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Negative Regulation of N-Cadherin-Mediated Cell–Cell Adhesion by the Estrogen Receptor Signaling Pathway in Rat Pituitary GH_3 Cells

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The ability of the estrogen receptor signaling pathway to regulate cell–cell adhesion, and N-cadherin and ß-catenin expression was examined in rat somatolactotropic GH_3 cells cultured in serum-free, phenol red-free medium (SFM). Estradiol-17ß (E_2) promoted a nonadherent phenotype, whereas the steroidal antiestrogen, ICI 182,780, induced the formation of tightly adherent aggregates of cells. The antiestrogen-induced cell–cell adhesion was associated with the presence of adherens junctions, and was Ca^{2+}-dependent. E_2 reduced surface N-cadherin protein to barely detectable levels, whereas ICI 182,780-treated cells displayed abundant punctate immunoreactive N-cadherin. Antiestrogen failed to induce adhesion in the presence of a blocking antibody to N-cadherin. ICI 182,780 increased the protein levels for N-cadherin and the cadherin-binding protein, ß-catenin, by twofold over SFM controls or E_2-treated samples. ICI 182,780 also increased the mRNA levels for N-cadherin and ß-catenin by two- to fivefold. In GH_3 cells cultured in growth medium, ICI 182,780 increased N-cadherin and ß-catenin levels by twofold over untreated controls, and inhibited cell proliferation by 53%. These results provide the first demonstration of the regulation of N-cadherin-mediated cell–cell adhesion by the estrogen receptor (ER) signaling pathway in pituitary somatolactotrophs through the coordinate regulation of N-cadherin and ß-catenin expression. The inverse relationship between ICI 182,780-induced adhesion and proliferation raises the possibility that these two processes are functionally related.

Key Words: N-cadherin; ß-catenin; estrogen; lactotrope; pituitary; cell–cell adhesion.

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Introduction

The pituitary lactotrope, which represents one of three Pit-1-dependent cell lineages in the pituitary, synthesizes and secretes prolactin (PRL) as its primary differentiated function *(1)*. During its existence, the pituitary lactotrope performs at least four fundamental cellular functions which are likely to require, and be regulated by, specific cell-matrix and cell–cell interactions. The first of these is cellular migration. In the mouse, lactotropes emerge within the anterolateral aspect of the pars distalis, and subsequently migrate as postmitotic cells to spread throughout the pars distalis *(2)*. Based on our understanding of how other cells (e.g., neural crest) migrate *(3)*, the movement of lactotropes is likely to require cell-matrix and cell–cell adhesion. The development of preferential cell–cell associations also facilitates paracrine signaling. In the adult, a subpopulation of lactotropes develops a preferential association with gonadotropes *(3,4)*. This lactotrope-gonadotrope association inhibits PRL secretion via a calcitonin-mediated paracrine mechanism *(5)*. The configuration of numerous lactotropes within a cluster has also been described *(6)*, and this may facilitate autocrine or paracrine signaling among lactotropes, such as galanin-mediated signaling during estrogen-induced proliferation *(7)*. There is also evidence that cell–cell adhesion regulates prolactin gene expression *(8,9)*.

Cell adhesion also modulates proliferation. Lactotropes represent a dynamic population of cells whose proliferative activity changes in response to fluctuating levels of estrogen, as well as other hormones and growth factors, during normal reproductive cycles *(10)* and during pregnancy, lactation and weaning *(11,12)*. Chronic estrogen treatment can induce lactotrope-derived pituitary tumors *(12)*. The proliferative potential of lactotropes likely contributes to the development of prolactinomas, which are the most common class of pituitary tumors *(13)*. Based on studies in other cell and tissue types *(14–16)*, the proliferative capacity of lactotropes may be modulated by both cell-matrix and cell–cell contacts.

Programmed cell death represents the fourth aspect of lactotrope function likely to be associated with cell–cell

and cell-matrix interactions. Evidence suggests that the estrogen-induced hyperplasia of lactotropes that occurs during pregnancy and lactation is, to some extent, reversed by programmed cell death of lactotropes at weaning *(17)*. Estrogen withdrawal in chronically estrogen-treated rats induced apoptosis of lactotropes *(18)*. Other studies indicate that apoptosis occurs after weaning, long after the precipitous drop in estrogen at parturition *(17)*. It is likely that cell-matrix and cell–cell adhesion influences the determination of which lactotropes will survive and which will die *(19)*.

Although estrogen regulates numerous aspects of lactotrope function in addition to its stimulatory effect on PRL gene transcription, there has been limited study of estrogen regulation of cell-matrix or cell–cell interactions in the pituitary. This prompted us to examine whether estrogen regulates cell–cell adhesion, using a rat pituitary tumor cell line, the GH_3 cell, which represents a bipotential somatolactotrope, producing both PRL and growth hormone (GH; *20*). In the present study, we demonstrate that perturbation of the estrogen receptor (ER) signaling pathway alters N-cadherin-mediated cell-cell adhesion in GH_3 cells, and that antiestrogen enhancement of cell–cell adhesion is associated with a decrease in proliferation.

Results

ER Signaling Pathway Regulates Cell-Cell Adhesion in GH_3 Cells

Initial experiments examined the effects of ICI 182,780 vs E_2 on cell–cell adhesion in GH_3 cells cultured in a phenol-red free, serum-free medium (SFM). In SFM, cells were loosely adhered to each other and formed two layers- a lower layer attached to the dish and an upper layer of rounded cells loosely adhered to cells in the lower layer (Fig. 1A). Treatment with 50 n*M* E_2 had little effect on the appearance of cells after 1 d (Fig. 1B), although longer treatments resulted in more cells in the upper layer (data not shown). Treatment with 100 nM ICI 182,780 induced the formation of tightly adherent aggregates consisting of flattened cells with obscured boundaries and little intercellular space. Very few cells were found in the upper layer (Fig. 1C; Fig. 2A). In the presence of 50 n*M* E_2, ICI 182,780 induced cell-cell adhesion at 500 n*M* (i.e., 10-fold molar excess) but not at 100 n*M* (i.e., twofold molar excess) (Fig. 1D and E).

