Gonadotropins Regulate N-cadherin-mediated Human Ovarian Surface Epithelial Cell Survival at Both Post-translational and Transcriptional Levels through a Cyclic AMP/Protein Kinase A Pathway*

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Gonadotropins are the major regulators of ovarian function and may be involved in the etiology of ovarian cancer. In this study, we report a new mechanism whereby gonadotropins regulate the survival of human ovarian surface epithelium (OSE), the tissue of origin of epithelial ovarian carcinomas. Our results indicate that disruption of N-cadherin-mediated cell-cell adhesion is an important molecular event in the apoptosis of human OSE. Treatment with surge serum concentrations of gonadotropins reduced the amount of N-cadherin with a concomitant induction of apoptosis, and this effect was mediated by a cAMP/protein kinase A pathway but not the ERK1/2 and protein kinase C cascades. We further demonstrated that activation of the gonadotropins/cAMP signaling pathway in human OSE led to a rapid down-regulation of N-cadherin protein level followed by a reduction at the level of N-cadherin mRNA, indicating that expression of N-cadherin was regulated by post-translational and transcriptional mechanisms. The former mechanism was mediated by increased turnover of N-cadherin protein and could be reversed by inhibition of proteasomal or matrix metalloproteinase (MMP-2) activity. On the other hand, at the transcriptional level, the addition of actinomycin D abolished the cAMP-mediated decrease in N-cadherin mRNA but did not change its stability. Inhibition of protein kinase A or expressing a dominant negative mutant of cAMP-response element-binding protein blocked this decrease of N-cadherin mRNA. Together, the combined operation of post-translational and transcriptional mechanisms suggests that regulation of N-cadherin is a crucial event and emphasizes the important role that N-cadherin has in controlling the survival capability of human OSE.

The surface of the human ovary is covered with a single layer of flat-to-cuboidal mesothelial cells, the ovarian surface epithelium (OSE).1 During each reproductive cycle, the OSE takes part in the cyclical ovulatory ruptures and repair. OSE on the preovulatory follicle undergoes apoptosis at the time of ovulation and then proliferates rapidly to repair the ruptured follicle and reconstitutes an intact mesothelium (1). However, factors regulating cell death and growth in human OSE are poorly understood. Because OSE is the source of epithelial ovarian carcinomas, which is the leading cause of death among all the human gynecological neoplasms, understanding the mechanisms that regulate OSE cell survival, apoptosis, and cell growth is of great clinical importance because an aberrant signaling of any of these pathways is likely to be involved in epithelial ovarian cancer.

The cadherins are a family of cell surface glycoproteins that function in promoting calcium-dependent cell-cell adhesion and play crucial roles in the maintenance of structure, differentiation, and function of reproductive tissues (reviewed in Ref. 2). In general, cadherins are expressed in a cell-specific manner within specific compartments of the ovary, and their expression shows changes during different differentiation or stages related to functions of ovarian cells (3, 4). Numerous types of cadherins were identified in ovarian tissues of different mammalian species. N-cadherin, the most abundant member of the classical cadherin family, has been observed on the surface of granulosa cells and OSE. In contrast to cultured rat OSE, which expresses both N- and E-cadherin together, human OSE only expresses N-cadherin (5, 6). Such differences in cadherin expression may be related to the structural and physiological differences among OSE from different species. N-cadherin has been shown to protect rat OSE from apoptosis (7, 8), but whether N-cadherin has a similar function in human OSE cells is not known.

Numerous papers have dealt with the regulation of E-cadherin expression in OSE and ovarian epithelial carcinogenesis and specifically with the acquisition of this marker as an early event in transformation (6, 9–11). In contrast, the mechanism underlying the regulation of N-cadherin in OSE has remain unknown. Several lines of evidence prompted us to investigate the possibility that one of the mechanisms regulating N-cadherin expression might involve cAMP. First, pituitary gonadotropins are the major regulators of ovarian function, and the responses to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are mediated by a cAMP/protein kinase A pathway but not the ERK1/2 and protein kinase C cascades. We further demonstrated that activation of the gonadotropins/cAMP signaling pathway in human OSE led to a rapid down-regulation of N-cadherin protein level followed by a reduction at the level of N-cadherin mRNA, indicating that expression of N-cadherin was regulated by post-translational and transcriptional mechanisms. The former mechanism was mediated by increased turnover of N-cadherin protein and could be reversed by inhibition of proteasomal or matrix metalloproteinase (MMP-2) activity. On the other hand, at the transcriptional level, the addition of actinomycin D abolished the cAMP-mediated decrease in N-cadherin mRNA but did not change its stability. Inhibition of protein kinase A or expressing a dominant negative mutant of cAMP-response element-binding protein blocked this decrease of N-cadherin mRNA. Together, the combined operation of post-translational and transcriptional mechanisms suggests that regulation of N-cadherin is a crucial event and emphasizes the important role that N-cadherin has in controlling the survival capability of human OSE.

