Chlamydia trachomatis Disturbs Epithelial Tissue Homeostasis in Fallopian Tubes via Paracrine Wnt Signaling

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The obligate intracellular pathogen *Chlamydia trachomatis* (*Ctr*) is a major cause of sexually transmitted disease and infertility worldwide. Ascending genital infections cause inflammation of fallopian tubes and subsequent scarring and occlusion. The cellular basis for such sequelae remains undetermined. We used confocal immunofluorescence microscopy to show that *Ctr* disrupts epithelial homeostasis in an *ex vivo* infection model of human fallopian tubes. *Ctr* triggered loss of polarity of inclusion harboring cells and of neighboring uninfected cells, as shown by subcellular redistribution of adhesion and polarity (occludin) markers. β -catenin (a component of the adherens junction and a Wnt signaling transducer) was recruited to the bacterial inclusion, suggesting a role for Wnt signaling in *Ctr*-mediated tissue damage. Comparative microarray analysis of infected epithelium in the presence of the Wnt secretion inhibitor (IWP2) demonstrated that the transcriptional response to *Ctr* infection was highly dependent on active Wnt secretion, moreover IWP2 reversed *Ctr*-induced tissue phenotypes. Notably, we observed the up-regulation of differentiation and proliferation biomarkers olfactomedin 4 and epithelial cell adhesion molecule, and also *Ctr*-induced proteolytic activation of epithelial cell adhesion molecule. Thus, acute *Ctr* infection activates the paracrine Wnt signaling pathway, leading to profound disruption of epithelial structure and function that facilitates the dissemination of damage beyond that of infected cells. (*Am J Pathol* 2012, 180: 186–198; DOI: 10.1016/j.ajpath.2011.09.015)

Chlamydia trachomatis (*Ctr*) is an obligate intracellular pathogen and a major cause of sexually transmitted disease worldwide. Recurrent *Ctr* infections increase the risk of infertility by 20% due to tubal occlusion and pelvic inflammatory disease.¹ An estimated 5% to 10% of the sexually active population in industrialized countries is exposed to *Ctr* each year (National Institute of Allergy and Infectious Disease and Centers for Disease Control and Prevention). Understanding the mechanisms of *Ctr*-induced disease pathology, therefore, remains a matter of global public health importance.

Patent fallopian tubes provide an environment for fertilization and transport of the developing blastocyst to the uterus, and are thus essential for successful reproduction. A simple columnar epithelium builds the mucosal surface, which resides on a layer of parenchyme (lamina propria) and two layers of muscles, which enable the tube to contract. Two types of epithelial cells are found within the fallopian tubes: secretory (peg) cells and ciliated cells. Peg cells produce tubular fluid whereas ciliated cells facilitate the movement of the oocyte and spermatozoa pre-fertilization, and the zygote postfertilization.^{2,3}

Ctr invades, and replicates within, the epithelium of the genital tract. Its unique biphasic life cycle is restricted to epithelial cells. On invasion, the metabolically inactive elementary bodies differentiate into reticulate bodies and establish a replicative niche, termed an inclusion, within the cytoplasm of the host epithelial cell. Cell culture models show that *Ctr* actively modulates numerous cellular processes, including vesicle trafficking, signaling, and apoptosis, to ensure successful completion of its developmental cycle.^{4–8} However, it remains unclear to what

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extent these *in vitro* findings are truly representative of the *in vivo* events. Furthermore, due to the absence of tissue regulatory mechanisms and architecture, *in vitro* cell culture models are inadequate for studies on the impact of *Ctr* on epithelial homeostasis *in vivo*.

The paracrine Wnt signaling pathway is not only a major player in developmental morphogenesis of epithelia,⁹ it is also required for tissue renewal and stem cell maintenance.¹⁰ Morphogenic gradients of soluble secreted Wnt proteins coordinate the response of individual epithelial cells, depending on concentration and receptor availability.¹¹ Wnt ligands, therefore, facilitate cell-cell communication within epithelia, and thus influence fundamental processes, such as cell migratory potential, and differentiation and proliferation rates. Mutations in Wnt pathway components are known to result in cellular transformation in several human cancers.¹² The major intracellular transducer of the Wnt pathway is β -catenin, which, on activation, translocates to the nucleus and forms an active transcriptional complex with TCF/LEF family members.¹³ β -catenin is also indispensable for cell adhesion, and therefore functionally integrates cell-cell communication with events in the nucleus. Although individual cell response to external Wnt-mediated stimuli depends on β -catenin signaling function, cell-cell communication and epithelial integrity are maintained by the adhesive function of β -catenin at the basolateral membranes. In contrast to the cancer cell lines routinely used in cell culture models, the epithelial layer of the fallopian tube represents a polarized, organized structure of non-transformed cells. The strength of the adherens junction, zonula adherens, is determined by Ca^{2+} dependent, homophilic interactions between E-cadherin (Cdh1) molecules from neighboring cells.¹⁴

Considering the tight regulatory control of epithelial layer maintenance and renewal, it is pertinent to consider that the intracellular accommodation of *Ctr* may directly disrupt the homeostasis of surrounding noninfected cells. Using electron microscopy, previous *ex vivo* *Ctr* infection models of fallopian tubes have provided descriptive insights into general morphological changes, such as loss of cilia, which occur within the mucosa during infection.¹⁵ Prolonged inflammation, marked by tissue infiltrating T cells and cytokine release¹⁶, is presently thought to be the main cause of the long-term tissue damage observed in *Ctr*-infected patients.¹⁷ However, to date, no studies have dealt with the direct response of the mucosal epithelial layer to *Ctr* infection by analyzing tissue integrity and functionality at the cellular and molecular levels. Here we show, using an *ex vivo* human fallopian tube model of *Ctr* infection, in concert with high-resolution confocal microscopy and tissue microarray expression profiling, that pleiotropic effects occurring within the epithelium lead to the disruption of epithelial homeostasis, and profound epithelial structural changes. Importantly, we found that paracrine Wnt signaling was activated in the infected tissue. Increased numbers of mitotically active epithelial cells were detected during infection, and notably the increase in proliferation could be suppressed with the addition of the Wnt secretion inhibitor, IWP2. Infected mucosa showed strong up-regulation and acti-

vation of cell adhesion regulators and stem cells biomarkers, including epithelial cell adhesion molecule (EpCAM) and olfactomedin 4 (OLFM4).

Materials and Methods

Human Samples

Fallopian tubes were provided by the Department of Gynecology, Charité University Hospital, Campus Virchow Clinic, Berlin. Scientific usage of the samples for experimental purposes was approved by the Ethics Commission of the Charité, Berlin (EA1/002/07). Fragments were sourced from standard surgical procedures for benign gynecological diseases. Only anatomically normal fallopian tubes were used for experiments. Tubes were transported and dissected within 2 to 3 hours of removal.