The addition of 0.5 m*M* EGTA to cells treated with ICI 182,780 disrupted the tightly adherent aggregates to loosely adhering aggregates with clear cell–cell boundaries and easily observable intercellular spaces within 30 min (Fig. 2). Electron microscopic examination of these aggregates revealed closely apposed membranes containing adherens-like junctions (Fig. 3). These findings suggest that estrogen down-regulates the expression and/or activity of one or more Ca^{2+}-dependent cell–cell adhesion molecules, such as cadherins, and/or cadherin-associated proteins (e.g., the catenins) in GH_3 cells.

Regulation of N-Cadherin and ß-Catenin Levels by the ER-Signaling Pathway

Preliminary studies indicated that N-cadherin, but not E-cadherin, is expressed in GH_3 cells. We examined the effects of ICI 182,780 versus E_2 on the level of N-cadherin using confocal immunofluorecent microscopy. In cells treated with ICI 182,780 for 2 d, punctate, membrane-associated fluorescence was readily detectable (Fig. 4). In contrast, surface N-cadherin was barely detectable in E_2-treated cells (Fig. 4).

To test the functional importance of surface N-cadherin in antiestrogen-mediated cell–cell adhesion, cells were treated with ICI 182,780 alone, or in the presence of affinity purified IgG fraction of a polyclonal goat anti-human N-cadherin antibody (1 μg/mL). As shown in a representative of three experiments, the presence of anti-N-cadherin antibody dramatically reduced the cell–cell adhesive effects of antiestrogen, whereas the same concentration of affinity purified non-immune goat IgG fraction had no effect on cell-cell adhesion (Fig. 5, right panel). Quantification of these results revealed that anti-N-cadherin antibody reduced the percentage of cells in tight aggregates by about 45% ($p < 0.05$), and approximately doubled the percentage of single and loosely adherent cells over a 24-h period ($p < 0.05$; Fig. 5, left panel), relative to no antibody and nonimmune IgG controls.

The steady-state levels of N-cadherin and the cadherin-binding protein, ß-catenin, were examined by immunoblot. As shown in a representative experiment in Fig. 6, ICI 182,780 increased N-cadherin and ß-catenin levels by approx twofold over SFM controls or E_2-treated samples after 24 h. In three separate experiments, ICI 182,780 increased N-cadherin and ß-catenin levels 2.0-fold and 1.8-fold, respectively ($n = 6$; $p < 0.05$ for both proteins), over E_2-treated samples. The effect of 100 n*M* ICI 182,780 was blocked by 50 n*M* E_2.

In order to assess whether the rise in N-cadherin and ß-catenin levels was a primary response to ICI 182,780, or a secondary response to the formation of stable adherens junctions, we examined the effects of ICI 182,780 on protein levels at time points prior to cell aggregation. The ICI 182,780-induced increases in the levels of N-cadherin and ß-catenin occurred in a concerted fashion, and were observed as early as 4 h after cells were transferred from a serum- and estrogen-containing environment to a serum- and estrogen-free environment containing ICI 182,780 (Fig. 7). Levels of the protein tyrosine kinase, Lck, did not change in response to ICI 182,780, demonstrating the specificity of the effect of ICI 182,780 on N-cadherin and ß-catenin (*see also* Fig. 9). No change in N-cadherin or ß-catenin levels was observed in cells transferred from serum- and estrogen-containing media to serum-free media containing 50 n*M* E_2 (data not shown).

We next examined whether antiestrogen or estrogen treatment altered the mRNA levels for N-cadherin and ß-