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ing hormone (LH/human chorionic gonadotropin (hCG) are mediated mainly by cAMP signaling. Normal OSE and ovarian carcinomas have specific receptors for gonadotropins, indicating a role of these factors in normal OSE physiology and in the transformation and progression of ovarian cancers (12–15). A number of epidemiological studies have demonstrated increased occurrence of ovarian cancer with exposure to high levels of gonadotropins during menopause or infertility therapy, whereas a reduced risk of ovarian cancer is associated with multiple pregnancy, breast feeding, and oral contraceptive use, which results in reduced exposure to gonadotropins (16–18). Second, studies of granulosa cells have shown that cAMP plays a critical role in promoting apoptosis (19–22). Additionally, CAMP has the capability to enhance the expression of E-cadherin, an epithelial cadherin (23, 24).

In this study, we first intended to verify the anti-apoptotic effect of N-cadherin in human OSE cells and, more significantly, to address potential molecular mechanisms involved in regulation of the gene. Our experiments revealed that expression of N-cadherin is an important mediator of cell survival in human OSE cells. The findings also demonstrate a potentially important role for gonadotropins and cAMP in regulating N-cadherin at multiple levels, and the findings identify components of a molecular mechanism by which this adhesion protein is regulated in human OSE. These results are physiologically and clinically important in understanding the regulation and function of human OSE.

***EXPERIMENTAL PROCEDURES***

**Experimental Reagents**—Compounds added to culture experiments included the following: FSH, hCG, and MDL12330, which were purchased from Sigma; Bt2cAMP was from Roche Applied Science; and (R,R)-cAMPS, H89, GF109203X, PD98059, MMP-2 inhibitor I (OA-Hy), and actinomycin D were obtained from Calbiochem. Cycloheximide was purchased from Sigma. Annexin V-FITC/John staining kit was from Roche Applied Science. Protein A/G-arosage beads were obtained from Santa Cruz Biotechnology. An antibody against N-cadherin (mouse) was from Zymed Laboratories Inc., and antibodies against CREB (rabbit) and MMP-2 (mouse) were purchased from Calbiochem. The antibody against phospho-CREB (mouse) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-ubiquitin (P4D1) was from Santa Cruz Biotechnology. Anti-N-cadherin neutralizing antibody (GC4) (mouse) and polyclonal antibodies against β-actin (rabbit) were from Sigma, and peroxidase-conjugated secondary antibodies were purchased from Bio-Rad. Small interfering RNA (siRNA) oligonucleotide targeting human N-cadherin was obtained from Santa Cruz Biotechnology. cDNA for dominant negative CREB (Ser-133 was replaced by alanine) (Invitrogen). Cells transfected with siRNA duplex oligonucleotides at 37 °C in a humidified incubator containing 95% room air and 5% CO2 were added. Cells were then maintained in the medium for 30 min prior to the addition of 1.5 mM Bt2cAMP. To inhibit adenylyl cyclase, cAMP, (GC4) (mouse) and polyclonal antibodies against bit) and MMP-2 (mouse) were purchased from Calbiochem. The anti- was purchased from Sigma, and peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

**TUNEL Assay**—Further characterization of apoptosis was achieved using a commercially available In Situ Cell Death Detection kit (Roche Applied Science) to find DNA strand breaks according to the manufacturer’s instructions. Briefly, cells were grown on glass cover slips until confluent, fixed in cold (–20 °C) methanol, and incubated with the TUNEL reaction mixture at 37 °C for 1 h. The reaction was stopped by rinsing slides with PBS. Omission of the enzyme in the TUNEL reaction was used as the negative control, and cells treated with DNase I were used as the positive control. The number of TUNEL-positive cells was counted in five different fields, and representative fields were photographed.

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation of ubiquitinated N-cadherin, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, supplemented with protease inhibitors of 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Cell lysates (400 μg) were preincubated by 20 μl of 1:1 slurry of protein A/G-agarose beads for an additional 2 h. The beads were collected by centrifugation and washed four times with lysis buffer. The immunoprecipitates were recovered by boiling in sample buffer, separated on SDS-PAGE, and subjected to immunoblotting. For Western blot analysis, proteins were extracted with RIPA buffer containing complete protease inhibitor mixture. 20 μg of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 3% nonfat dry milk in PBS with 0.05% Tween 20 and then incubated with the following primary antibodies: monoclonal anti-N-cadherin (1:1000), anti-phospho-CREB (0.5 μg/ml), rabbit anti-CREB (1:1000), polyclonal anti-ubiquitin (1:1000), or anti-β-actin (1:1000), in the same solution at 4 °C under constant agitation and followed by the addition of 20 μl of protein A/G-agarose beads for an additional 2 h. The beads were collected by centrifugation and washed four times with lysis buffer. The immune proteins were recovered by boiling in sample buffer, separated on SDS-PAGE, and subjected to immunoblotting. For Western blot analysis, proteins were extracted with RIPA buffer containing complete protease inhibitor mixture. 20 μg of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 3% nonfat dry milk in PBS with 0.05% Tween 20 and then incubated with the following primary antibodies: monoclonal anti-N-cadherin (1:1000), anti-phospho-CREB (0.5 μg/ml), rabbit anti-CREB (1:1000), polyclonal anti-ubiquitin (1:1000), or anti-β-actin (1:1000), in the same solution at 4 °C overnight. Bound antibodies were detected with an enhanced chemiluminescence system (Amersham Biosciences). The density of the bands was quantified by densitometric analysis using an Image Tool (version 3.0) System. Results were expressed as the ratio of N-cadherin/β-actin.

