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CD147 induces epithelial-to-mesenchymal transition by disassembling CAS/E-cadherin/β-catenin complex in human endometriosis

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Running head: Roles of CD147 and CAS in endometriosis

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Abstract

Epithelial-to-mesenchymal transition (EMT) is postulated to be a prerequisite for the establishment of endometriosis (EMS), a common reproductive disorder in women. Our previous studies have demonstrated the elevated expression of transmembrane glycoprotein CD147 and its pro-survival effect on abnormal cells in endometriosis. Intriguingly, CD147 is known to promote EMT in cancers. However, the involvement of CD147 in EMT during the establishment of endometriosis remains incompletely understood. Here, we showed that CD147 promotes EMT in human endometrial adenocarcinoma cell line Ishikawa (ISK). We identified a novel CD147-interacting partner, cellular apoptosis susceptibility protein (CAS), which stabilized the interaction between E-cadherin (E-cad) and β-catenin (β-cat) by forming CAS/E-cad/β-cat complex. Down-regulation of CAS led to the release and nuclear translocation of β-cat from E-cad, resulting in the overexpression of EMT-promoting gene *SNAIL*. Interestingly, overexpression of CD147 impaired the interaction between CAS and E-cad and triggered the release of β-cat from CAS/E-cad/β-cat complex, which in turn led to EMT. Furthermore, CAS was down-regulated in EMS with elevated levels of CD147 and nuclear β-cat. These findings suggest a previously undefined role of CAS in regulating EMT and reveal the involvement of a CD147-induced EMT signaling pathway in pathogenic progression of EMS.

Introduction

Endometriosis (EMS) affects 6% to 10% of women of reproductive age with pelvic pain and infertility [1-3]. It originates from retrograde menstruation of endometrial cells, which migrate through the fallopian tubes and invade into the peritoneal cavity to establish ectopic lesions [1, 4]. Although EMS is considered a benign disease, several studies suggested that the enhanced cell migration and invasion during EMS progression is similar to that observed in cancer metastasis [5, 6].

Epithelial-to-mesenchymal transition (EMT) is a critical step in cancer metastasis. It is a process in which polarized epithelial cells are converted into mesenchymal-like cells with stronger motility and invasiveness. β-catenin (β-cat) signaling pathway and cadherin-mediated cell adhesion both play important roles in regulating EMT [7]. The interplay between these two pathways regulates the activity of β-cat. In cell adhesion, the interaction between cadherin and β-cat maintains cell polarity. Loss of cadherin-mediated adhesion promotes the nuclear translocation of β-cat that in turn activates set of EMT-promoting transcription factors, including Snail, Slug, and Twist. These transcription factors subsequently lead to down-regulation of epithelial marker E-cadherin (E-cad) and up-regulation of mesenchymal markers (eg, Vimentin (Vim) and N- cadherin (N-cad)), which are considered the hallmarks of EMT [7-11]. Since ectopic lesions in EMS demonstrated similar biological properties as cancer metastasis in terms of cell survival and cell migration, EMT has been postulated to be involved in the establishment of EMS. In fact, studies have demonstrated the loss of E-cad expression in ectopic lesions of EMS [6]. However, the molecular mechanisms underlying the regulation of EMT in EMS remain largely unknown.

Cluster of differentiation 147 (CD147) is a transmembrane glycoprotein mainly expressed in the reproductive tracts, eye, brain, and muscles in normal individuals [12]. Despite the restricted expression in normal individuals, its expression is widely elevated in different types of cancer cells. CD147 is known to act as pro-survival and pro-migration factor in various physiological and pathological processes, such as spermatogenesis and tumor metastasis [13-18]. The pro-migration property of CD147 can be attributed to its ability in promoting the production of matrix metalloproteinases (MMPs) and in mediating EMT [15, 19-21]. However, the molecular mechanism underlying the regulation of EMT by CD147 remains elusive.

Our previous studies have shown that CD147 is elevated in human EMS [17]. Elevated level of CD147 promotes the abnormal cell survival through up-regulation of Bcl-2 [18]. On the other hand, it promotes the migration of endometrial cells in an MMP2-independnet manner [17]. However, the potential involvement of CD147-mediated EMT in the establishment of EMS has not been explored. In the present study, we identified a CD147-interacting partner cellular apoptosis susceptibility protein (CAS) and showed that CD147 and E-cad formed a complex through CAS. Furthermore, elevated level of CD147 impaired the binding between CAS and E-cad, leading to the release of β-cat that subsequently promotes EMT. In addition, the expression of CAS was down-regulated in endometriosis lesions with elevated levels of CD147 and nuclear β-cat. Together, our results suggest that the interaction between CD147 and the CAS/E-cad/β-cat complex is an important regulator of EMT during progression of EMS.