Chemical Treatment

EGTA (Sigma-Aldrich, St. Louis, MO) was added to the tissue pieces at a final concentration of 10 mmol/L and incubated for 48 hours. Lithium chloride was added at a final concentration of 20 mmol/L and added to tissue samples in parallel with bacteria. IWP2 inhibitor (Calbiochem, San Diego, CA) was used at the final concentration of 5 $\mu\text{mol/L}$. Samples of chemically treated fragments were processed in the same way as infected samples and controls.

Antibodies

Antibodies used were EpCAM (Cell Signaling 2929, Danvers, MA), β -catenin, (Sigma-Aldrich C2206), phosphorylated glycogen synthase kinase 3 β (GSK3 β) (Cell Signaling 9336), pHistone3 (Ser10) (Millipore CS20166, Billerica, MA), E-cadherin (BD Transduction Laboratories 610181, Dorchester, England), cytokeratin-8 (Santa Cruz Biotechnology sc-8020, Santa Cruz, CA), acetylated tubulin (Sigma 611B1), *C. trachomatis* (AbD Serotec OBT0978, Dusseldorf, Germany), HSP60 (Alexis ALX-804-072, Lausen, Switzerland), β -actin (Sigma A5441), EpCAM intracellular domain (Epitomics 1144-1 Burlingame, CA), adenomatous polyposis coli (APC) (Chemicon MAB3786, Temecula, CA), Muc1 (Cell Signaling 4538), and *C. trachomatis* major outer membrane protein (University of Washington KK12).

Tissue Dissection and Infection

Fallopian tube tissue was dissected to remove extra mesenteric connective tissue. Tubes were rinsed continuously with PBS during preparation. Before infection, the tube lumen was washed with PBS using a G23 syringe. Transverse, 2- to 3-mm thick sections of the tissue (closed tube) (~50 mg) were stored in MEM-Earl's medium + 10% fetal calf serum + L-glutamine. On the same day, tissue samples were infected with *C. trachomatis* serovar D (5×10^6 inclusion forming units per sample) in RPMI 1640 medium + 5% fetal calf serum and incubated

at 35°C 5% CO₂ in a humidified incubator. For detecting infectious elementary bodies in the tissue culture, supernatants of the tissue culture containing mature, released *Ctr* infectious particles, elementary bodies, were removed after 3 days and titrated on Hela cell monolayers, and further incubated as previously indicated. Chlamydia inclusions were confirmed by phase contrast microscopy at 48 hours postinfection.

Paraffin Embedding and Microtome Sections

Tissues were washed with PBS, fixed in 4% paraformaldehyde for 16 to 24 hours, washed twice with PBS, and then stored in PBS at 4°C until use. Tissues were dehydrated via alcohol series and perfused with paraffin in a Shandon Citadel 1000, with the following steps: 60% (1 hour), 70% (1 hour), 80% (1 hour), 90% (1 hour) ethanol, 2× 96% ethanol (2 hours), 2× isopropanol (2 hours), 2× acetone (2 hours), and 2× paraffin (2 hours). Tissues were embedded in paraffin blocks using a Microm Paraffin Console and cut with a Microm Paraffin Rotation Microtome into 5 μm thick slices. Sections on object slides were incubated overnight at 37°C before use.

Immunofluorescence Staining of Fixed Paraffinized Tissue

For de-waxing and antigen retrieval of paraffin tissue sections, sample slides were placed in a glass slide holder, incubated twice with Xylene (each 5 to 10 minutes) with agitation at regular intervals, then twice with 100% ethanol (10 to 20 seconds) and once in 90%, 70%, and 50% ethanol, each for 10 to 20 seconds, followed by two washes in water. Slides were transferred into preheated (95°C) slide holders filled up with Dako target retrieval solution and incubated for 30 minutes at 95°C, 20 minutes at room temperature, and then 5 minutes under running water. Immunofluorescence labeling of tissue sections was performed as previously described.¹⁸ Briefly, slides were removed from running water and wiped around the section. PBS (100 μL) was added to the section and a DAKO Pen (DAKO, Tokyo, Japan) was used to make rings around the sections. The slide was shaken to remove excess PBS and 100 μL of blocking solution (PBS + 1% bovine serum albumin + 2% fetal calf serum, filtered through 0.2-μm filter) was added, and slides were placed in a moist chamber to prevent drying. Primary antibodies, diluted in blocking solution, were added for 90 minutes. After washing three times with PBS for 5 minutes, the fluorescently labeled secondary antibodies (1:200) and Draq5 (1:800), diluted in blocking solution, were incubated for 60 minutes in the dark. All steps were performed at room temperature using 20 to 40 μL volumes, depending on the size of the sections. Samples were washed three times with PBS for 5 minutes and once with distilled water, and were then mounted with Mowiol (Sigma-Aldrich St Louis, MO). Dried samples were stored at 4°C in the dark.

Microscopy

Confocal images were acquired with a Leica TCS SP-1 microscope (Leica Microsystems GmbH, Wetzlar, Germany) using Leica Confocal Software (Leica Microsystems GmbH).

Microarray Analysis

Microarray experiments were performed as dual-color hybridizations. To compensate for dye-specific effects, an independent dye-reversal color-swap was applied.¹⁹ Quality control and quantification of total RNA was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop 1000 spectrophotometer (Kisker Biotech, Steinfurt, Germany). RNA labeling was performed with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). In brief, mRNA was reverse transcribed and amplified using an oligo-dT-T7-promotor primer and the resulting complementary RNA was labeled either with Cyanine 3-CTP or Cyanine 5-CTP. After precipitation, purification, and quantification, 1.25 μg of each labeled complementary RNA was fragmented and hybridized to whole human genome 4 × 44k multipack microarrays (AMADID 014850), according to the supplier's protocol (Agilent Technologies). Scanning of microarrays was performed with 5-μm resolution and extended range using a G2565CA high resolution laser microarray scanner (Agilent Technologies). Raw microarray image data were analyzed with the Image Analysis/Feature Extraction software G2567AA (version A.10.5.1, Agilent Technologies) using default settings and the GE2_105_Jan09 FE protocol. The extracted MAGE-ML files were further analyzed with the Rosetta Resolver Biosoftware, Build 7.2.2 SP1.31 (Rosetta Biosoftware, Cambridge, MA). Ratio profiles comprising single hybridizations were combined in an error-weighted fashion to create ratio experiments. A 1.5-fold change expression cut-off for ratio experiments was applied together with anti-correlation of ratio profiles rendering the microarray analysis highly significant (*P* value > 0.01), robust, and reproducible. Microarray data presented in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO at <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO Series accession number GSE26692.