**Subcellular Fractionation**—Crude purification of membrane-bound and -unbound fractions of cadherin was performed as described essentially in Gottardi et al. (26). In brief, control and treated cells were...
Quantitative Real Time PCR—Total RNA was extracted by the Trizol reagent, and reverse transcription (RT) was performed using the SuperScript II kit (Invitrogen) following the manufacturer’s instruction. Quantitative analysis of cDNA amplification was assessed by incorporation of SYBR Green (Bio-Rad) into double-strand DNA. PCR primers for N-cadherin are as follows: forward primer, 5'-CAGTGTCTC-AGGACCCAGAT-3', and reverse primer, 5'-TAAAGCGAGTGATGTTGTAACCA-3'. All samples were analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (forward, 5'-ATGTGGCTATGGGTG-TGAACCA-3', and reverse, 5'-TGCCAGCTTTTCCTAGACGGCAG-3') in the same run as internal controls. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. Relative mRNA expression was determined by dividing the threshold of each sample by the threshold of the internal control GAPDH. These experiments were carried out in duplicate and independently repeated three times.

Semi-quantitative RT-PCR—0.5 μl of the RT products was amplified in a 25-μl PCR reaction mixture containing 1x buffer A, 1.5 mM MgCl2, 1 μM dNTPs, and 2.5 units of DNA Taq polymerase (Promega), with 10 pmol of each set of 20–21-mer oligonucleotide primers either for N-cadherin, as described above, or MMP-2 (sense, 5'-GGGCCCCGTCACTC-CTGAGAT-3', and antisense, 5'-GGGATCACCTAATGGGGA-3'). The corresponding cDNA fragments were denatured at 94°C for 45 s, annealed at 60°C for 45 s, and extended at 72°C for 90 s. Semi-quantitative RT-PCR was conducted using the housekeeping gene GAPDH as an internal standard. The number of amplification cycles, during which PCR product formation was limited by template concentration, was determined in pilot experiments. After 21 (for N-cadherin and GAPDH) and 28 (for MMP-2) cycles of amplification, the PCR products were analyzed on a 1% agarose gel.

Gelatin Zymography—Equal total protein (10 μg) from conditioned media was loaded on 7.5% SDS-PAGE gels containing 0.1% gelatin under nonreducing conditions. After electrophoresis, the gels were washed twice with renaturing buffer containing 2.5% (v/v) Triton X-100 for 1 h at room temperature to remove SDS and three times with H2O to remove Triton X-100. The gels were then incubated overnight at 37°C in the developing buffer containing 50 mM Tris-HCl and 20 mM CaCl2, pH 7.4. The proteinase activity was visualized by staining the gel with 0.5% Coomassie Brilliant Blue R-250 in 30% (v/v) methanol, 10% acetic acid (v/v) for 6 h and, subsequently, destaining the gel until the bands became clear. To test the effect of the MMP-2 inhibitor, OSE cells were preincubated with the inhibitor before stimulation, and conditioned media were analyzed by zymography as described.

Statistical Analysis—All experiments were performed using all three immortalized human OSE lines in duplicate and repeated at least two to three times with each experiment yielding essentially identical results. Data were expressed as mean ± S.D. Statistical comparisons of control groups with treated groups were carried out using the unpaired t test. Comparisons among three or more groups were made by one-way analysis of variance followed by Tukey’s least significant difference test for post hoc analysis (GraphPad software, San Diego). p < 0.05 was considered statistically significant.

RESULTS

Blocking N-cadherin-mediated Cell-Cell Adhesion Promotes Apoptosis in OSE—We first determined whether calcium-dependent cell-cell interactions were involved in regulating OSE survival. OSE cells were cultured with serum in the presence of the calcium chelator EGTA (final concentration, 4 mM). Treatment of cells with EGTA caused cells to detach from one another (data not shown) and clearly decreased N-cadherin expression in immortalized OSE cell lines (Fig. 1A). Along with decreases in N-cadherin expression, apoptotic activity was observed. The activity occurred as early as 3 h and with peak time at 24 h after EGTA treatment (Fig. 1, B and C). To ascertain that apoptosis was attributable to interference with N-cadherin-mediated interactions, we then examined whether an adhesion blocking anti-N-cadherin antibody, GC4, which recognizes the extracellular domain of N-cadherin, could induce apoptosis in human OSE cells. Fig. 2 shows that untreated OSE cells displayed a low percentage of apoptotic cells (5.5 ± 2.2%). Treatment with GC4 (1:100) resulted in a significant reduction in cell-cell contact, and these cultures had an increased number of single cells, compared with the IgG-treated controls (Fig. 2B, left panel), as manifested by DAPI staining (Fig. 2A). The histogram summarizes the mean percentage of apoptotic cells over time as indicated. Error bars indicate the S.D. of the mean. The asterisk indicates significant difference from 0 h with p < 0.05.