Materials and methods

Human tissue collection

In total, 59 women undergoing gynecologic laparoscopic surgery were recruited to the study, 47 with ovarian EMS (aged 25 to 42 y), and 12 without EMS (aged 26 to 45 y). Endometriotic tissues were collected from the walls of endometriomas in patients with EMS, and normal endometrial tissues (confirmed by pathologic diagnosis) were collected by curettage in women with infertility. All tissues were taken at proliferative phase of the menstrual cycle, and all women with or without EMS had regular menstrual cycles. Individuals receiving hormone therapy or anti-inflammatory agents were excluded. All samples were collected with informed consent from each woman and approval by the institutional review board of the Second People's Hospital of Shenzhen (201306015) [17].

Cell culture, plasmid and siRNA transfection, lentivirus transduction, and anti-CD147 treatment

ISK cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin in 5% CO_2 incubators at 37 °C. For overexpression of CD147, ISK cells were seeded in 6-well plate at 5×10^5 cells/well. The pcDNA3.0-CD147 plasmid was provided by Dr. Z.N. Chen [22]. Transfection was performed using 8 μL Lipofectamine 2000 (Invitrogen, USA) and 5 μg DNA of pcDNA3.0-CD147 or pcDNA3.0 according to the manufacturer's instructions. The transfected cells were selected by 400 μg/mL G418 for 10 days. For knockdown CAS in ISK cells, the cells were transfected with 50 nM CAS siRNA as above. The three CAS siRNAs were purchased from Invitrogen (Cat. # 1299001). The protein was extracted

with RIPA buffer 5 days after transfection. The lentiviral CD147 shRNA and CAS overexpression lentiviruses were purchased from GenePharma (Shanghai). For lentiviral transduction, ISK cells were seeded into 24-well plate at 1×10^4 cells/well with 200 μL DMEM/RPMI-1640 plus 10% FBS. After one day in culture, the medium was replaced with fresh medium and cells were transduced with 5×10^6 TU lentiviruses plus 0.1 % polybrene. Stable transduced cells were selected by adding 4 μg/mL puromycin after 48 h transfection. For anti-CD147 antibody treatment, cells were seeded in 6-well plate at 5×10^4 cells/well. After 24 h culture, the medium was replaced by fresh medium containing 10 μg/mL mouse anti-CD147 monoclonal antibody (Table 1), and normal mouse IgG at the same concentration was used as negative control. After 48 h antibody treatment, total cell protein was extracted with RIPA buffer.

RNA extraction and quantitative real-time PCR

Total RNA from cultured cells and human specimens was extracted using Trizol regent (Invitrogen, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg total RNA with iScript cDNA synthesis kit (Bio-Rad, USA), as per the manufacturer's instructions. The RT-qPCR for *Snail* was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA) in a 10 μL total volume on Applied Biosystems 7500 Fast Real-time PCR System as described previously [18]. The sequences of the primers are listed as follows: *SNAIL* (forward 5'-TTTACCTTCCAGCAGCCCTA-3'; reverse 5'-CCCACTGTCCTCATCTGACA-3'; product size 207 bp) and *GAPDH* (forward 5'-AACGACCCCTTCA TTGAC-3'; reverse 5'-TCCACGACATACTCAGCAC-3'; product size 190 bp). The RT-qPCR for *CAS*

expression was performed using TaqMan primers (Applied Biosystems). TaqMan GenExpression Inventoried Assay (Assay ID: Hs00354853_m1 hCAS) and TaqMan Universial PCR Master Mix (Applied Biosystems) were used in real time PCR reaction. S18 rRNA (Applied Biosystems) was used as the endogenous control. The PCR programs were: stage 1, 50 °C for 2min; stage 2, 95 °C for 10min; stage 3, 95 °C for 15sec, 60 °C for 1min. Stage 3 was repeated for 40 cycles. The PCR assays were performed in separate tubes and relative quantitation of mRNA was determined using standard curve method according to the manufacturer's instructions.

Cell migration and invasion assay

Cell migration assay was performed by live imaging system (Carl Zeiss, Germany) [23]. Briefly, 1×10^6 cells were cultured in a well of the 6-well plate until they reached a confluent monolayer. This monolayer was subsequently scratched with a 20 μL culture tip, and the medium was replaced with fresh medium plus 1% FBS. Cellular migration from the two wound fronts was tracked and recorded over a 24 h period using a live imaging system. At least five random imaging views were investigated on each plate to quantify the distance of migration. For invasion assay, the Corning® BioCoat™ Matrigel® Invasion Chambers with 8.0 μm PET Membrane (Corning, USA) were used, according to the manufacturer's instructions. Briefly, 1×10^5 cells were re-suspended in RPMI-1640 medium without FBS and plated into the upper inserts. RPMI-1640 medium containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, non-invasive cells were removed from the upper chamber with a cotton swab.