Quantification of Cell Counts, Adhesion Phenotype, and Statistical Analysis

Fallopian tubes were dissected, infected, fixed, and stained under standard experimental conditions, as previously described. Microscopic slides were analyzed by epifluorescence using a Leica DMR microscope. Mean numbers of Ser10 phosphorylated form of histone H3 (H3P)-positive cells and proliferating cell nuclear antigen positive cells were calculated from 20 optical fields per sample (magnification 63×) and normalized to the number of counted nuclei per field. Exposure time was kept constant to ensure unbiased identification of H3P- or proliferating cell nuclear antigen-positive cells. Signifi-

cance ($P < 0.05$) was determined by paired Student's *t*-test comparing variation in counts per field of infected/treated samples to noninfected sample. Frequency of adhesion impaired cells in the epithelial layer was calculated as a ratio of cells that exhibit phenotype to polarized cells in the optical field, counting 20 random fields per sample. Normalization was performed to the mean value of uninfected sample. Significance ($P < 0.05$) was calculated by paired Student's *t*-test.

Protein Lysates and Immunoblot Analysis

Organ pieces were transferred into 4 mL Greiner tubes (Greiner Bio-One, Frickenhausen, Germany) on ice with 1 mL ice-cold protein preparation buffer PPB (PPB: 50 mmol/L Hepes, 330 mmol/L sucrose, pH 7.4, 1× "complete" protease inhibitor filtered through 0.2 μ m filter). Tissue was homogenized by 3 × 15 seconds pulses with an Ultra-turrax (IKA, Staufen, Germany) at maximum speed on ice; lysates were centrifuged for 10 minutes at 20,000 × *g* at 4°C. Protein concentration in the lysates was determined by BCA protein assay reagent (Thermo Fisher Scientific, Rockford, IL).

Samples were boiled after addition of 1× SDS loading dye to the supernatants and analyzed on a reducing SDS-polyacrylamide gel. After separation, samples were transferred to a polyvinylidene difluoride membrane and incubated with specific antibodies. After binding of horseradish peroxidase-conjugated secondary antibodies, ECL reagent (GE Healthcare, Amersham, England) was used for detection on X-ray films.

RNA Isolation and Real-Time PCR

Organ pieces were transferred into 2 mL tubes on ice with 0.4-mL ice-cold RLT buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany) and homogenized with three 20-second pulses of an Ultra-turrax (IKA) at maximum speed, on ice. Lysates were centrifuged for 10 minutes at 20,000 × *g* at 4°C and RNA isolated from resulting supernatants, according to the manufacturer's protocol (Qiagen). Real-time PCR was performed using Quantitect SYBR Green RT-PCR kit (Qiagen), on ABI Prism 7900 H sequence detection system (Applied Biosystems, Darmstadt Germany). Reactions performed in 25 μ L contained 100 ng RNA, 12.5- μ L SYBR Green mix, 0.25- μ L RT mix, and 0.4- μ mol/L per primer.

Program: 30 minutes 50°C; 15 minutes 95°C; followed by, 45 × cycles of 20 seconds 94°C/40 seconds 60°C/40 seconds 72°C. Primers: glyceraldehyde-3-phosphate dehydrogenase forward 5'-GGTATCGTGGAAGGACTCATGAC-3', reverse 5'-ATGCCAGTGAGCTTCCCGTTCTAG-3'; EpCAM forward 5'-AATGTGTGTGCGTGGA-3', reverse 5'-TTCAAGATTGGTAAAGCCAGT-3'; OLFM4 forward 5'-GCTC-CAGCCGACGCTTAG-3', reverse 5'-GGAGCCGGTGGA-AATTGGAA-3'; DEF4 forward 5'-TCCAGTCTTTTGC-CCTAGAAGGT-3', reverse 5'-TGGCTTTTTCAGCATTTT-G-3'; SP100 forward 5'-TCTGGCCTCCAAGTCTTGA-3', reverse 5'-ACCCGAAGGTGGCCAAGT-3'; TCF2 forward 5'-GAGACAAGGAACCAAGAG-3', reverse 5'-TCGCATCAGTTTGTTCGATGA-3'; octamer binding tra-

nscription factor 4 forward 5'-GATGTGGTCCGAGTGTG-GTTCT-3', reverse 5'-TTGTGCATAGTCGCTGCTTGA-3'; LCN2 forward 5'-GAACCTCATCCGCTTCTCCAA-3', reverse 5'-GTCGATTGGGACAGGAAGA-3'.

For each oligonucleotide pair, the sample reaction was performed in triplicate. Relative expression level of the target gene was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase in each individual sample amplification plots were analyzed with the ABI Prism SDS Software package (version 2.2.2; Applied Biosystems). Relative quantification of gene expression was performed by the comparative Ct (threshold cycle)²⁰ calculating $\Delta\Delta$ Ct. The parameter Ct is defined as the cycle number at which fluorescence passes the fixed threshold. Statistical analysis was performed by calculating *P* value (paired Student's *t*-test) for mean Δ Ct values between different biological replicates. Significance was set at $P < 0.05$.

siRNA Transfection

Small-interfering RNAs (siRNAs) were purchased from Qiagen and were validated at the Max Planck Institute for Infection Biology (Berlin, Germany) for their ability to knock-down mRNA expression of target genes by more than 70% in comparison to cells transfected with a control siRNA against luciferase. Cells were seeded in 12-well plates, grown to 50% to 70% confluency, and transfected using HiPerFect (Qiagen). Six μ L of siRNA (si β -cat, siLuci) diluted to 2 μ mol/L in serum-free medium was mixed with 100 μ L serum free medium and 6 μ L HiPerFect and incubated for 10 minutes at room temperature. The mixture was added dropwise to cells in 1 mL culture media. Cells were trypsinized after 24 hours and seeded into new plates. Infection was performed 72 hours after siRNA transfection.

Infectivity Assay

Infectious progeny were harvested from infected cells 48 hours after the primary infection by cell lysis with glass beads and titration of the supernatant containing released elementary bodies onto a HeLa cell monolayer, seeded the previous day. Cells were incubated for 24 hours at 35°C, 5% CO₂, fixed in methanol, stained for chlamydial major outer membrane protein and inclusions were counted as an average of 10 optical fields per sample. Inclusion numbers after knockdown of target genes were calculated as inclusion forming units and expressed as a percentage of the luciferase siRNA control.