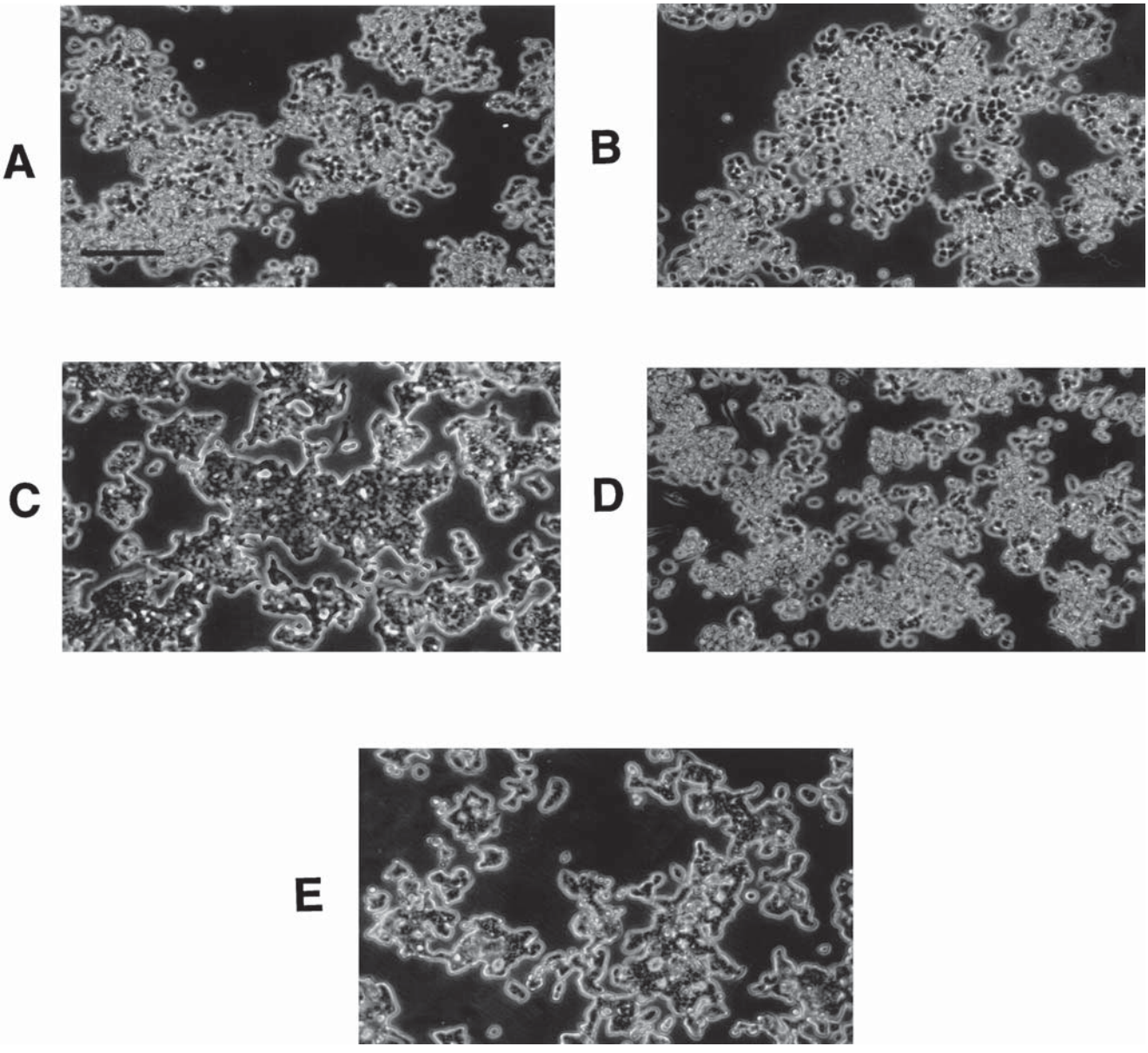


Fig. 1. ICI 182,780 induces Ca^{2+}-dependent cell-cell adhesion in GH_3 cells. Cells were treated for 1 d in SFM alone **(A)**, 50 n*M* E_2 **(B)**, 100 n*M* ICI 182,780 **(C)**, 50 n*M* E_2 plus 100 n*M* ICI 182,780 **(D)** or 50 n*M* E_2 plus 500 n*M* ICI 182,780 **(E)**. Bar (A) = 100 μm.

catenin. After 24 h, the level of both mRNAs were low in SFM controls. ICI 182,780 increased N-cadherin and ß-catenin mRNA levels by two- to fivefold (Fig. 8). As expected, ICI 182,780 strongly inhibited and E_2 increased PRL gene expression. In the experiment shown in Fig. 8, E_2 increased PRL mRNA 43-fold over ICI 182,780-treated samples.

ICI 182,780 Inhibits GH_3 Cell Proliferation

In order to examine the effect of antiestrogen on GH_3 cell proliferation, cells were treated with 200 n*M* ICI 182,780 in serum-containing growth medium (GM). Under these conditions, cell-cell adhesion was visibly enhanced by 72 h (data not shown). ICI 182,780 increased the levels of N-cadherin and ß-catenin by 2.3-fold and 2.1-fold, respectively ($n = 4$; $p < 0.05$ for both proteins), over those of untreated controls (Fig. 9A). In addition, ICI 182,780 caused a 53% reduction ($n = 4$; $p < 0.05$) in total cell number as compared to untreated controls over a 5-d period (Fig. 9B). Examination of hydroethidine-stained cells did not reveal evidence of increased cell death in ICI 182,780-treated cells.

Discussion

Estrogen regulates fundamental aspects of lactotrope function, including hormone and growth factor production, proliferation and cell death, which are potentially influenced by cell–cell contact. In the present study, we have established conditions which allow for the examination of

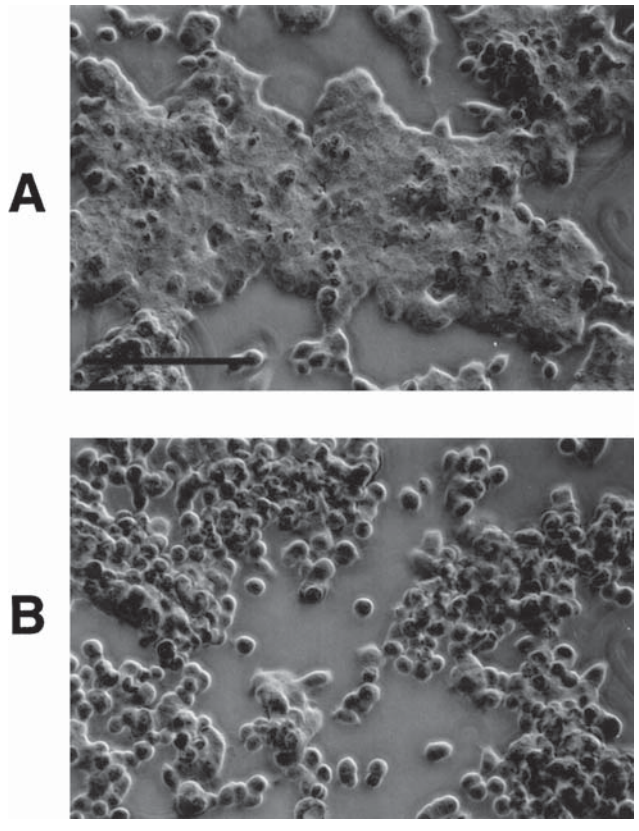


Fig. 2. ICI 182,780-induced cell–cell adhesion is reversed by EGTA. Cells were treated for 24 h with 100 n*M* ICI 182,780 **(A)**. After being photographed, 0.5 m*M* EGTA was added and the cells incubated for an additional 30 min **(B)**. Bar = 100 μm.

the regulation of lactotrope cell–cell adhesion by the ER signaling pathway using the GH_3 cell line. Effective inhibition of ER-dependent signaling was achieved by culturing cells in a serum-free and phenol red-free medium in the presence of ICI 182,780. ICI 182,780 is a pure estrogen antagonist *(21)*, which has been reported to increase ER turnover *(24)*, and to significantly slow the formation of ER-DNA complexes *(25)*. ICI 182,780 resulted in a dramatic reduction of PRL gene expression, which is consistent with the well-established fact that the high level of PRL gene transcription in lactotropes is ER-dependent (e.g., *26*), and indicates that ICI 182,780 acts as an anti-estrogen in GH_3 cells. ICI 182,780 also induced cell-cell adhesion when present at a 10-fold molar excess over E_2 , indicating that this effect of ICI 182,780 is mediated through its anti-estrogen actions. However, ICI 182,780 has been reported to have effects on ER-negative cells (e.g., *27*), and we cannot rule out that ICI 182,780 regulates cell-cell adhesion through ER-independent pathways.