The cells on the lower surface of the insert were fixed by 4% PFA and stained with 0.5% crystal violet. The invading cells were counted under microscopy.

Co-Immunoprecipitation, silver staining, and mass spectrometry

Co-Immunoprecipitation (Co-IP) was conducted according to the manufacture's instructions (GE Healthcare, USA). The appropriate antibody (25 μg) was added in binding buffer, and 20 μL magnetic bead slurry medium was equilibrated using 500 μL binding buffer. After equilibration, the antibody solution was added in binding buffer with magnetic bead, incubated for 30 min. After antibody binding, the beads were washed using 500 μL binding buffer, then the protein sample (lyzed with the IP lysis buffer) was added into the antibody-bond beads, and incubated overnight at 4 °C. Then, the beads were washed three times with wash buffer. The proteins bound to the beads were eluted by 20 μL 1×SDS loading buffer. For silver staining, after running the SDS-PAGE gel for the IP protein products, the gel was fixed with fixation solution I (50% methanol and 10% acetic acid) for 20 min, and then fixed with fixation solution II (5% methanol and 1% acetic acid) for 10 min. The gel was washed three times with ddH_2O for 5 min. The gel was sensitized with 0.02% thiosulfate solution for 90 sec, and then washed three times with ddH_2O for 30 sec. After sensitization, gel was stained with 12 mM silver nitrate for 30 min, and then washed three times with ddH_2O for 1 min. The gel was developed in 0.04% formaldehyde with 2% sodium carbonate for 10 min. Each of the specific protein bands was cut from the gel, and analyzed by MALDI-TOF/TOF tandem mass spectrometry. The mass spectrometry analysis was conducted in the Centre

for Genomic Sciences at the University of Hong Kong. The antibodies used in Co-IP assay are listed in Table 1.

Western blotting

Cells and human tissues were lyzed with RIPA buffer. Western blotting analysis was performed as described previously [24]. Briefly, cell protein (30 μg per lane) was subjected to SDS-PAGE electrophoresis and was transferred onto nitrocellulose membranes. The transferred membrane was blocked with 5% skim milk in TBST for 1 h, and was incubated with primary antibodies (Table 1) at 4 °C, overnight. Then the membrane was washed three times with TBST and incubated with appropriate peroxidase-conjugated secondary antibodies for 1 h. The membrane was washed three times with TBST and then detected by enhanced chemiluminescence (GE Healthcare). GAPDH and β-actin were used as loading control.

Immunofluorescent staining

Human samples or coverslips with cells (fixed with 4% PFA in PBS for 15 min) were incubated for 1 h in blocking solution (5% normal donkey serum and 1% BSA diluted in PBS). Diluted primary antibody of rabbit anti–β-catenin (1:200, cell signaling technology, USA) was added on specimen overnight at 4 ºC. The primary antibody solutions were removed from the slides and then the slides were washed three times (5 mins each) with 0.05% Tween-20 in 1×PBS. Secondary antibodies (Alexa 488-conjugated donkey-anti-rabbit, 1:500, Invitrogen, USA) were added and incubated for 1 h at room temperature. The slides were mounted with ProLong Gold Antifade Reagent with TO-PRO-3

(Invitrogen, USA) and visualized on NIKON microscope (NIKON Corp, Japan) using 40× objectives.

Statistical analysis

All data were collected blindly. Statistical significance for comparison between two measurements was determined by *t*-test. One-way analysis of variance was used for evaluation of three measurements. All statistical analyses were performed using Prism 6.0 (GraphPad, USA). Differences were considered to be statistically significant at $P< 0.05$.

Results

CD147 promotes endometrial EMT-associated cell migration

The involvement of CD147 in EMT of endometrium was first examined. CD147 or immunodepleted CD147 was overexpressed in human endometrial adenocarcinoma cell line Ishikawa (ISK) and EMT assessed by monitoring cell migration, morphological changes, and EMT markers expression. Overexpression of CD147 dramatically promoted cell migration in ISK cells (Fig. 1A). The average migration distance in 24 h was significantly longer in CD147-overexpressing cells (211.6 ± 9.0 μm) compared with vector control (127.3 ± 7.5 μm). CD147-overexpressing ISK cells transited from cuboidal morphology to a spindle-like, elongated shape, compared with the control (Fig. 1B). Western blotting results showed that CD147 overexpression decreased E-cad, and increased the expression of N-cad and Vim (Fig. 1C). The RT-qPCR result demonstrated that the expression of EMT-associated gene *SNAIL* increased in the CD147-

overexpressing ISK cells (Fig. 1D). In contrast, blocking CD147 function with a characterized anti-CD147 neutralizing antibody significantly reduced cell migration (Fig. 1E) and N-cad expression (Fig. 1F). Consistently, knockdown of CD147 with lentiviral shRNA in ISK cells up-regulated E-cad (Fig. 1G). Together, these results indicate that CD147 promotes EMT-associated migration in endometrial cells.