Gonadotropic Effects on N-cadherin—To identify the upstream signaling molecules that regulated N-cadherin activity in this process, we initially examined the effect of gonadotropins (FSH and hCG), which are major physiological regula-
tors of ovarian function (27), in modulating N-cadherin-mediated survival signals in human OSE. Cells were grown in a number of concentrations of FSH and hCG, spanning systemic levels at low, mid, and high doses found in humans during the ovulatory cycle (16). FSH was used at 2, 20, and 200 ng/ml, and hCG was used at 0.01, 0.1, and 1 IU/ml. As shown in Fig. 3, we found that low or mid doses of FSH (2–20 ng/ml) and hCG (0.01–0.1 IU/ml) had no significant effect on N-cadherin protein expression, whereas high doses of FSH (200 ng/ml) and hCG (1 IU/ml) significantly decreased N-cadherin protein expression. The levels of N-cadherin were decreased to 18 (p < 0.05 versus control) and 41% (p < 0.05 versus control), respectively, in response to treatment with 200 ng/ml FSH and 1 IU/ml hCG (Fig. 3). In accordance with this, trypan blue exclusion assay showed a 2–3-fold increase in cell death compared with the control (p < 0.05) (Fig. 4A). To determine whether apoptosis accounted for the loss of viability in FSH- or hCG-treated cells, we first examined the presence of nuclear and DNA fragmentation using DAPI and TUNEL assays. Fig. 4 shows that the percentage of DAPI- and TUNEL-positive cells was significantly higher in FSH- or hCG-treated cultures compared with cells grown in control medium. We also examined the reactivity to annexin V-FITC in conjunction with PI to detect exposure of dislocated phosphatidylserine to the external face of the plasma membrane, a process regarded as a marker of apoptosis. In view of the cells that are positive for both annexin V and PI may also be cells that are undergoing necrosis, only the percentage of early apoptotic cells (annexin V-positive and PI-negative) was quantified from three individual experiments and shown in Fig. 4A. Treatment of cells increased the fraction of apoptotic cells to 18.7% (p < 0.05 versus control) (Fig. 4A). This magnitude of change was comparable with the fold induction detected by DAPI and TUNEL assays. The slight variation using different techniques can be
cAMP antagonist (R)-cAMPs and the PKA inhibitor H89 for 30 min before treatment with 1.5 mM Bt2cAMP (dbcAMP). After 24 h, cell death was determined by trypan blue exclusion assay, and apoptosis was measured by DAPI, TUNEL, and annexin V staining. B, left panel, apoptotic cells exhibited positive for TUNEL staining. No positive staining was observed in untreated control. Right panel, the histogram summarizes the mean percentage of apoptotic cells counted in four fields in replicate wells from one experiment. Bars represent S.D. The asterisks indicate significant differences from controls with p < 0.05. C, cells were stained with FITC-annexin V and PI and analyzed by flow cytometry. Viable cells are PI− cells (lower left quadrant). Early apoptotic cells are defined as FITC+PI− cells (lower right quadrant); late apoptotic or necrotic cells are defined as FITC+PI+ cells (upper right quadrant). Numbers in the quadrant give the percent of FITC−/PI− cells.

accounted for by their different sensitivity and detection of specific markers in the apoptotic pathway. Together, these data demonstrate that gonadotropins can induce apoptosis of human OSE by down-regulating N-cadherin.

cAMP-mediated Inhibition of N-cadherin Expression—As an initial attempt to examine the gonadotropin-mediated signaling pathway used in OSE cells to regulate N-cadherin expression, cells were pretreated with an adenylyl cyclase inhibitor (ACI) MDL12330 for 30 min before gonadotropin treatment. Our results indicated that the gonadotropin (FSH)-induced decrease in N-cadherin expression was abolished in the presence of ACI, whereas ACI alone had no effect (Fig. 5A). Furthermore, treatment of cells with follicular concentration of cAMP analogues (19), dibutyryl cAMP (1.5 mM), also significantly decreased N-cadherin protein, implicating a role of the adenylyl cyclase/cAMP pathway in regulating N-cadherin expression in OSE (Fig. 5B). In parallel experiments, we found that this cAMP-induced reduction of N-cadherin correlated with a decrease in cell viability and an increase in apoptotic activity in these cells (Fig. 4). Because the major signaling pathway that mediates responses to cAMP involves activation of the PKA, a cell-permeable, metabolically stable cAMP antagonist ((R)p-cAMPs) and the PKA inhibitor H89 were used to determine whether PKA was required for cAMP-mediated decrease of N-cadherin gene expression. As shown in Fig. 5B, exposure of cells to either (R)p-cAMPs or H89 completely abolished the ability of Bt2cAMP to inhibit N-cadherin expression, whereas inhibitors alone had no observable effect (Fig. 5B). Collectively, these results suggest that gonadotropins induce apoptosis of human OSE by decreasing the level of N-cadherin protein via activation of the cAMP signaling pathway.