Although a previous study reported that antiestrogen treatment resulted in apparent apoptotic death of GH_3 cells *(28)*, ICI 182,780-treated cells remained healthy in our experiments. In fact, exposure of GH_3 cells to E_2 in SFM conditions decreased cell–cell and cell–matrix contact, and led to the appearance of rounded cells on the surface of underlying cells that were attached to the culture dish. These rounded cells showed signs of apoptosis (e.g., condensed nuclei and/or apoptotic bodies) after 2–3 d of culture (data not shown). This apparent apoptosis may be a consequence of the inability of estrogen to fully drive the cells through the cell cycle in the absence of other growth factors *(29)*.

ICI 182,780 coordinately enhanced the levels of N-cadherin and ß-catenin within a few hours, which then led to the formation of tightly adherent GH_3 cells within 24 h. The importance of elevated N-cadherin levels in the induction of cell-cell adhesion is supported by our finding that the ability of ICI 182,780 to induce cell-cell adhesion was significantly reduced by anti-N-cadherin antibodies. A relatively modest (i.e., about twofold) increase in ß-catenin and N-cadherin levels was accompanied by a dramatic enhancement of cell–cell adhesion. One possible explanation for this is that ICI 182,780 allowed N-cadherin and ß-catenin protein to exceed a critical threshold level. In this light, it is interesting to note that modest changes in ß-catenin expression have been shown to dramatically alter the intracellular distribution of this protein *(30)*. Thus, ICI 182,780 may elevate these proteins to a level sufficient to maintain the stable adherens junctions that were clearly observed by transmission EM. This scenario is supported by the confocal immunofluorescent microscopy, which demonstrated a disproportional increase in cell surface N-cadherin in response to ICI 182,780 treatment.

It is also possible that ICI 182,780 regulates adhesion molecules other than N-cadherin. Although the anti-N-cadherin antibody blocking study indicates that N-cadherin makes a significant contribution to ICI 182,780-induced cell-cell adhesion in GH_3 cells, other cadherins have been detected in the pituitary and in lactotrope cell lines. Expression of PB-cadherin and P-cadherin has been described in pituitary and pituitary cell lines *(9,31,32)*, and we have observed E-cadherin by immunohistochemical staining in normal pituitaries (data not shown). Although our data indicate that N-cadherin is the primary cadherin expressed in GH_3 cells under our culture conditions, we have detected low and variable levels of R-cadherin mRNA and protein in GH_3 cells. Further work is needed to fully characterize the range of cadherin expression in pituitary and pituitary cell lines, and to determine whether other cadherins are also regulated by the ER signaling pathway. ICI 182,780 may also regulate the expression or activity of Ca^{2+}-independent adhesion molecules, or the effects of ICI 182,780 on N-cadherin may be amplified by a cooperativity between N-cadherin and a preexisting Ca^{2+}-independent adhesion molecule. Cooperativity between Ca^{2+}-dependent and Ca^{2+}-independent adhesion molecules has been demonstrated in other cell types *(33)*. In the pituitary and GH_3 cells, neural-cell adhesion molecule (N-CAM) is abundantly expressed *(34)*. Thus, Ca^{2+}-dependent N-cadherin-mediated adhesion may cooperate with or allow the

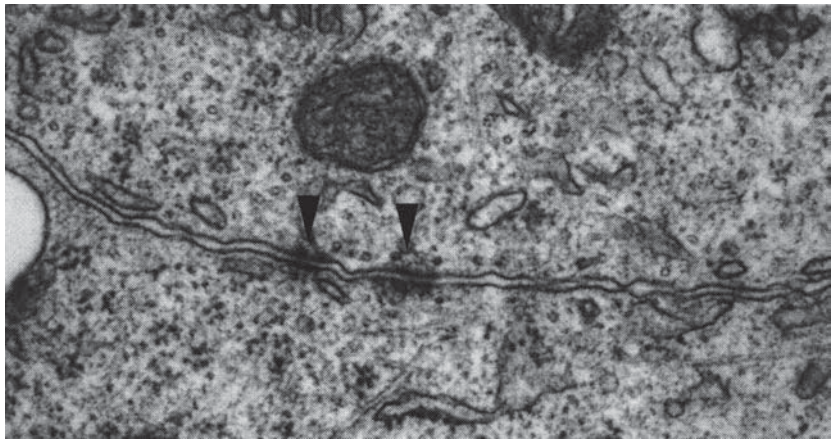


Fig. 3. ICI 182,780-induced aggregation is associated with the presence of adherens junctions (arrows). Magnification; x36,000.