CD147 interacts with CAS

CD147 is required for germ cell migration during both spermatogenesis and EMS [14, 17]. To further investigate the protein-protein interaction networks underlying CD147-induced cell migration, CD147-interacting partners were pulled down by Co-IP, followed by mass spectrometry identification in spermatogenic GC-2 cells (Supplemental fig. S1 and Supplemental table S1). From these experiments, CAS was identified as a novel interacting partner of CD147. Interestingly, CD147 also interacts with CAS in ISK cells (Fig. 2A).

CAS negatively regulates EMT in endometrium

Since the role of CAS in EMT has not been explored, the effect of CAS on EMT were determined. CAS was silenced in ISK cells with three different siRNAs, resulting in down-regulation of E-cad and up-regulation of Vim (Fig. 2B) as well as increased mRNA expression of EMT-promoting genes *SNAIL* (Fig. 2C). In contrast, overexpression of CAS led to the inhibition of cell migration and invasion (Fig. 2D and E). Taken together, these results indicated that CAS negatively regulates EMT.

CD147 induces EMT by disrupting CAS/E-cad/β-cat complex

During lung tumor metastasis, CD147 induces EMT by activating β-cat signaling pathway [20]. Interestingly, CAS has been shown to maintain the polarity of human colon epithelial cells by enhancing interaction between E-cad and β-cat [25]. Our present finding showed that CD147 and its interacting partner CAS had the opposite effects on EMT. Thus, we speculated that excess CD147 might impair the interaction between CAS and E-cad and lead to the release of β-cat from E-cad. This in turn facilitates the nuclear translocation of β-cat and activation of β-cat signaling pathway. To test this, the interactions between CAS, E-cad, and CD147 were first examined in ISK cells by Co-IP assay using anti-CD147 and anti-CAS antibodies, respectively. CAS and E-cad could be pulled down by anti-CD147 antibody, and E-cad and CD147 could be pulled down by anti-CAS antibody, suggesting that the three proteins were associated with each other (Fig. 3A). To investigate the interaction dynamics among CD147, CAS, and E-cad, CD147 was overexpressed in ISK cells and the total protein was collected at 48 h after transfection. The total protein expression levels of CAS, E-cad, and β-cat in the CD147-overexpressing ISK cells were comparable to the vector control (Fig. 3B). However, the Co-IP results showed that anti-CAS antibody could pull down more CD147, less E-cad as well as less β-cat in the CD147-overexpressing ISK cells, compared with the vector control (Fig. 3C), suggesting that overexpression of CD147 blocked the interaction between CAS and E-cad, leading to the release of β-cat from E-cad. To further investigate the interaction between these proteins, CAS was silenced by siRNA and the total cell protein was collected at 48 h after transfection. Knockdown of CAS did not affect the total protein levels of E-cad and β-cat compared to the negative control in 48 h

(Fig. 3D), but reduced the interaction between E-cad and β-cat (Fig. 3E). Silencing of CAS also attenuated the interaction between CD147 and E-cad (Fig. 3F), suggesting that CD147 was associated with E-cad through CAS. Notably, overexpression of CD147 led to the decreased cytoplasmic β-cat and increased nuclear β-cat (Fig. 4 A and B). Similarly, knockdown of CAS also caused decreased cytoplasmic β-cat and increased nuclear β-cat (Fig. 4 C and D). Taken together, these results suggest that CAS interacts with E-cad to enhance the interaction between E-cad and β-cat, which can be impaired by excess CD147, resulting in release and nuclear translocation of β-cat.

Down-regulation of CAS is associated with decreased E-cad in human endometriotic lesions

During EMS progression, loss of cell polarity and E-cad of endometrial cells enhanced cell migration and invasion, resulting in the establishment of ectopic lesions [5, 6]. Thus, we speculated that the loss of cell polarity and E-cad in EMS might be associated with the alteration of CAS. To test this, the mRNA levels of *CAS* were examined in 47 endometriotic lesions and 12 normal endometria from women without EMS by RT-qPCR analysis. The results showed a significant decrease in the mRNA expression of *CAS* in the endometriotic lesions, with nearly a 50% decrease as compared to the normal controls (Fig. 5A). Consistently, the western blot results showed the decreased protein levels of CAS in the endometriotic lesions in comparison with the normal controls (Fig. 5B). In addition, decreased E-cad was also observed in the endometriotic lesions (Fig. 5B) with increased nuclear β-cat (Fig. 5C). These results suggest that down-regulation of CAS promotes EMT of endometriotic cells through β-cat signaling.