Down-regulation of N-cadherin Is Independent of MAPK and PKC—Several studies have shown that cAMP can modulate the Ras/MAPK signaling pathways and that it can cross-talk with the PKC (15, 28), prompting us to investigate the possible roles of these signaling molecules on N-cadherin expression. Our results indicated that neither the MAPK nor PKC pathways were involved in regulating N-cadherin expression, as the pretreatment of cells with PD98059 (inhibitor of MEK1, an activator of ERK1/2 kinases) and GF109203X (specific inhibitor of PKCa and PKCβ) had no observable effect compared with control cells (Fig. 5C).

Post-translational and Transcriptional Down-regulation of N-cadherin—To examine the mechanism by which gonado-
Gonadotropins and cAMP promoted down-regulation of the N-cadherin level in human OSE, cells were cultured in the presence of 200 ng/ml FSH, 1 IU/ml hCG, or 1.5 mM Bt2cAMP for 2, 4, 8, 16, and 24 h, and the expression of N-cadherin was examined by Western blot and real time PCR analyses in parallel sets of experiments. Remarkably, the decrease of N-cadherin protein was already maximally evident (~80%) as early as 4 h after treatment and diminished gradually afterward, yet was prolonged and maintained up to 32–44% reduction even 24 h later (Fig. 6). This decrease within the first 4-h period, however, was not the result of changes in N-cadherin mRNA since gonadotropins and Bt2cAMP did not significantly alter the steady state levels of the mRNA for this same period (Fig. 7). Longer treatment (8–24 h) caused a drop at the level of N-cadherin mRNA and a concomitant reduction in the amount of its corresponding protein (Figs. 6 and 7). These results suggest that two mechanisms are operated sequentially; the initial effect of gonadotropins/ cAMP on N-cadherin is elicited at the post-translational level and a later response at the transcriptional level.

Gonadotropins and cAMP Increase N-cadherin Turnover— One post-translational mechanism by which cAMP could possibly down-regulate the levels of N-cadherin protein is by enhancing its degradation. To examine this possibility, OSE cells were treated with or without Bt2cAMP in the presence of the protein synthesis inhibitor cycloheximide in a time course experiment, and cell lysates were analyzed by Western blot. As shown in Fig. 8, in the absence of Bt2cAMP, the half-life of N-cadherin protein was estimated to be ~6 h. In the presence of Bt2cAMP, the half-life of N-cadherin protein decreased to ~4 h. These results indicate that cAMP increases the turnover of N-cadherin.

cAMP Promotes N-cadherin Degradation via a Proteasome-dependent Pathway— Because cadherins can be degraded by proteasome-mediated proteolysis, we therefore wished to determine whether proteasomal activity is required for cAMP-mediated increase in N-cadherin turnover. OSE cells were treated with or without 1.5 mM Bt2cAMP in the presence of proteasome inhibitor MG132, and the level of N-cadherin protein was examined by Western blot. As shown in Fig. 9A, blocking of proteasomal activity by MG132 markedly stabilized N-cadherin protein in both untreated and Bt2cAMP-treated OSE cells. Because N-cadherin was degraded by the proteasome, we asked whether cAMP promoted ubiquitination of N-cadherin. OSE cells were treated with or without Bt2cAMP in the presence or absence of MG132 for 2 h, and N-cadherin was immunoprecipitated and immunoblotted using anti-ubiquitin antibody to detect ubiquitin-conjugated N-cadherin. Fig. 9B showed that untreated control contained small amounts of ubiquitinated N-cadherin proteins. Treatment with MG132 alone increased the amount of the ubiquitinated form of N-cadherin, suggesting that N-cadherin levels are regulated by the ubiquitin-proteasome-mediated protein degradation. Exposure of cells to Bt2cAMP only had a slight effect on ubiquitination of N-cadherin, presumably because of the reduced levels of N-cadherin at the time of analysis. Cotreatment of cells with Bt2cAMP and MG132 dramatically increased the amount of ubiquitinated N-cadherin (Fig. 9B). Thus, these results suggest that cAMP promotes N-cadherin turnover by increasing its ubiquitination and its degradation via the proteasomes.

Inhibitor of Matrix Metalloproteinase Inhibits the cAMP-mediated Degradation of N-cadherin— Several studies have demonstrated the capacity of human OSE to secrete MMPs at the time of ovarian rupture during ovulation (29, 30). Moreover, MMPs can regulate cadherin expression or function through proteolytic degradation (31, 32). Thus, we sought to investigate the possible link between MMPs and the down-regulation of N-cadherin in human OSE. To do so, we first examined the expression of MMP-2 and MMP-9, which have been identified in follicular fluid during follicle growth and ovulation (33, 34) in OSE. As shown in Fig. 10A, semi-quantitative RT-PCR analysis indicated high levels of MMP-2 but negligible expression of MMP-9 in all three OSE cell lines, indicating MMP-2 is the major gelatinase secreted by human OSE. The presence of gelatinase activity and its regulation by cAMP in OSE cells were investigated by zymography. Fig. 10B shows that MMP-2 activity was significantly higher in Bt2cAMP-treated cultures compared with cells grown in control medium. The identity of MMP-2 zymographic activity was further confirmed by Western blot analysis using the anti-MMP-2 antibody (Fig. 10C). To examine whether MMP-2 might play a functional role in the