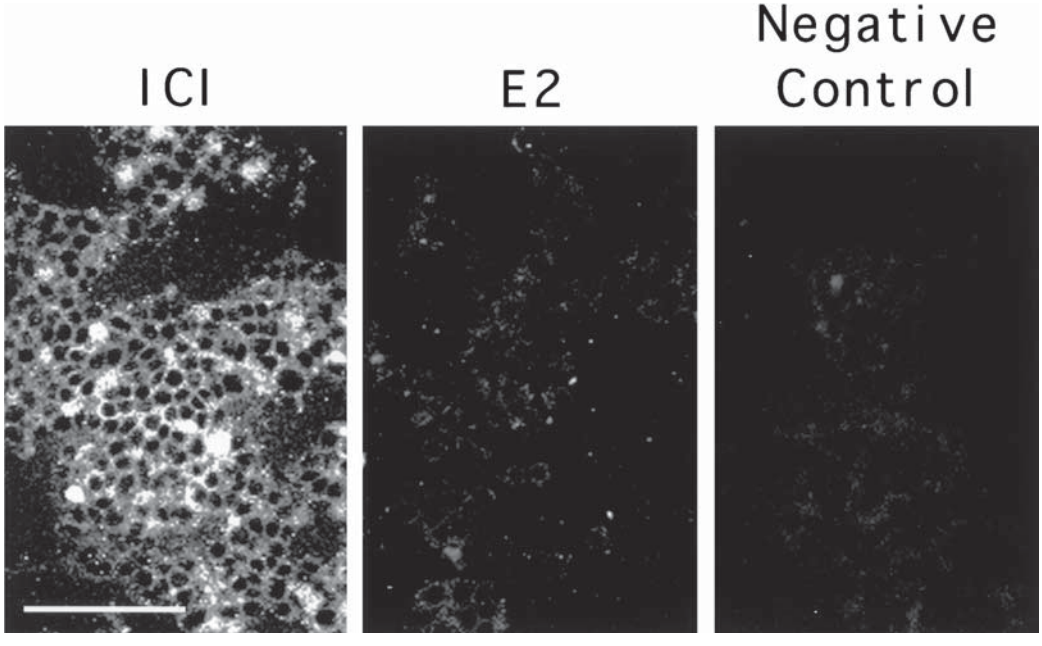


Fig. 4. ICI 182,780 increases cell surface immunoreactive N-cadherin. GH_3 cells were treated essentially as described for Fig. 1, fixed in formalin and processed for immunofluorescent confocal microscopy as described in *Materials and Methods*. Negative control refers to cells treated with ICI 182,780, but processed without primary antibody. Bar = 100 μm.

formation of Ca^{2+}-independent NCAM-mediated adhesion. In this regard, it is striking that transmission EM revealed relatively few adherens-type junctions associated with long stretches of closely apposed cell membranes from adjacent cells.

The fact that the ER is a member of the nuclear hormone transcription factor gene family prompted us to examine whether E_2 repressed N-cadherin and/or catenin gene expression. Indeed, estrogen regulation of cadherin gene expression has been previously reported in other tissues. Estrogen decreased E-cadherin mRNA levels in an endometrial cancer cell line *(35)*, and upregulated N-cadherin and E-cadherin mRNA expression in mouse uterine and ovarian cells *(36–38)*. Our study indicates that ICI 182,780 increases N-cadherin and ß-catenin levels pretranslationally. In contrast, other studies have provided evidence that estrogen regulates cadherins and/or catenins by modulating their stability. For example, estrogen was previously shown to increase E-cadherin degradation in mouse uterine epithelium *(39)*. Similarly, retinoids increased ß-catenin stability and cell-cell adhesion in the SKBR3 breast cancer cell line without affecting ß-catenin mRNA levels *(40)*. Also, the dexamethasone-induced decrease in cell-cell adhesion in 235-1 lactotropes was associated with no change in N-cadherin or P-cadherin mRNA levels, but was correlated with a decrease in α-catenin and ß-catenin protein levels *(9)*.

We also observed that ICI 182,780 enhanced N-cadherin and ß-catenin levels and cell-cell adhesion in GH_3 cells

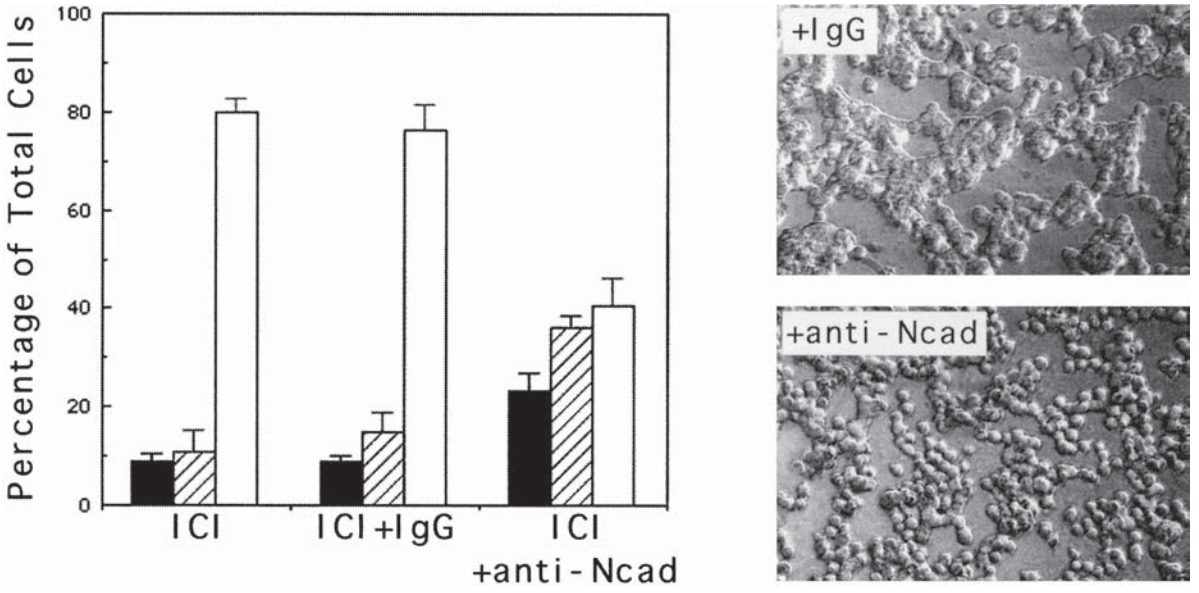


Fig. 5. Anti-N-cadherin antibody inhibits ICI 182,780-induced cell-cell adhesion. Cells were treated with 100 n*M* ICI 182,780 alone (ICI), or in the presence of 1 μg/mL of nonimmune IgG (ICI + IgG) or anti-N-cadherin antibody (ICI + anti-Ncad). The percentages of single (black bar), loosely-adherent (hatched bar) or tightly-adherent (white bar) cells were determined as described in *Materials and Methods*. All three categories of cells in ICI + anti-Ncad cultures were significantly different ($p < 0.05$) from either ICI- or ICI plus IgG-treated cells. Images of cells at right show that ICI 182,780 was able to induce extensive cell-cell adhesion in the presence of nonimmune IgG (+ IgG), but not in the presence of an anti-N-cadherin antibody (+ anti-Ncad).