Discussion

CAS is primarily recognized as a nuclear transport factor, subsequent studies showed that it is also associated with cell polarity [25, 26]. The present study identified CAS as a CD147-interacting partner, which negatively regulates EMT by maintaining the stability of E-cad/β-cat complex. The elevated CD147 in EMS promotes EMT by disrupting CAS/E-cad/β-cat complex, leading to activation of β-cat pathway (Fig. 6). These findings were supported by the analysis of endometriotic samples showing that down-regulation of CAS was associated with decreased E-cad and increased nuclear β-cat.

Over-activation of β-cat pathway has been shown to be associated with the development of EMS [4, 27-29]. Matsuzaki et al [4] showed the active β-cat in superficial peritoneal EMS. In addition, the numbers of invasive endometriotic cells were decreased by blocking the transcriptional function of β-cat complex in nucleus, suggesting that abnormal activation of the β-cat signaling pathway is involved in the invasive phenotype of endometriotic cells [29]. Taken together with our previous findings of CD147 up-regulation in endometriotic samples [17], both down-regulation of CAS and up-regulation of CD147 contribute together to invasive phenotype of endometriotic cells by activation of β-cat pathway. Therefore, CD147 and CAS might serve as new biomarkers for early diagnosis of EMS. The alterations of CD147 and CAS in EMS might be caused by different reasons, which need further study.

CD147 is well known to promote cancer metastasis by inducing EMT in colorectal cancer cells, hepatocellular carcinoma cells and lung carcinoma cells [15, 19, 20]. CD147 regulates the migration of endometrial epithelial cells in an MMP-2 independent manner [17]. In the present study, we demonstrated that CD147 regulates EMT by modulating the

interaction dynamics of CAS/E-cad complex in endometrium. CD147 is well characterized to stimulate the production of MMPs that digest the extracellular matrix to facilitate cell migration. However, how CD147 regulates the level of MMPs remains largely unknown. To this end, it is intriguingly to note that MMPs are also the downstream targets of β-cat signaling [30]. Thus, the present findings of the CD147/CAS-regulated β-cat signaling might reveal an alternative MMP induction mechanism by CD147.

In summary, we propose that this CD147/CAS/E-cad/β-cat signaling axis is a critical pathogenic pathway for EMS progression. The present study provides insights into the biological roles of CD147 and CAS in regulating EMT. The capability of CD147 and CAS to regulate β-cat signaling pathway, as we showed here, may have far-reaching implications beyond EMS progression, as CD147 and CAS are widely distributed throughout the body and β-cat signaling is important for many pathological and physiological processes, such as tumor metastasis and spermatogenesis.

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H.C., C.Q.W., and H.C.C. conceived and designed the experiments. C.Q.W., H.C., J.T.Z., and L.L.T. performed the experiments and analyzed the data. M.Y. and J.N.L. performed experiments for revised manuscript. F.H.L. and Z.J.Z. provided intellectual advice. C.Q.W., H.C., K.L.F., and H.C.C. wrote the paper.

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Figure legends

Figure 1. CD147 induces epithelial-to-mesenchymal transition (EMT) in human endometrial cells. A: Representative photographs and statistical analysis of migration of CD147-overexpressing ISK cells at 24 h. Cells transfected with pCDNA 3.0 were used as the vector control (VC). The transfected cells were selected by 400 μg/mL G418 for 10 days. **B:** Morphology of CD147-overexpressing ISK cells with spindle-shape. Scale bar = 10 μm. **C:** Western blotting CD147, E-cad, N-cad, and Vim in CD147-overexpressing ISK cells. **D:** RT-qPCR analysis of mRNA levels of *SNAIL* expression in CD147-overexpressing ISK cells. The experiments were repeated three times. **E:** Representative photographs and statistical analysis of the migration of anti-CD147–treated ISK cells. Cells were treated with 10 μg/mL of anti-CD147 antibody or normal IgG for 24 h. **F:** Western blotting of N-cad in anti-CD147–treated ISK cells. **G:** Western blotting of CD147 and E-cad in CD147 knockdown ISK cells. ISK cells transduced with the CD147 shRNA lentivirus and stable cell line were selected by 4 μg/mL puromycin. Values represent the mean ± SEM. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$. GAPDH was used as the loading control.