Results

Ex Vivo Fallopian Tube Infection Model

To delineate the influence of *Ctr* on the integrity of epithelial tissues, we established an *ex vivo* infection model using human fallopian tubes and analyzed samples by high-resolution, immunofluorescence confocal microscopy. We used *Ctr* serovar D, which is one of the genital serovars (D-K) known to be causative agents of sexually

Figure 1. *Ex vivo* model of *Ctr* infection in fallopian tubes. **A:** Schematic representation of Fallopian tube tissue architecture. The mucosal epithelium (endosalpinx) envelops the inner surface of the fallopian tube and resides on connective tissue, the lamina propria. Two layers of visceral muscles in the muscularis (myosalpinx) provide mechanical support and contractility of the tube (arrows). Within the mucosal epithelium two types of epithelial cells can be discriminated: secretory cells and ciliated cells. **B:** Tissue sample fixed after three days in culture and processed for indirect immunofluorescence of anti E-cadherin (Cdh1) (green); Overlay with transmission light microscopy picture. Cdh1 marks the basolateral cell outlines of the epithelium exclusively. **C:** Higher magnification of a tissue sample stained for Cdh1 (green) and DNA (Draq 5) (blue); basolateral cell outlines of epithelial cells are readily visible. **D:** Tissue sample stained for cytokeratin-8 (green) and DNA (Draq 5) (blue); cytokeratin-8 accumulates at the apical plasma membrane. **E:** Tissue sample stained for the cilia marker acetylated α -tubulin (Ac-Tubulin) (green) and the basolateral marker β -catenin (β -Cat) (red). **F:** Tissue sample incubated with *Ctr* for 24 hours stained for Cdh1 (green), *Ctr* (red) and DNA (Draq 5) (blue); *Ctr* is detected only in epithelial cells. Scale bar = 16 μ m.

transmitted disease. The primary host cell tissue, the mucosal epithelial layer, was rendered accessible for *Ctr* infection by limited dissection of tubal fragments (Figure 1A), which were obtained as remnants from unrelated gynecological procedures. Epithelial viability and function, as well as preservation of structural tissue integrity,

were determined for five days in culture. The epithelial layer was visualized with the cell type-specific markers Cdh1 and cytokeratin-8. Cdh1 localized to the basolateral plasma membrane of epithelial cells (Figure 1, B and C) and all cells within the epithelial layer expressed cytokeratin-8 (Figure 1D), indicating preservation of intact tissue architecture. Staining for acetylated tubulin discriminated ciliated from secretory cells (Figure 1E). *Ctr* elementary bodies, retrieved from tissue culture supernatants of infected fallopian tubes readily infected HeLa cells (data not shown), confirming the ability of the pathogen to successfully complete its developmental cycle in *ex vivo* tissue culture. Furthermore, single-cell analyses revealed that *Ctr* was located exclusively within the single layered cuboidal mucosal epithelium of fallopian tubes and did not infect the underlying lamina propria (Figure 1F); features that are consistent with previous observations.¹⁵

Ctr Infection of Fallopian Tubes Disrupts Epithelial Cell Adhesion and Polarity

Loss of epithelial adhesion is the first step in the process of epithelial mesenchymal transition, a cellular mechanism critical for tissue development and a hallmark of tissue injury, fibrosis, and carcinogenesis.²¹ To elucidate a molecular basis for the development of pathology during *Ctr* infection, we aimed to identify early events in the infected fallopian tube epithelia. Confocal imaging of specific epithelial markers revealed substantial phenotypic changes in the epithelium of all analyzed tissue specimens ($n = \sim 60$) after two days of *Ctr* infection. Functional integrity of the epithelium can be shown by immunostaining against Cdh1, the main component of zonula adherens (Figure 2A). In contrast to the restricted basolateral distribution of Cdh1 in noninfected tissue (Figure 2B, left), Cdh1 was frequently distributed throughout the whole plasma membrane in *Ctr* infected samples (Figure 2B, right, arrows); a phenotype reminiscent of the breakdown of organized cell contacts and the loss of the apical plasma membrane domain. We also observed a multi-layering of the epithelium in infected tissue, a hallmark of apical-basal polarity loss within mucosal surfaces.²² Intriguingly, a similar cell morphology and redistribution of Cdh1 were observed in neighboring uninfected cells (Figure 2B right). To quantify the effect of *Ctr* infection on epithelial cell adhesion disruption we determined the percentage of cells with clearly defined polarity, per field of view, using apico-basal orientation, visible lateral Cdh1 domains, and absence of apical Cdh1 signal as criteria. Although occasional cells that are not polarized are found in noninfected mucosa, likely due to cell division or apoptosis, the frequency of the adhesion disrupted phenotype is significantly increased in *Ctr* infected samples (fivefold to sevenfold increase) (Figure 2C). This phenotype could also be induced by treatment of noninfected samples with the Ca^{2+} -chelating agent EGTA (10 mmol/L; Figure 2D), which inhibits Cdh1-dependent cell adhesion in cultured epithelial monolayers.²³ To confirm the loss of cell polarity, we determined the distribution of occludin, an integral membrane protein

Figure 2. *Ctr* infection triggers loss of cell adhesion and polarity in fallopian tube epithelium. **A:** Schematic representation of ultrastructural organization within the epithelium. Polarity is maintained along the apico-basal axis. Protein complexes, components of tight junctions, and zonula adherens junctions form a contact zone with neighboring cells and separate apical and basolateral domains of the plasma membrane. In the extracellular space, adhesion is facilitated through homotypic interactions of E-cadherin (Cdh1) (blue) in the presence of Ca^{2+} ions. β -catenin (red) provides the link between Cdh1 and the actin cytoskeleton. **B:** Analysis of Cdh1 staining in NI and *Ctr* infected (3 dpi) fallopian tubes. (Cdh1; green; DNA; blue; *Ctr*; red) (left) A basolateral distribution of Cdh1 is observed in NI tissue. (right) Cdh1 is detected along the plasma membrane and cells exhibit rounded cell morphology in infected tissues (arrowheads). **C:** *Ctr* infection increases number of detected cells with impaired adhesion, as a fraction of total epithelial cell count in the optical field, normalized to NI sample. Data are representative of two different infections of independent tissue samples. Significant difference of $*P < 0.05$; paired Student's *t*-test. Errors are $\pm$ SD and are calculated based on the internal variation adhesion impaired cells/polarized cells in individual sample. **D:** Loss of adhesion phenotype is induced by adding 10 mmol/L EGTA to the tissue culture in the absence of infection. The Ca^{2+} -chelating substance causes disruption of Cdh1 interactions and loss of cell adhesion (arrowheads). **E:** Localization of the tight junction protein occludin (green). (left) In NI samples, occludin is exclusively found at tight junctions (arrowheads); (right) during *Ctr* (red) infection, occludin is lost from subapical regions, indicative of a loss of cell polarity. Phase contrast images of tissue samples reveal global morphological changes in mucosa induced by *Ctr* infection. Scale bar = 16 μm . NI, noninfected; dpi, days postinfection.

localizing to the tight junctions.²⁴ Immunostaining revealed a distinct loss of occludin from subapical regions in *Ctr*-infected mucosa, as compared to noninfected epithelium (Figure 2E). Thus, we observed that during *Ctr* infection of the fallopian tubes, both cell polarity and Cdh1-mediated cell adhesion, were disrupted. Most interestingly, these changes were not restricted to infected cells, but extended toward the neighboring tissue, suggesting that *Ctr* infected cells engage in the stimulation of a paracrine signaling pathway.