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**Fig. 6.** Time-dependent changes of N-cadherin protein by gonadotropins and cAMP. OSE cells were treated with 200 ng/ml FSH (A), 1 IU/ml hCG (B), or 1.5 mM Bt2cAMP (C) for 2, 4, 8, 16, and 24 h. Left panel, cell lysates were prepared at the times shown and then subjected to immunoblotting with antibodies against N-cadherin (N-cad) and actin. Right panel, immunoblots were quantified by densitometry and expressed as the ratio of N-cadherin relative to β-actin for each sample. Error bars represent S.D. (n = 3). dBcAMP, Bt2cAMP.

**Fig. 7.** Time-dependent changes of N-cadherin mRNA by gonadotropins and cAMP. In parallel experiments, total RNA of OSE cells treated with 200 ng/ml FSH, 1 IU/ml hCG, or 1.5 mM Bt2cAMP (dbcAMP) was isolated at the times shown, and quantitative real time PCR was performed with N-cadherin and GAPDH sequence-specific primers. The signal intensity was determined by densitometry, and the amount of N-cadherin mRNA was normalized for the amount of GAPDH present. Error bars represent S.D. (n = 3).

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**Fig. 8.** Time-dependent changes of N-cadherin protein by gonadotropins and cAMP. OSE cells were treated with 200 ng/ml FSH, 1 IU/ml hCG, or 1.5 mM Bt2cAMP for 4, 8, 16, and 24 h. Left panel, cell lysates were prepared at the times shown and then subjected to immunoblotting with antibodies against N-cadherin (N-cad) and actin. Right panel, immunoblots were quantified by densitometry and expressed as the ratio of N-cadherin relative to β-actin for each sample. Error bars represent S.D. (n = 3). dBcAMP, Bt2cAMP.
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**Fig. 8. cAMP increases the rate of N-cadherin turnover.** Left panel, OSE cells were pretreated with 5 μg/ml cycloheximide (CHX) for 30 min followed by the addition of 1.5 mM Bt2cAMP (dbcAMP) over a 6-h time course. Left panel, total cell lysates were prepared at the times shown, and immunoblotting was performed with anti-N-cadherin (N-cad) and anti-β-actin antibodies. β-Actin works as a loading control. Right panel, immunoblots were quantified by densitometry and expressed as the ratio of N-cadherin relative to β-actin for each sample. Data points shown represent the mean, and the error bars represent S.D. (n = 2).

**Fig. 9. cAMP induces degradation of N-cadherin protein via a ubiquitin-proteasome pathway.** A, OSE cells were pretreated with the proteasome inhibitor 20 μM MG132 for 30 min before the addition of 1.5 mM Bt2cAMP (dbcAMP). After 2 h, total lysates were prepared and then subjected to immunoblotting with antibodies against N-cadherin (N-cad) and actin. Lower panel, immunoblots were quantified by densitometry and expressed as the ratio of N-cadherin relative to actin for each sample. Data points shown represent the mean, and the error bars represent S.D. B, whole cell extracts were immunoprecipitated with anti-N-cadherin antibodies. The immune proteins were resolved on SDS-PAGE and subjected to Western blot analysis with anti-ubiquitin and anti-N-cadherin antibodies. Nonimmune mouse serum was included as a control. The blot shown is a representative of two independent experiments. IP, immunoprecipitation; WB, Western blotting; Ab, antibody.

regulation of N-cadherin expression, the cells were preincubated with the MMP-2 inhibitor OA-Hy (35). Addition of OA-Hy completely blocked the Bt2cAMP-induced decrease of N-cadherin (Fig. 10D). Zymogram analysis verified an inhibitory effect of OA-Hy on MMP-2 activity (Fig. 10B, lane 4). As yet, by using an anti-N-cadherin antibody (GC4) that specifically recognizes the extracellular domain of the molecule, we were unable to detect any apparent cleavage product of N-cadherin in the conditioned media (data not shown). However, we demonstrate that Bt2cAMP induced a decrease in membrane-associated N-cadherin and OA-Hy inhibited it, indicating a role for MMP-2 in the regulation of the surface expression of N-cadherin (Fig. 10E). Immunoblotting of β-actin confirmed equivalent loading on this membrane.