Figure 2. CAS interacts with CD147 and negatively regulates epithelial-to-mesenchymal transition (EMT). A: Western blot analysis of CAS and CD147 in the IP products from ISK cells. IP assay was conduceted with anti-CD147 antibody. **B:** Silencing of CAS-induced EMT in ISK cells. ISK cells were transfected with three differently designed CAS siRNAs at a 50 nM final concentration and the total protein was extracted five days after transfection. Reduced E-cad and increased Vim is observed

in CAS-silenced ISK cells, compared with the cells transfected with negative control (NC) siRNA. **C:** mRNA levels of *SNAIL* in CAS-silenced ISK cells were determined by RT-qPCR. The experiments were repeated three times. Representative photographs and statistical analysis of migration (**D**) and invasion (**E**) of CAS-overexpressing ISK cells at 24 h. ISK cells transduced with the CAS overexpression lentivirus and vector lentivirus control (VC), and stable cell line was selected by 4 μg/mL puromycin. Values represent the mean ± SEM. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001.

Figure 3. CD147 is associated with CAS/E-cadherin/β-catenin complex. A: Western blot analysis of the IP products from ISK cells. IP assay was conduceted with anti-CD147 and anti-CAS antibodies, and CD147, CAS, and E-cad were detected by western blot. **B:** Input control for **C**, showing no change in total protein levels of CAS, E-cad, and β-cat in ISK cells after CD147-overexpressing plasmid transfection at 48 h. CD147-overexpressing plasmid was introduced into ISK cells, and total cell lysate was collected at 48 h transfection. **C:** Western blot analysis of the IP products from CD147-overexpressing ISK cells at 48 h after transfection. IP assay was conduceted with anti-CAS antibody, and CAS, CD147, E-cad, and β-cat were analyzed by western blot. **D:** Input control for **E** and **F**, showing no change in total protein levels of CD147, E-cad, and β-cat in CAS-silenced ISK cells at 48 h after siCAS transfection. Cells were transfected with CAS siRNA1/2/3 or negative control siRNA, and maintained for 48 h. **E:** Western blot analysis of β-cat in the IP products from CAS-silenced ISK cells at 48 h after transfection. IP assay was conduceted with anti–E-cad antibody. **F:** Western blot analysis of E-cad in the IP products from CAS knockdown ISK cells at 48h after

transfection. IP assay was conduceted with anti-CD147 antibody. ISK cells were transfected with CAS siRNA1/2/3, and total cell lysate was collected at 48 h transfection.

Figure 4. Overexpression of CD147 and knockdown of CAS increases nuclear β-catenin. A: Western blot analysis of active β-cat in the nuclear extracts (NE) and cytoplasmic extracts (CE) of CD147-overexpressing ISK cells. Cells transfected with pCDNA 3.0 were used as the vector control. The transfected cells were selected by 400 μg/mL G418 for 10 days. **B:** Immunofluorescent staining of β-cat in CD147-overexpressing ISK cells. **C:** Western blot analysis of active β-cat in the NE and CE of CAS-silenced ISK cells. Cells were transfected with CAS siRNA#1/2/3 or negative control siRNA, and maintained for three days. **D:** Immunofluorescent staining of β-cat in CAS-silenced ISK cells. β-actin and histone H3 were used as loading control. The arrows shows the nuclear β-cat. Nuclei were counterstained with TO-PRO-3. Scale bar: 20 μm.

Figure 5. Down-regulation of CAS is associated with decreased E-cadherin in human endometriotic lesions. A: mRNA levels of *CAS* expression in human endometriotic tissues (EMS, n=47) were determined by RT-qPCR, compared with normal endometria (NE, n=12), ***$P < 0.001$ vs control. The experiments were repeated three times. Values represent mean ± SEM. **B:** Representative western blot results show the protein levels of CAS and E-cad are significantly down-regulated in the ovarian ectopic endometria from women with EMS, compared with NE from women without EMS. **C:** Immunofluorescent staining of β-cat in NE and EMS. The arrows show the nuclear β-cat. Nuclei were counterstained with TO-PRO-3. Scale bar: 20 μm..

Figure 6. Working model depicting the CD147/CAS regulated epithelial-to-mesenchymal transition (EMT) in endometrial cell. In normal endometrium, CAS binds to E-cad for enhancing the E-cad/β-cat complex, preventing the nuclear translocation of β-cat and maintaining cell-cell junctions. The basal level of CD147 cannot affect the interaction between CAS and E-cad. Excess CD147 impairs the interaction between CAS and E-cad, and down-regulation of CAS further reduces the interaction between E-cad and β-cat, resulting in release and nuclear translocation of β-cat. In nucleus, β-cat can enhance the expression of EMT-associated genes. The endometrial cells undergoing EMT subsequently lose their membrane junctions and transform into motile mesenchymal cells during the progression of endometriosis. The figure was drawn with the softwares ChemBioDraw 14.0 and Adobe Photoshop CS4.

