper panel) ($n \geq 6$ per group). (C) Effect of *Lcn2* siRNA treatment on cell migration and invasion under stress conditions. Endometrial epithelial cells ($\sim 1.5 \times 10^6$) were transfected with *Lcn2* siRNA vectors and subjected to migration and invasion assays. Cells transfected with an empty vector served as a control. The numbers of migrating cells and invasive cells are expressed relative to the control (100%). *** $P < 0.001$; $n \geq 3$ per group.

the endometrial epithelial cells cultured in nutrient-deprived medium. On the basis of the data of Fig. 3, we found an increase in fibronectin, which is involved in cell adhesion and supports the cell movement (Mao & Schwarzbauer 2005); it can be interpreted that EMT occurs under stress conditions. The cells also displayed morphological and physiological changes associated with cell migration and relocalization to the peritoneum (Mathias *et al.* 2010). The effects of LCN2 on EMT and cell migration of nutrient-stressed endometrial epithelial cells were abrogated by anti-LCN2. Together, these findings suggest that LCN2 promotes the implantation of ectopic endometrial tissue by inducing EMT.

Endometriosis, such as tumorigenesis, requires angiogenesis for the ectopic mass to expand (Vargha *et al.* 2008). As Yang's report mentioned, LCN2 may regulate angiogenesis in human breast cancer, although LCN2 levels merely correlated with vessel formation and tumor growth in breast cancer cells (Yang *et al.* 2013). The finding supports a relationship between LCN2 and angiogenesis during the growth of endometrial tissue within the peritoneum. We have previously shown that LCN2 stimulates the secretion of angiogenesis-related cytokines from an endometrial cell line (RL95-2; Lin *et al.* 2011). Our current study showed time-dependent production of LCN2, which supports the idea that LCN2 stimulates the expansion of ectopic endometrial tissue in the peritoneum. Anti-LCN2 eliminated the induction of EMT in stressed endometrial epithelial cells and inhibited LCN2-dependent development of autotransplanted endometrial tissue, suggesting that endometriosis occurs as a consequence of LCN2-induced EMT.

In conclusion, our results demonstrate a role for LCN2-induced EMT in the development of endometriosis and provide molecular details concerning the development of endometriosis under stress conditions. As far as we know, this is the first report that demonstrates a role for LCN2-induced EMT in endometriosis development. Because mice with endometriosis had elevated serum levels of LCN2, LCN2 may represent a useful biomarker for endometrial disorders. Furthermore, as LCN2 is overexpressed in patients with endometrial hyperplasia (Liao *et al.* 2012), LCN2 may serve as a therapeutic target for endometrial disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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