**Transcriptional Down-regulation of N-cadherin in OSE—** The results of Fig. 7 indicate that prolonged exposure to Bt2cAMP decreased N-cadherin mRNA. This decrease could be the result of decreased stability and/or synthesis of the transcript. To examine the effect of Bt2cAMP on the stability of N-cadherin mRNA, OSE cells were treated for 16 h with 1.5 mM Bt2cAMP and then in the presence or absence of RNA polymerase II inhibitor actinomycin D known to inhibit transcription (36) for 1, 2, 4, and 6 h. Total RNA was extracted at the indicated times, and RT-PCR was performed. As can be seen in Fig. 11A, there was no difference in the half-life of N-cadherin mRNA (4 h) in control and Bt2cAMP-treated cultures, showing that Bt2cAMP did not change the stability of N-cadherin mRNA. To test whether the decreased N-cadherin mRNA by Bt2cAMP was because of decreased synthesis of the gene, OSE cells were pretreated with or without actinomycin D for 30 min to inhibit new mRNA synthesis before the addition of 1.5 mM Bt2cAMP for 24 h. Fig. 11B showed that pretreatment with actinomycin D completely abolished the ability of Bt2cAMP to decrease N-cadherin mRNA. Thus, these results show that the decreased level of N-cadherin mRNA in response to cAMP treatment is primarily the result of decreased transcription of the N-cadherin gene.

To determine whether the activity of PKA was involved in the effect of Bt2cAMP on N-cadherin, cells were treated with or without H89 for 30 min before the addition of 1.5 mM Bt2cAMP. Fig. 12A shows that pretreatment with H89 totally blocked the effect of Bt2cAMP on decreased expression of N-cadherin mRNA, suggesting that PKA may be involved in the regulation of N-cadherin in human OSE. To assess further the activation of transcription factors by Bt2cAMP, we determined the levels of phosphorylated CREB at Ser-133, which were related to activation on the Western blots. Fig. 12B demonstrates that treatment with Bt2cAMP caused a significant increase in phosphorylated CREB levels. Furthermore, expressing a dominant negative mutant of CREB, where Ser-133 was replaced by Ala (S133A), reverted the suppression of N-cadherin gene expression in OSE cells (Fig. 12A), further confirming that the inhibition of N-cadherin by cAMP was associated with the transcription factor CREB.

**DISCUSSION**

A role for the OSE in ovulation has been debated for many years. Despite direct observations demonstrating that the OSE contains lysosome-like inclusions and produces proteolytic en-
zymes, this concept has been questioned because of inconsistencies in the timing of the appearance of the dense lysosome-like granules in the OSE, their biochemical nature, and the observation that follicles denuded of overlying OSE can also rupture (37–40). The present study clearly demonstrates that human OSE cells express high levels of MMP-2 but little or no MMP-9. B, cells were treated with either vehicle as control, Bt,cAMP (dbcAMP) (1.5 mM), or cells were pretreated with the MMP-2 inhibitor OA-Hy (10 μM) before stimulation. Conditioned media were collected after treatment and analyzed by zymography. The clear zone at 72 kDa represents MMP-2 digestion. C, in parallel, anti-MMP-2 antibody was used to detect and react with MMP-2 by immunoblotting. D, cells were treated with OA-Hy for 30 min prior to adding Bt,cAMP for 2 h. Immunoblotting was performed with anti-N-cadherin (N-cad) monoclonal and anti-β-actin polyclonal antibodies. β-Actin acts as a loading control. Immunoblots were quantified by densitometry and expressed as the ratio of N-cadherin relative to β-actin for each sample. Values shown in the figures are the means ± S.D. (n = 3). E, fractionation of N-cadherin into membrane (ConA-bound) (B) and cytosolic (−bound) (UB) fractions using the lectin ConA.

**FIG. 11.** cAMP decreases N-cadherin mRNA synthesis but not N-cadherin mRNA stability. A, cells were pretreated with 1.5 mM Bt,cAMP for 16 h followed by the post-treatment of 4 μg/ml actinomycin D (Act D) over a time course of 1, 2, 4, and 6 h. B, cells were treated with or without 4 μg/ml actinomycin D for 30 min prior to adding 1.5 mM Bt,cAMP (dbcAMP) for 24 h. Total RNA was isolated at the times shown, and RT-PCR was performed with N-cadherin (N-cad) and GAPDH sequence-specific primers. Right panel, the signal intensity was determined by densitometry, and the amount of N-cadherin mRNA was normalized for the amount of GAPDH present. Error bars represent S.D. (n = 2). The asterisk indicates significant difference from control with p < 0.05.

**FIG. 12.** CREB-mediated inhibition of N-cadherin mRNA expression. A, OSE cells were pretreated with H89 or transfected with a dominant negative mutant of CREB, where Ser-133 was replaced by Ala (S133A), and then treated with 1.5 mM Bt,cAMP (dbcAMP). After 24 h, cells were stimulated with Bt,cAMP (1.5 mM) or vehicle. Total RNA was extracted for RT-PCR. The signal intensity was determined by densitometry, and the amount of N-cadherin (N-cad) mRNA was normalized for the amount of GAPDH present. Data points shown represent the mean, and the error bars represent S.D. of observations from three experiments. B, Western blot of cell lysates from equal protein of OSE samples treated with vehicle (Me2SO) or 1.5 mM Bt,cAMP. Immunoblotting was performed with anti-phospho-CREB (at Ser-133) and anti-CREB antibodies. The active phosphorylated form is indicated (pCREB).