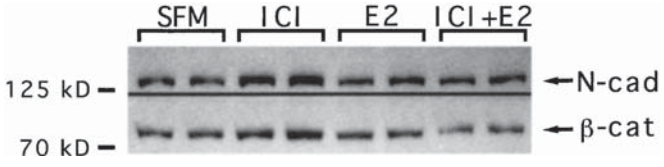


Fig. 6. ICI 182,780 increases levels of N-cadherin and ß-catenin protein. Duplicate cultures of GH_3 cells were treated in SFM alone or containing 100 n*M* ICI 182,780, 50 n*M* E_2 or both. Cell lysates were assayed by Western blot as described in Materials and Methods. No bands were detected in the absence of primary antibody (data not shown). Solid line denotes the position of where the blot was cut after transfer (*see* Materials and Methods).

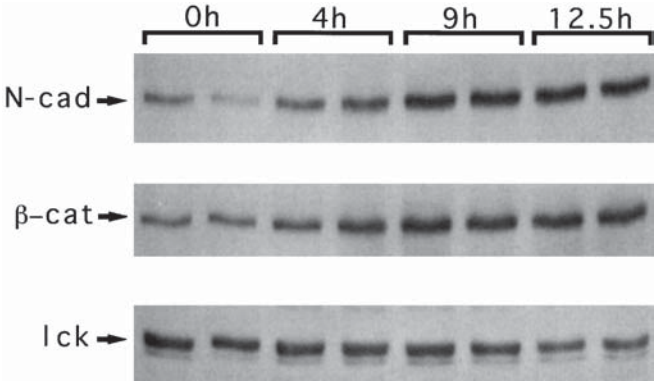


Fig. 7. N-cadherin and ß-catenin levels increase within 4 h after addition of ICI 182,780. Growth medium was removed from GH_3 cells and replaced with serum-free medium plus 100 nM ICI 182,780. Duplicate cultures were lysed at the indicated time points and assayed by Western blot.

cultured in a serum-containing medium. These effects were inversely correlated with those on cell proliferation, raising the possibility that the ICI 182,780 inhibits cell proliferation in part through its effects on cell-cell adhesion. In other cell types, forced expression of vascular endothelial- (VE) cadherin and N-cadherin repressed cell proliferation *(16)*, and disruption of cadherin-catenin complex function has been related to loss of density-dependent growth regulation *(41)*. In a recent study, E-cadherin overexpression induced cell-cell adhesion which was accompanied by a decrease in proliferation and an induction of the cyclin-dependent kinase inhibitor, $p27^{kip1}$ *(42)*. Further, ß-catenin has recently been identified as a protooncogene, which, on heterodimerization with members of the Tcf/Lef transcription factor family, regulates the expression of specific genes *(43–45)*, such as c-myc *(46)*. Thus, it is tempting to speculate that the downregulation of N-cadherin-mediated cell-cell contact and changes in ß-catenin localization by estrogen is functionally related to the ability of estrogen to increase lactotrope proliferation and promote the development of prolactinomas *(47)*.

Materials and Methods

Cell Culture

GH_3 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM/F12 (Sigma, St. Louis, MO) supplemented with Pen-Strep (100 U Penicillin G and 100 mg Streptomycin per ml of media),

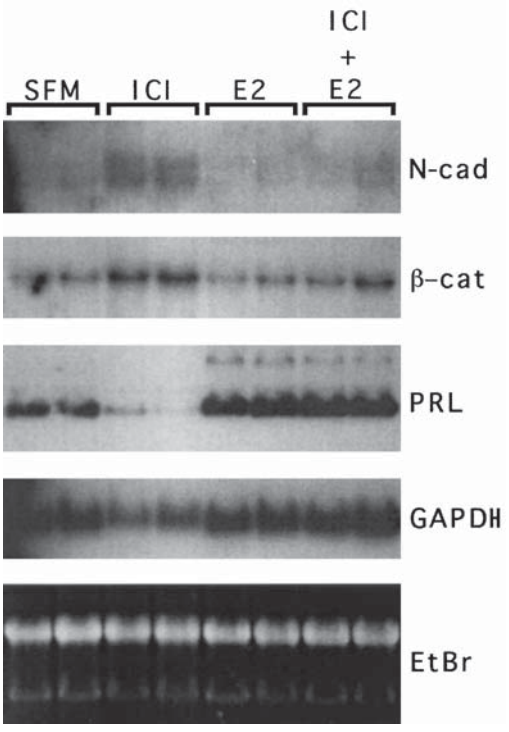
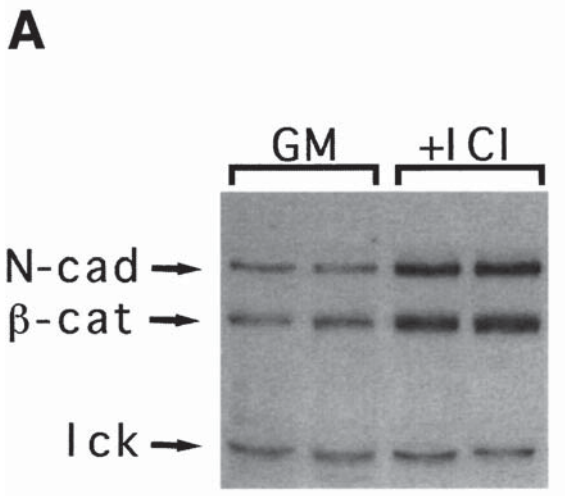


Fig. 8. ICI 182,780 increases N-cadherin and ß-catenin mRNA levels. Duplicate cultures of GH_3 cells were treated for 1 d in SFM with 100 n*M* ICI 182,780 (ICI), or 50 n*M* E_2 (E2). RNA was isolated and equal amounts (10 µg) were assayed by Northern blot hybridization for N-cadherin, ß-catenin, PRL, and GAPDH mRNAs. Ethidium bromide staining is shown from the filter consecutively probed for ß-catenin and GAPDH.