β -Catenin Is Recruited to the Ctr Inclusion

To address the molecular mechanisms underlying the loss of cell adhesion on *Ctr* infection, we analyzed the distribution of β -catenin, a major cytoplasmic interaction partner of Cdh1 that mediates cell adhesion and is also critical for Wnt signaling transduction.²⁵ In noninfected cells, β -catenin localized to the same basolateral membrane domain compartment as Cdh1 (Figure 3); however, in infected cells β -catenin strongly co-localized with internalized *Ctr* at the

lateral plasma membranes of epithelial cells in the proximity of zonula adherens after 8 hours of infection (Figure 3A, upper row). At later infection times (24 to 48 hours) we observed strong co-localization of β -catenin with the inclusion surface (Figure 3A, middle and bottom row). Interestingly, in cultured HeLa cells the knockdown of β -catenin using siRNA ($\sim 75\%$ at the protein level determined by Western blot, data not shown) resulted in a twofold reduction of *Ctr* infectivity (Figure 3B), indicating that β -catenin recruitment to the *Ctr* inclusion is beneficial for the pathogen. In turn, the observed disturbances of host cell polarity and cell adhesion in the infected tissue could be explained by changes to membrane adhesion complexes of which β -catenin is an important component.

Wnt Signaling Is Deregulated on Ctr Infection

The cytoplasmic pool of β -catenin is tightly regulated and is readily degraded by the Wnt pathway inhibitory complex.²⁶ Thus, given our observation that β -catenin is recruited to the *Ctr* inclusion, we reasoned that infection

Figure 3. β -catenin is recruited to the *Ctr* inclusion and is required for bacterial replication. **A:** β -catenin (green) and *Ctr* (red) localization in epithelial cells over an infection time course of 8 to 48 hours. Scale bar = 16 μ m. **(A, middle and lower panels)** At later infection time points of 24 and 48 hours, patches of β -catenin are associated with inclusions membranes (arrowheads). Scale bar = 16 μ m. **B:** Chlamydial infectivity on small-interfering RNA (siRNA)-mediated knockdown of β -catenin is reduced twofold in comparison to cells treated with a nonspecific siRNA (Luci RNAi). Numbers of infectious progeny calculated as inclusion forming units (IFU)/mL and expressed as a fraction of control (Luci RNAi). Data are representative of three independent experiments. Error is mean $\pm$ SD calculated on the variation of inclusion count per optical field. Significant difference of $*P < 0.05$; paired Student's *t*-test. **C:** *Ctr* infection leads to a fivefold increase in the number of histone H3 (H3P)-positive cells, similar to lithium chloride treatment. Treatment of infected tissue with 20 mmol/L lithium chloride shows a mild additive, mitosis-inducing effect. Data are representative of three independent experiments; $n = 20$ fields of view per sample, normalized to the number of nuclei per field. Significant differences of $*P < 0.05$; paired Student's *t*-test. **D:** To identify actively dividing cells in infected fallopian tubes, samples were stained for the mitosis marker Ser10 phosphorylated form of H3P (green). Confocal imaging shows increased frequency of H3P-positive cells in infected epithelia in comparison to noninfected (NI) (control) tissues (arrowheads) (*Ctr*; red). Scale bar = 16 μ m. **E:** Immunoblot analysis of protein lysates for presence of the inactive form of glycogen synthase kinase 3 β (GSK3 β), ie, phosphorylated glycogen synthase kinase 3 β (pGSK3 β), and the *Ctr* heat shock protein 60 (HSP60) in *Ctr* infected, noninfected, and heat-treated (inactive) samples. Infected tissue lysate is positive for HSP60. pGSK3 β levels are elevated in *Ctr* infected lysates. Images in **D** and blots in **E** are representative of experiments with five independent patient samples.

could alter its steady state equilibrium in the cytoplasm, not only affecting adhesion of *Ctr* infected cells, but also deregulating Wnt signaling in the tissue. Indeed, we observed prominent adhesion changes in noninfected cells, which were accompanied by increased cytoplasmic levels of β -catenin (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). These changes were indicative of activation, rather than inhibition of Wnt signaling, and were suggestive of Wnt signaling disruption in the entire epithelium, including both infected and noninfected cells.

A hallmark of Wnt deregulation is an alteration of cell proliferation rates within diseased tissue.²⁷ To quantify mitotic rate in the *Ctr*-infected fallopian tube samples, we immunostained tissue sections for mitotic marker, H3P.²⁸

Ctr infection led to an approximate sixfold increase in the number of H3P-positive epithelial cells per optic field in comparison with noninfected control tissue (Figure 3, C and D), suggesting increased proliferation and altered epithelial cell homeostasis. The *Ctr*-dependent effect was comparable to the mitotic induction observed after treatment with lithium chloride for 24 hours (Figure 3C). Lithium chloride is a chemical inhibitor of GSK3 β ,²⁹ which is a negative regulator of β -catenin stability.²⁶ Thus, *Ctr*-induced remodeling of the epithelial monolayer is reminiscent of other effectors known to cause aberrant activation of the Wnt signaling pathway.

To investigate the mechanism underlying the observed *Ctr*-dependent proliferation of epithelial cells, we deter-

Figure 4. Blocking of paracrine Wnt release inhibits tissue response to infection, while *Ctr* replication is enhanced. **A:** Venn diagram of differentially regulated genes in the presence of Wnt inhibitor IWP2 during *Ctr* infection (48 hours postinfection). *Ctr* infection drives strong transcriptional responses in the fallopian tube. Response is strongly suppressed if Wnt signaling transmission is interrupted by IWP2. **B:** Cellular pathway generated by Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) representing selected regulated target genes (green) and their relationship to β -catenin (CT-NNB1, yellow) in the center. Candidates were found to be up-regulated in normal *Ctr* infection, but not regulated in *Ctr*-infected samples in the presence of IWP2. **C:** Quantitative analysis of proliferating cell nuclear antigen (PCNA)-positive cells per field view = 10. Experiment is representative of three independent experiments. Errors are $\pm$ SD and are calculated based on the internal variation of PCNA positive cells/nuclei in individual samples. Significant differences $^*P < 0.05$; paired Student *t*-test. **D:** Western blot of protein samples from epithelial cell lysates determining *Ctr* bacterial load in fallopian tube $\pm$ IWP2 with anti-HSP60 as a marker. *Ctr* replication is increased in tubes treated with IWP2. Blot is representative of three independent experiments with three different samples/infections. NI, noninfected.