Table 1. Antibodies used in this study.

Name	Experiment	Vendor	Cat.	Dilution
Anti-CD147 (M6/1)	Functional blocking	SCB	sc-51591	10 μg/mL
Anti-CD147 (N19)	WB/IP	SCB	sc-9752	1:500 (WB); 25 μg (IP)
Anti-E-cadherin	WB	CST	9662	1:1000
Anti-N-cadherin	WB	CST	9664	1:1000
Anti-Vimentin	WB	CST	4927	1:1000
Anti-CAS	WB	CST	9429	1:1000
Anti-β-catenin	WB	SCB	sc-25780	1:500
Anti-active-β-catenin	WB	CST	9544	1:1000
Anti-Histone H3	WB	CST	4712	1:1000
Anti-GAPDH	WB	SCB	sc-47724	1:2000
Anti-β-actin	WB	Sigma	A1978	1:2000

Figure 1.

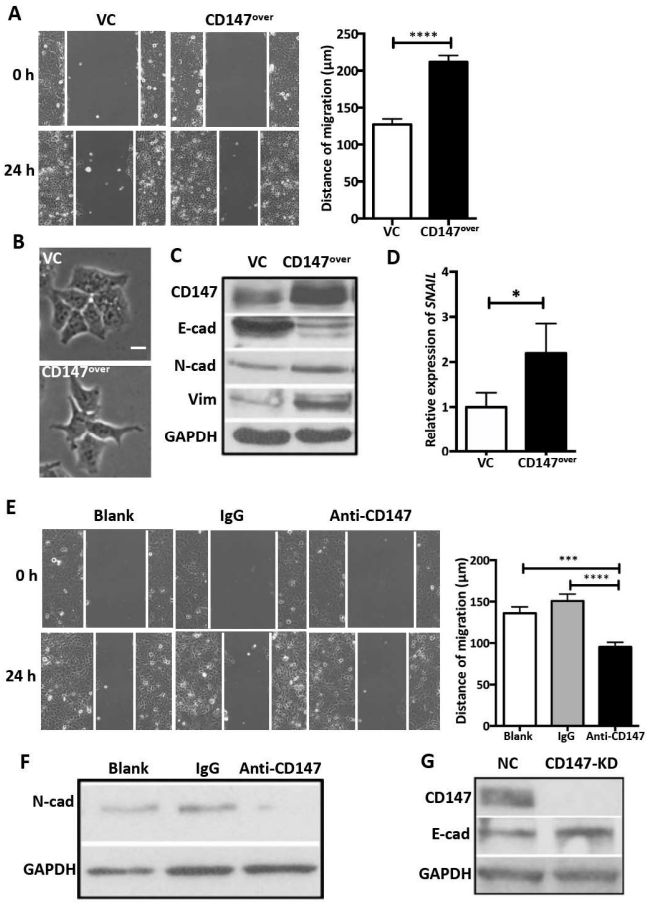
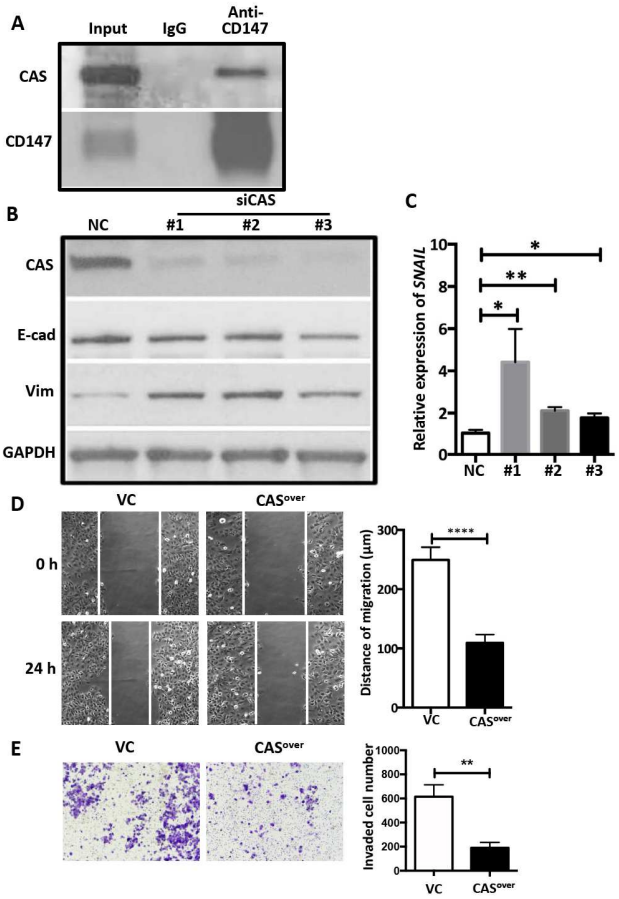


Figure 2.