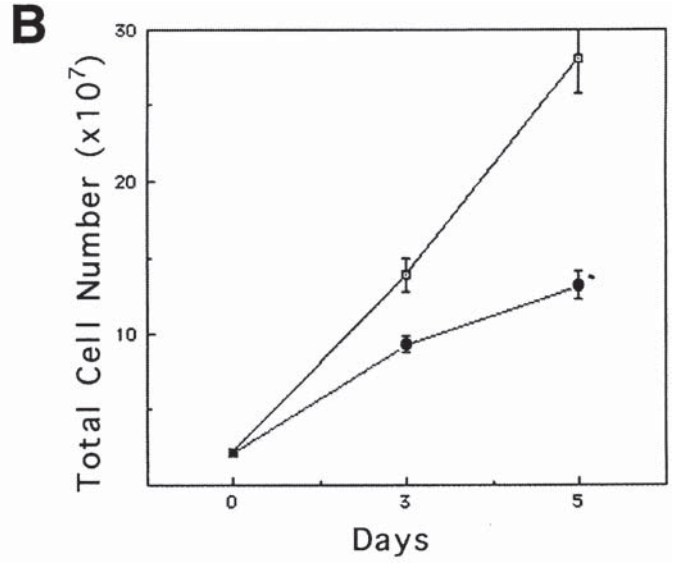


Fig. 9. (A) ICI 182,780 increases N-cadherin and ß-catenin levels in cells cultured in serum-containing GM. Duplicate cultures of GH_3 cells were treated with 200 n*M* ICI 182,780 for 3 d in GM. *(B)* ICI 182,780 inhibits GH_3 cell proliferation. Cells were cultured as in A and cell counts obtained from quadruplicate culture dishes on d 0 and 5, and from triplicate culture dishes on d 3. Values represent the mean +/- SEM from cells cultured in GM only (open squares) and in GM plus ICI 182,780 (closed circles).

10% horse serum and 2.5% fetal bovine serum (GM). For experiments, cells were removed by trypsinization and plated onto 60 mm tissue culture dishes in GM. The following day, GM was removed and the cells gently washed with 2 mL of phenol red-free, serum-free Ham's F-12 (SFM; Life Technologies, Gaithersburg, MD). Cells were examined and photographed (*see* Fig. 1) under phase microscopy using a Zeiss IM microscope. Cells were then treated with antiestrogen and/or estrogen in SFM for up to 48 h. For the proliferation studies, cells were maintained in GM, and cell counts obtained using a hemacytometer on d 0, 3, and 5 after treatment. The details for each experiment are described in the legend for the corresponding figure. Water soluble ß-estradiol (E_2) was obtained from Sigma Chemical Co. (St. Louis, MO). The steroidal antiestrogen, compound ICI 182,780 *(21)*, was generously provided by Zeneca Pharmaceuticals, Chesire, England. A stock solution was prepared by dissolving ICI 182,780 in 100% DMSO at a concentration of 2 m*M*.

Electron Microscopy

GH_3 cells were cultured for 24 h in SFM plus 10 n*M* ICI 182,780, then fixed for 1 h in 2.5% glutaraldehyde in 0.1 *M* cacodylate (CAC) buffer, pH 7.4, rinsed in CAC buffer, and processed for transmission electron microscopy (TEM). Cells were postfixed in 1% osmium tetroxide with 0.8% potassium ferricyanide in 0.1 *M* CAC buffer, rinsed, stained in block with 0.5% aqueous uranyl acetate, dehydrated in EtOH, and embedded in Polybed resin (Polysciences, Warrington, PA). Thin sections were cut with a diamond knife, examined after staining with aqueous uranyl acetate and lead citrate, and observed in a Philips CM-TEM.

Immunofluorescent Confocal Microscopy

GH_3 cells were plated in 35 mm culture dishes and treated with either ICI 182,780 or E_2 for 48 h. The cells were then

washed in PBS, incubated in PBS containing 1 m*M* EGTA for 30 s at room temperature, and then fixed in 10% buffered formalin for 10 min and stored in PBS at 4°C. Cells were stained for N-cadherin by outlining two randomly-selected areas using a hydrophobic slide marker (Pap Pen, Research Products International Corp, Mount Prospect, Il). One area was incubated with anti-N-cadherin antibody (A-CAM; Clone GC-4; Sigma Chemical Co., St. Louis, MO) at a dilution of 1:100 for 2 h at 37°C. The remaining area was incubated with PBS and served as a negative control. All the cells were then washed three times with PBS and incubated for 30 min at room temperature with an anti-mouse IgG conjugated with FITC (Sigma Chemical Co., St. Louis, MO) using a 1:50 dilution. Cells were washed three times with PBS and observed under a Zeiss confocal laser scanning microscope (CLSM 410) using an oil achrostigmat 40X (1.3 n.a.) objective (excitation at 488 nm with an LP 515 filter). The presence of N-cadherin was detected as bright green fluorescence.