mined the activity of GSK3 β in infected tissues. The phosphorylated form of GSK3 β serves as a marker of its inactivation.³⁰ Immunoblotting of whole tissue lysates showed increased levels of phospho-GSK3 β in infected samples, as compared to control tissues (noninfected and heat-inactivated Ctr) (Figure 3E). Interestingly, we observed redistribution of APC protein in infected tissue, particularly in neighboring, uninfected cells (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). APC is the key component of the β -catenin degradation complex and one of the most well-characterized tumor suppressor genes, with significance for the etiology of colorectal cancer.³¹ In noninfected mucosa, APC localized to the subapical region of epithelial cells, and exhibited a distinct granular pattern. By contrast, staining of infected samples revealed diffuse distribution of APC throughout the cytoplasm. Taken together, the increased proliferation of epithelial cells, the suppression of GSK3 β activity, and the re-localization of APC in infected tissues were consistent with enhanced Wnt signaling during Ctr infection.

Paracrine Wnt Signaling Stimulates Transcriptional Response of Fallopian Tube Epithelium to Ctr Infection

To test the hypothesis that *Ctr* infection activates the Wnt pathway in the surrounding epithelium via a paracrine route, we aimed to disrupt Wnt signaling in our tissue to compare the outcome of infection on epithelial homeostasis. Recently, a class of potent small molecule compounds has been identified that specifically inhibit secretion of Wnt and antagonize the Wnt pathway.³² IWP2 specifically inhibits the maturation of Wnt proteins by

blocking porcupine acetyltransferase. Porcupine acetyltransferase localizes to endoplasmic reticulum membranes and modifies Wnt proteins before secretion. IWP2 does not, however, affect general protein secretion or the activity of other paracrine signaling pathways, such as Notch.³² We isolated total RNA from noninfected and *Ctr* infected tubal sections and compared gene expression profiles in the presence and absence of IWP2 (5 μ mol/L) using microarray analysis. RNA was retrieved from two independent experiments with samples from two female donors to ensure identification of *Ctr* regulated genes independent of host genetic polymorphisms. We identified 2279 genes to be differentially regulated in response to *Ctr* infection (Figure 4A). The up-regulated genes not only comprised pro-inflammatory determinants, such as interleukin, cytokine, and immune response mediator genes; but also numerous genes involved in control of cell growth, proliferation, adhesion, and differentiation.

Intriguingly, blocking the Wnt pathway by adding IWP2 during *Ctr* infection reduced the number of significantly regulated genes in the infected fallopian tubes to 1056. Importantly, inhibitor alone exhibited only minor influence on gene expression in the fallopian tube tissue samples (Figure 4A) with 35 genes found to be differentially regulated also in *Ctr* infection. Therefore, the strong inhibition of *Ctr* induced gene activation by IWP2 cannot be attributed to a pathogen-independent inhibitor action. The differentially regulated gene sets were uploaded to the software platform Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) for biomining and functional analysis. Data analysis and network assembly for selected hits showed that genes regulated in infection and responsive to IWP2 were clearly linked with β -catenin

mediated Wnt signaling, either as direct targets or downstream of directly regulated proteins (Figure 4B). All genes shown in green were found to be up-regulated during *Ctr* infection and regulation was inhibited in the IWP2-treated samples. Next, we tested if IWP2 responsive gene expression changes were reflected at the tissue level during infection. Cell division rates in the tissue were analyzed by staining the samples with antibody against proliferating cell nuclear antigen, another marker of actively dividing cells. Indeed, blocking Wnt activity and secretion using IWP2 led to a marked reduction (~two-fold) of the increased mitosis observed during *Ctr* infection (Figure 4C). Interestingly, IWP2-mediated inhibition of Wnt secretion had a positive effect on the replication of *Ctr*, observed by comparison of the levels of the *Ctr* structural protein, HSP60, in *Ctr*-infected samples, compared with noninfected samples (Figure 4D). Therefore, the marked suppression of transcriptional response and reduction of mitotic rate by IWP2 cannot be attributed to an IWP2-mediated inhibition of *Ctr* infection. On the contrary, activation of Wnt secretion in response to *Ctr* infection appeared to be the causative signaling event that mediated a pleiotropic response over the whole mucosa constraining pathogen replication within the infected host tissue.

Wnt-Dependent Up-Regulation of the Stem Cell Marker OLFM4 in Infected Fallopian Tubes

Among the genes identified by microarray as being differentially regulated during *Ctr* infection of fallopian mucosa, one of the top hits exhibiting ~132-fold induction was *OLFM4*. *OLFM4* is a glycoprotein that localizes to different intracellular compartments and also to the extracellular space.³³ It binds cadherins and lectins, and thereby modulates cellular adhesion. Furthermore, *OLFM4* is found to be highly enriched in adult stem cells, in particular in Lgr5⁺ populations of intestinal and gastric mucosa.³⁴ We validated the induction of *OLFM4* in the fallopian tube *Ctr* infection model by real-time PCR with samples from three different women and three independent *Ctr* infections. Overall, the level of *OLFM4* mRNA was increased in *Ctr* infected samples ~100-fold. Interestingly, the up-regulation mechanism also appeared to be dependent on Wnt pathway activity because IWP2 suppressed induction ~10-fold (Figure 5A). Induction of *OLFM4* in fallopian tube epithelium was confirmed by confocal imaging of infected samples using anti-*OLFM4* (Figure 5B). In noninfected epithelial cells, signal intensity for anti-*OLFM4* was very low, consistent with low mRNA levels identified by real-time PCR. By comparison, *Ctr* infection caused a visible increase in signal intensity for *OLFM4* in all epithelial cells (ie, infected and noninfected). Notably, *OLFM4* immunostaining was uniform throughout the epithelium, while we observed that immunostaining of the submucosa was limited to isolated cells. In addition to *OLFM4* up-regulation, we also validated the Wnt dependent up-regulation of other selected hits from the microarray analysis using real-time PCR: *DEF4*, *LCN2*,

Figure 5. Stem cell marker olfactomedin 4 (*OLFM4*) is significantly up-regulated in the infected fallopian tube in a paracrine fashion. **A:** Real-time validation of *OLFM4* expression. *Ctr* induces strong up-regulation in *OLFM4* transcription with average change of ~100-fold. Addition of IWP2 reduces this effect to ~10-fold. Error bars $\pm$ SD from three independent experiments, with calculations based on variation in Δ Ct among independent biological samples. Significant difference of $*P < 0.05$ versus noninfected (NI) sample (paired Student's *t*-test). **B:** Confocal images of *Ctr*-infected cells stained against anti-*OLFM4* (green); *Ctr* (red), *OLFM4* is detected in low amounts in epithelial cells in NI fallopian mucosa. *Ctr*-infected tissue shows higher expression of *OLFM4* signal in all epithelial cells. This is especially prominent in the granular staining pattern in the enlarged cytoplasm of phenotypically altered epithelial cells from infected samples (arrowheads).