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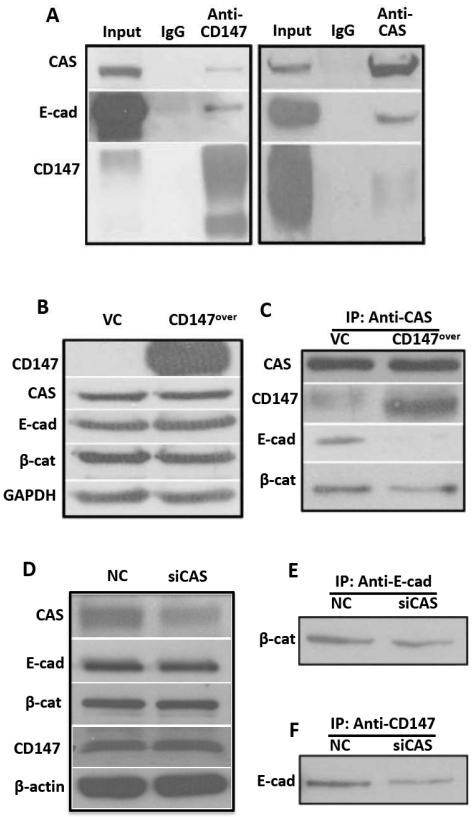

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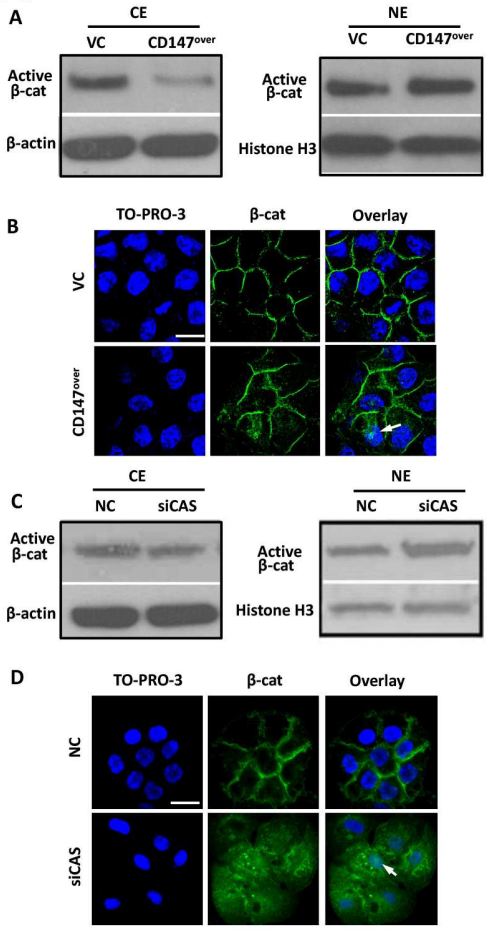

Figure 5.

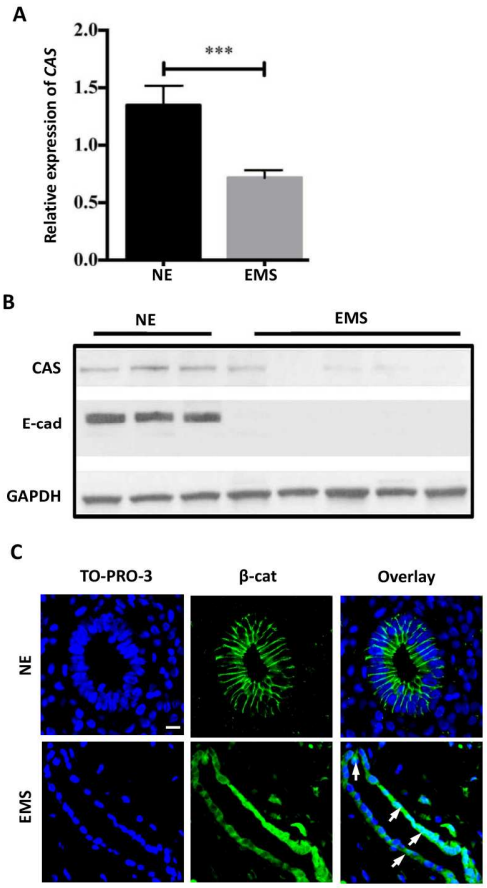

Figure 6.