Blocking Antibody Studies

Cells were cultured as described above in eight-well LabTek plastic chambered slides (Nunc, Naperville, IL), either in the presence of ICI 182,780 alone, or in combination with 1 μg/mL of affinity purified nonimmune goat IgG or affinity purified polyclonal goat anti-human N-cadherin antibody (both from Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The percentage of single, loosely adherent and and tightly adherent cells were determined from counts of 100 cells in three separate fields for each treatment. A loosely adherent cell was defined as a rounded cell that touched another cell at a point on its membrane. In contrast, tightly adherent cells in mats were rectangular (or polygonal) flattened cells with most or all of their membrane tightly adhered to surrounding cells.

RNA Isolation

Cytoplasmic RNA was isolated using Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX). Medium was decanted and cells immediately lysed by addition of 500 μL of Ultraspec reagent per 60 mm tissue culture dish. Cells were scraped, transferred to a microcentrifuge tube and RNA isolated according to manufacturer's instructions. Poly (A)+ RNA was isolated using the PolyATract mRNA Isolation System IV (Promega, Madison, WI) following the kit directions. mRNA was then precipitated with 0.1 volume of 3 *M* sodium acetate and 1.0 volume of isopropanol at –20°C overnight. Precipitated mRNA was then centrifuged for 30 min at 4°C at 12,000*g*.

DNA Probes

The PRL and GAPDH cDNA probes have been previously described *(22)*. The rat N-cadherin probe was generated by reverse transcription-polymerase chain reaction (RT-PCR) cloning. Total GH_3 cytoplasmic RNA (10 μg) was primed with oligo d(T) and reverse transcribed (RT) with MMLV reverse transcriptase (Life Technologies, Gaithersburg, MD) in a 50 mL reaction. One-tenth of the RT-reaction was used as the template for PCR. Oligonucleotide primers (National Biosciences, Inc., Plymouth, MN or Life Technologies, Gaithersburg, MD) used for amplifying the approximately 576 bp N-cadherin fragment correspond to nucleotide positions 1699-1716 and 2257-2274 of mouse N-cadherin sequence (accession number M31131). N-cadherin primer sequences were: sense, 5' GGA TGT TTG TCC TTA CTG 3'; antisense, 5' TAG TCA CTG GAG ATA AGG 3'. PCR was performed by an initial 5 min denaturation at 95°, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min and denaturation at 95° or 1 min. The N-cadherin cDNA fragment was cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequencing showed 98% identity at the predicted amino acid level with mouse N-cadherin. A 323 bp ß-catenin cDNA probe was generated by RT-PCR, using the same reaction parameters as described for N-cadherin. The cDNA used as a template was made using poly(A)-selected RNA and oligo d(T). The ß-catenin primers corresponded to positions 2120-2339 and 2424-2443 of the mouse ß-catenin mRNA sequence (GenBank accession number M90364). The ß-catenin primer sequences were: sense, 5' TCA GTC GAG CTG ACC AGT TC 3'; antisense, 5' TTA CAG GTC AGT ATC AAA CCA GG 3'. The PCR generated a single band of the correct size. Two diagnostic restriction digests with EcoR V or Alw I generated fragments of correct sizes (data not shown). Aliquots (2–5 μL) of subsequent PCR amplifications of the gel purified ß-catenin fragment, as well as the cloned cDNAs described above, were used for ^{32}P-labeling using Rad Prime reagents (Life Technologies, Gaithersburg, MD).

Northern Blot Hybridization

RNA was assayed by Northern blot hybridization essentially as described *(23)*. RNA samples were denatured and resolved in an agarose-formaldehyde gel using standard procedures *(23)*. Gels were stained with ethidium bromide, and the nitrocellulose filter was examined in order to confirm equal loading and transfer of RNA. The filter was then processed for hybridization using standard protocols *(23)*. Filters were exposed to BioMax MS film (Kodak, Rochester, NY) using a BioMax screen at –70°. The molecular size of specific bands were estimated using the 0.24–9.5 kb RNA ladder (Life Technologies, Gaithersburg, MD). The sizes of the major N-cadherin transcript and the ß-catenin transcript were approx 4.2 and 3.7 kb, respectively.

Immunoblots

Cells were lysed in 2.5% Triton-X-100, 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EGTA, 1 m*M* $MgCl_2$ and 100 μl/mL of protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein, as assayed by BCA Protein Assay (Pierce, Rockford, IL), were resolved by 10% polyacrylamide gel electrophoresis, and transferred to nitrocel-

lulose. Blots were stained with Ponceau S to confirm an even transfer, and blocked with 5% milk in PBS, 0.05% Tween 20 (PBS-T) for 1 h at room temperature. Blots were washed briefly, cut horizontally at postions corresponding to molecular weights of approx 65 and120 kD, and the individual strips incubated with either an antipan-cadherin antibody generated against a synthetic peptide representing the C-terminal domain of chick N-cadherin (Sigma Cemical Co, St. Louis, MO), anti-ß-catenin (Transduction Laboratories, Lexington, KY) or anti-Lck (Santa Cruz Biothechnology Inc, Santa Cruz, CA) diluted in 1% BSA in PBS-T for 1 h at room temperature. Following washes with PBS-T, blots were incubated with HRP-labeled secondary antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA or Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in 1% BSA in PBS-T. Blots were then subject to washes, and bands were detected using the Renaissance chemiluminescent kit (DuPont NEN, Boston, MA). Molecular weights were estimated using Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA). The anti-cadherin antibody detected a single band at approximately 135 kD, the anti-ß-catenin antibody detected a single band at about 92 kD, and the anti-Lck antibody detected a band at 56 kD.

Data Analysis

Images of gels and autoradiograms were captured using the Alpha Imager 2000 system (Alpha Innotech, San Leandro, CA), and bands were quantified using IPLab Gel (Vienna, VA). Figures were prepared using Adobe Photoshop 4.0 software (Adobe Systems, San Jose, CA). N-cadherin and ß-catenin protein levels were analysed statistically by *t*-test. Cell counts from the blocking antibody and cell growth studies were analyzed statistically by one-way analysis of variance followed by a Student-Newman-Keuls test.

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