SP100, *OCT4*, and *TCF2* (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

Ctr Infection Induces Up-Regulation and Activation of EpCAM

Because changes in cell adhesion, polarity, and proliferation are the main phenotypic characteristics of *Ctr*-infected fallopian tube epithelium, we searched for other known modulators of cell adhesion and proliferation that could contribute to the observed cellular changes. One of the major markers of ovarian neoplastic tissue is over-expressed EpCAM.³⁵ EpCAM is an adhesion protein that negatively modulates the strength of Cdh1-mediated cell adhesion.³⁶ Due to cellular changes in *Ctr*-infected tissue being consistent with reduced Cdh1-mediated cell adhe-

Figure 6. *Ctr* triggers up-regulation and activation of ovarian cancer biomarker EpCAM. **A:** Immunoblot shows increased levels of the epithelial cell adhesion molecule (EpCAM) full length (EpCAM FL) protein, as detected with a monoclonal antibody against the C-terminus (EpICD), in protein lysates from tissue samples of three different patients (I, II, and III). All tested lysates also showed the presence of the cleaved intracellular domain EpICD. The basal level of EpCAM protein differs, as longer exposure reveals the presence of a cleavage fragment in patient I. The C-terminal EpCAM fragment is also detected in tissues treated with LiCl (20 mmol/L, 24 hours), confirming the occurrence of proteolytic EpCAM activation in the tissue and suggesting that the mechanism of processing is dependent on activation of the Wnt pathway. **B:** Real-time PCR of EpCAM mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase in five patients. Error bars indicate $\pm$ SD. **C:** Immunofluorescence imaging of EpCAM distribution in fallopian tubes. EpCAM (green); DNA (blue); *Ctr* (red). Scale bar = 16 μ m. EpCAM localizes to the basolateral membrane in noninfected (NI) epithelium (**left panel**). *Ctr* infection triggers redistribution of membrane associated EpCAM; cytoplasmic aggregates are prominent in infected, as well as neighboring cells (**right panel, arrowheads**). **D:** Real-time PCR of samples showing reduction in EpCAM induction in the presence of IWP2. Error bars indicate $\pm$ SD and are calculated for the internal variation in *Ctr* values of representative experiment. **E:** Western blot of three different patient samples (IV, V, and VI) tested for EpCAM cleavage in *Ctr* infection $\pm$ and plus IWP2 (+I). All three samples show suppression of EpCAM activation in the presence of IWP2 during infection. Significance of transcriptional regulation in **B** and **D** calculated as $*P < 0.05$; paired Student's *t*-test. EpICD, epithelial cell adhesion molecule intracellular domain.

sion, we speculated that EpCAM might be de-regulated in *Ctr*-infected fallopian tubes. In addition, EpCAM has been previously identified as a Wnt pathway target in a hepatocellular carcinoma model.³⁷ *EPCAM* was found to be positively regulated by *Ctr* infection on the microarray, although it failed statistical inclusion in the final hit list. To further investigate the putative role of EpCAM, we quantified EpCAM mRNA and protein levels on independent *Ctr* infection of fallopian tubes from 10 different women. Full length EpCAM was elevated in *Ctr*-infected tissue samples from three separate donors, as compared to the respective noninfected tissues (Figure 6A). This finding was reproduced in seven further samples tested (data not shown); however, some natural variation of basal EpCAM expression levels was evident. Real-time PCR confirmed that *Ctr* infection resulted in the transcriptional induction of *EPCAM* (Figure 6B). EpCAM immunostaining in noninfected fallopian tube epithelium was similar to the distribution of Cdh1 along lateral cell membranes (Figure 6C, left). Cytoplasmic EpCAM-positive dots were detected in subapical regions. By contrast, infected tissue exhibited a marked redistribution of EpCAM, with a loss of basolateral restriction and dispersal of the immunofluorescence signal. Patches of EpCAM-positive aggregates were detected throughout the cytoplasm (Figure 6C, right). Notably, EpCAM staining was strongest in cells neighboring inclusion-containing cells. The change in staining pattern was indicative of EpCAM activation,

consistent with the often diffuse cellular distribution of EpCAM in histological tumor samples.³⁸

The oncogenic potential of EpCAM is not only mediated by increased expression levels, but also by proteolytic processing of the membrane bound form.³⁹ We observed EpCAM proteolytic cleavage in infected fallopian tube lysates (Figure 6A) using an antibody against the EpCAM intracellular domain, which detects both full length EpCAM protein and a cleaved cytoplasmic fragment. Although basal levels of EpCAM expression and rates of processing differed among samples taken from different individuals, likely due to different genetic backgrounds, we observed markedly increased levels of both full length EpCAM protein and its proteolytic fragment in *Ctr* infection in all tested samples (Figure 6, A and E). Lithium chloride treatment of noninfected samples also induced EpCAM up-regulation and activation, in a manner similar to that observed in *Ctr* infection, corroborating the notion that EpCAM protein levels are responsive to Wnt activity (Figure 6A). Interestingly, IWP2 reduced transcriptional induction of EpCAM approximately twofold (Figure 6D) indicating that the transcriptional response was dependent on active Wnt signaling. To confirm the contribution of paracrine Wnt secretion to the processing of EpCAM, we analyzed the effect of the inhibitor, IWP2. Protein extracted from three different fallopian epithelial samples infected with *Ctr* for 48 hours in the presence and absence of IWP2 were probed with anti-EpCAM in-

tracellular domain. In all cases, IWP2 inhibited EpCAM proteolytic processing during infection (Figure 6E). Thus, EpCAM regulation in the fallopian tube occurs downstream of Wnt activation.

Our data clearly indicate a general Wnt-dependent activation of EpCAM signaling in response to *Ctr* infection. Importantly, the upregulation and redistribution of EpCAM was pronounced in the noninfected surrounding epithelium representative of the paracrine influence of *Ctr* infection.