**Gonadotropins Regulate N-cadherin-mediated OSE Cell Survival**
Although N-cadherin is a major adhesion protein in human and rat OSE, the anti-apoptotic process by N-cadherin studied here is distinct from other, previously described, events in rat OSE (ROSE-179), based on several observations. First, we show that the anti-apoptotic effect in human OSE strictly depends on homophilic adhesive activity of N-cadherin based on the combined evidence from calcium depletion and antibody treatment. In contrast, cell viability in ROSE-179 is regulated independent of homophilic N-cadherin binding. Indeed, N-cadherin binds to the fibroblast growth factor receptor, which in turn leads to activation of the fibroblast growth factor receptor and transduces an anti-apoptotic signal (7, 8). Second, PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7). PKC activity has been proposed as part of the mechanism through which N-cadherin prevents apoptosis (7)

The initiation of ovulation is induced by gonadotropins (FSH and LH/hCG). Normal human OSE express specific receptors for FSH and LH/hCG both in vivo and in vitro (42–44). However, the findings regarding the effect of FSH and LH/hCG on OSE are inconsistent; treatment with gonadotropins stimulated OSE proliferation in some studies (43, 45–47) but was either noneffective (16) or inhibitory for other cases (48). In this study, we observed changes in N-cadherin levels at concentrations of FSH that correlate with the profile of serum concentration of FSH and LH/hCG in the normal reproductive cycle. We observed a significant drop in N-cadherin only at the highest concentration of FSH (Fig. 3). Based on this result, we postulate that, in response to the preovulatory surge of FSH and LH/hCG, N-cadherin-mediated cell-cell adhesion in the OSE is disrupted, and the OSE on the preovulatory follicle undergoes apoptosis. At lower FSH levels, which correspond to different phases in the reproductive cycle, FSH may have different effects, including stimulation of the OSE proliferation. Such biphasic concentration-dependent effects of hormones have been observed in many other systems (49–51). In particular, recent data indicate that FSH, depending on its concentrations, can influence oocyte growth by regulating the ratio of Kit ligand and bone morphogenetic protein (49). The variations in hormone concentration may in fact account for the contradictory reports of FSH effects on OSE (16, 43, 47–48) and provide a possible basis for the concept that FSH can stimulate growth as well as induce apoptosis in OSE cells.

The reasons why FSH would induce apoptosis selectively in those OSE cells, which overlie the preovulatory follicle, are not known, but they may involve factors such as reduced intercellular adhesion because of local stretching of these OSE cells and increased exposure to stromal and hormonal factors as a result of the thinning of the tunica albuginea overlying the preovulatory follicle. These events have been proposed to initiate genomic instability; there is evidence that the OSE surrounding the sites of ovulatory rupture is exposed to inflammatory cytokines or other factors such as reactive oxygen species produced during the mechanics of follicle rupture, which increase the chance of DNA damage (52). Thus, it is conceivable that subsequent proliferation in postovulatory repair of the OSE may promote the survival of OSE cells with accumulated mutations and ultimately lead to tumor formation. In support of this hypothesis, epidemiological data consistently indicate that the risk of ovarian cancer increases with the number of ovulatory events (18). Most interestingly, we find that although normal OSE cells require both adhesion and gonadotropins for cell survival, malignant OSE cells are able to bypass these needs. This could be related to the changes brought about during neoplastic transformation of normal OSE to malignant ovarian carcinoma cells, including decreased expression of LH and/or FSH receptors in human ovarian cancer (41) or the involvement of other signaling molecules, such as phosphatidylinositol 3-kinase/2 Y. L. Pon, N. Auersperg, and A. S. T. Wong, unpublished data.
Akt is frequently activated in ovarian cancer cells (53), and activation of this pathway could promote ovarian tumor cell growth by protecting against apoptosis or enhancing proliferation (54). The loss of adhesion-dependent control likely provides survival advantages for the malignant progression of OSE.

Our finding that the action of exogenous cAMP analogues operates within follicular concentrations of cAMP (22) suggests that this cyclic nucleotide is a potential physiological regulator for N-cadherin. Two mechanisms are operated in sequence as follows: the initial suppression of N-cadherin as a result of decreased protein stability, and the later inhibition of transcription to effectively mediate the reduction of N-cadherin protein. The rapidity with which N-cadherin is degraded by cAMP could provide a mechanism that allows OSE cells to specifically and rapidly respond to cyclical hormonal and environmental changes.

We also show that cAMP accelerates N-cadherin protein turnover. This suggests that enhanced proteolytic degradation either by increased rates of proteasome-dependent protein degradation or shedding of the cadherin extracellular domain may contribute to the reduced levels of N-cadherin. Our data demonstrate that the proteasome proteolysis pathway plays an important role in both basal and cAMP-mediated regulation of N-cadherin protein level and demonstrate that it depends on the addition of ubiquitin to N-cadherin. The proteasome-dependent protein degradation is also an important mechanism of E-cadherin regulation (55). An alternative way for the modulation of cell surface expression and function of cadherins is its processing through cleavage of the extracellular domain of the molecule through MMPs (31, 32). There is circumstantial evidence that OSE actively provides survival advantages for the malignant progression of OSE, which may have a role in the control of ovulation and ovarian carcinogenesis.

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