Discussion

Using an *ex vivo* human fallopian tube model to gain insight into the pathogenic mechanisms of *Ctr*, we observed a prominent disturbance of epithelial homeostasis during acute infection. Confocal immunofluorescence microscopy revealed substantial changes in epithelial cell adhesion and polarity, and increased proliferation, comparable to that observed with known inducers of Wnt secretion. Gene expression tissue profiling and protein analysis of *Ctr*-infected tubal fragments in the presence of a Wnt secretion inhibitor showed that *Ctr* induced changes were highly dependent on active Wnt signaling. We detected the oncogene markers, OLFM4 and EpCAM, as Wnt-regulated targets during *Ctr* infection. Interestingly, both the changes in tissue morphology and the Wnt-dependent expression of protein biomarkers extended to neighboring, uninfected cells suggesting a paracrine influence of *Ctr*-infected cells.

In the absence of a functional adaptive immune system in our *ex vivo* model, the observed epithelial rearrangements could be considered to result from an early pathogen triggered response. However, contrasting to other pathogens, *Ctr* is not effective in eliciting an early proinflammatory response. Typically, *Ctr* genital tract infections are asymptomatic with no significant cytokine release detected *in vivo* until 24 hours postinfection.⁴⁰ Extending this notion, Lad et al⁴¹ showed that NF- κ B activation is inhibited *in vitro* via *Ctr*-mediated p65 degradation, suggesting that *Ctr* down-regulates the early host inflammatory response. Our finding of a strong induction of OLFM4 corroborates the negative influence of *Ctr* on proinflammatory pathways: OLFM4 is known to suppress NF- κ B pathway activation and to down-regulate innate immunity in an *in vivo* mouse model of *Helicobacter pylori* infection.⁴² Histologically, *Ctr* infections of the genital tract can also be discriminated from those of other bacterial etiologies; for example, tissue samples from patients with *Ctr*-positive acute epididymitis are largely devoid of abscesses and exhibit minimal structural damage compared with those from *Escherichia coli* positive samples.⁴³ Consistent with our own observations, the same study reported increased proliferation of epithelial cells, which appeared to be *Ctr*-specific. These observations indicate that *Ctr*-mediated pathogenesis differs from the typical progressive inflammatory manifestation of infections in that epithelial homeostasis is disturbed before inflammatory destruction.

Our functional analysis of *ex vivo* fallopian tube *Ctr* infection, in accordance with *in vitro* studies by Prozialeck et al,⁴⁴ revealed that β -catenin was recruited by *Ctr* at the beginning of the cellular infection and was subsequently sequestered to the inclusion. β -catenin is a major transducer of the Wnt pathway within tissues²⁵, and because of this we hypothesized that its recruitment to the inclusion might alter canonical Wnt signaling. Interestingly, siRNA-mediated depletion of β -catenin in cell culture impaired bacterial growth, indicating an integral role for this molecule in *Ctr* replication, while IWP2-mediated inhibition of paracrine Wnt secretion stimulated *Ctr* replication. This suggests differential effects of β -catenin recruitment and Wnt signaling on *Ctr* replication within the primary infected cells. Host cell activation by Wnt, which promotes β -catenin nuclear translocation, might antagonize β -catenin recruitment to the inclusion.

Using the Wnt secretion inhibitor, IWP2, we were able to assess the dependence of the tissue transcriptional response during acute *Ctr* infection on paracrine Wnt signaling. Of 2279 infection-specific regulated genes, we found only ~400 genes were similarly regulated, independent of Wnt activity status. The role of Wnt signaling in tissue homeostasis and disease is well-documented.⁹ However, our study provides the first evidence for a direct link between pathogen colonization of a mucosal surface and activation of the Wnt pathway. This observation emphasizes the importance of cell-to-cell communications in complex biological systems and their relevance as targets of infection.

Our findings also reveal remarkable parallels between *Ctr*-induced changes in epithelial tissue homeostasis and various pathologies of the genital tract that are characterized by changes in Wnt regulation. For example, previous work has shown that inactivation of GSK3 β is one of the key events conferring resistance to cisplatin in an aggressive form of ovarian cancer.³⁰ In addition, in a model of xenografted human endometrial tissue, inactivation of GSK3 β triggered proliferation of epithelial cells.²⁹ This observation is in striking accordance with our finding of increased cell proliferation during *Ctr* infection and increased levels of phospho-GSK3 β . We also determined that increased cell proliferation could be partially inhibited by blocking Wnt transmission. Increased cell proliferation and loss of cell adhesion and polarity are generally associated with an increased risk of cellular transformation within the epithelium. Despite existing epidemiological evidence linking *Ctr* infection and cancer,^{45,46} a confirmed etiological link with genital cancer has not yet been established in a defined biological system. Our finding that *Ctr* infection both increased EpCAM expression and enhanced its proteolytic activation provide a first molecular link between *Ctr* infection and the capacity for cell transformation in the fallopian tube. Even if the acute infection process studied here is unlikely to cause cancerous lesions *in situ*, *Ctr* appears to trigger molecular changes in surrounding epithelium, which could favor transformation. Increases in EpCAM expression are likely to contribute to a loss of adhesion, due to a negative effect on the strength of Cdh1 homophilic interactions.³⁶ In addition, EpCAM proteolytic activation

could be part of the signaling network that transduces Wnt signals to the nucleus, because it has been shown that an EpCAM cleavage fragment can form a transcriptionally active complex with β -catenin and FH2 in the nucleus of activated cells.³⁹

Our identification of OLFM4 as a robust marker of *Ctr*-infected tissue implicates a potentially altered differentiation status of fallopian epithelial cells. OLFM4 is strongly enriched in grade I highly differentiated cancers, but absent from both normal tissue and grade III undifferentiated cancers.⁴⁷ There is also a correlation between OLFM4 expression and the differentiation status of gastric and colon cancers.⁴⁸ Perhaps of most relevance to this latest study is that OLFM4 expression and distribution is associated with the progression of intraepithelial neoplasia to invasive squamous cell carcinoma in the cervical epithelium.⁴⁸ Both EpCAM and OLFM4 have been implicated in malignant transformation, where their high expression levels are indicative of pluripotency and a less differentiated state in colon mucosa.^{49,50} In addition to OLFM4 and EpCAM up-regulation, we also validated the Wnt-dependent up-regulation of other cancer-associated determinants, including *LCN2*, *SP100*, *OCT4*, and *TCF2* (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). One might speculate that dysregulation of the expression of these factors in the female genital mucosa could also be involved in loss of differentiation/adhesion and increased proliferation^{51–53} and could jointly promote a pre-malignant environment.

In summary, our data obtained with an *ex vivo* tissue infection model point to a *Ctr*-induced shift in epithelial homeostasis toward decreased differentiation, accelerated proliferation, and a cell adhesion-defective state. Of particular interest from the perspective of pathology of potential long-term sequelae of *Ctr* infection, is the discovery of distal phenotypic and functional changes of noninfected cells. Thus, a better understanding of the communications between infected and noninfected cells appears to be essential to explain the mechanisms of *Ctr*-induced pathologies.

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