Research Communication

Human Chorionic Gonadotropin Decreases Human Breast Cancer Cell Proliferation and Promotes Differentiation

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Abstract

Human chorionic gonadotropin (hCG) is a glycoprotein produced by placental trophoblasts. Previous studies indicated that hCG could be responsible for the pregnancy-induced protection against breast cancer in women. It is reported that hCG decreases proliferation and invasion of breast cancer MCF-7 cells. Our research also demonstrates that hCG can reduce the proliferation of MCF-7 cells by downregulating the expression of proliferation markers, proliferating cell nuclear antigen (PCNA), and proliferation-related Ki-67 antigen (Ki-67). Interestingly, we find here that hCG elevates the state of cellular differentiation, as characterized by the upregulation of differentiation markers, β-casein, cytokeratin-18 (CK-18), and E-cadherin. Inhibition of hCG secretion or luteinizing hormone/hCG receptors (LH/hCGRs) synthesis can weaken the effect of hCG on the induction of cell differentiation. Furthermore, hCG can suppress the expression of estrogen receptor alpha. hCG activated receptor-mediated cyclic adenosine monophosphate/protein kinase A signaling pathway. These findings indicated that a protective effect of hCG against breast cancer may be associated with its growth inhibitory and differentiation induction function in breast cancer cells. © 2014 IUBMB Life, 66(5):352–360, 2014

Keywords: *human chorionic gonadotropin; breast cancer; cell differentiation; MCF-7 cells*

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Introduction

Human chorionic gonadotropin (hCG), which is produced by placental trophoblasts, is indispensable for human pregnancy because it controls implantation, decidualization, and placental development (1). The epidemiological data suggest that breast cancer risk decreases in women who complete full-term pregnancy at a young age and that hCG has a protective effect against carcinogen-induced mammary tumor formation in rodents (2). hCG inhibits carcinogen-induced rat mammary carcinogenesis by inducing programmed cell death in the mammary gland initiated in the carcinogenic process (3).

Previous studies indicated that hCG could inhibit the proliferation of breast cancer cells (4). hCG prevents the initiation and inhibits the progression of chemically induced mammary carcinomas by inducing differentiation of the mammary gland,

inhibiting cell proliferation, and increasing apoptosis (3). Alvarado et al. (5) have shown that very high hCG concentrations can inhibit proliferation, presumably via inducing the expression of inhibin in human breast epithelial cells. The treatment of MCF-7 cells with highly purified hCG resulted in a modest dose-dependent decrease in cell proliferation (6). hCG has been reported to have antiproliferative and anti-invasive effects in MCF-7 cells by inhibiting NF-κB and AP-1 activation (4).

High expression of the estrogen receptor alpha (ERα) is associated with a poor prognosis, which correlates closely with cellular proliferation in breast cancer. Up to now, it has been well reported that ERα has great potential to promote breast cancer cell motility and invasion (7–9). ERα plays a critical role in mammary gland biology as well as in breast cancer progression (9,10). It can also mediate estrogen-induced cell proliferation in ERα-positive breast cancer cell lines (7–10). This finding prompts us to study whether hCG achieves the effect of the inhibition of cell proliferation by inhibiting the expression of ERα.

Cell proliferation and differentiation are two mutually exclusive processes. Inhibited proliferation of primary tumors with therapeutic compounds can induce differentiation. Previous reports showed that hCG can influence the differentiation of mammary tissue (2,3,6); however, it is not clear whether hCG can promote differentiation of breast cancer MCF-7 cells. Moreover, how hCG regulates the balance of proliferation and differentiation is not well understood.

Kouros-Mehr et al. (11) confirmed that transcription factor GATA-3 regulates luminal breast cancer differentiation by upregulation of milk proteins (β-casein), basement membrane components (perlecan), and epithelial-specific cytokeratin-18 (CK-18). The MCF-7 cell line, which has a luminal epithelial-like phenotype, thus expresses CK-18 and β-casein (12,13). Indeed, downregulation of CK-18 has recently been shown to be associated with the progression of human breast cancer (14). It has been reported that increase of E-cadherin, CK-18, GATA-3, and desmoplakin 1 expression and induction of casein kinase were recognized as markers of MCF-7 cell differentiation (15). Thus, in this study, we have tested whether hCG could affect CK-18, perlecan, and E-cadherin expression and β-casein production.

It has been reported that some human breast cell lines, including MCF-7, MDA-MB-231, and HBL-100 cells, contain functional luteinizing hormone/hCG receptors (LH/hCGRs) (16). The MCF-7 cells contain higher hCG/LH receptor levels than MDA-MB 231 and normal human breast epithelial HMEC cells (17). Previously reports show that activation of LH/hCGRs results in tissue-specific responses that can be collectively described as anticancer regulator (18,19). The growth inhibition action of hCG was abrogated in MCF-7 cells when receptor synthesis was inhibited by treatment with antisense LH/hCGRs phosphorothioate oligodeoxynucleotide (4). Here, we also investigated the role of LH/hCGRs in hCG-mediated MCF-7 cell differentiation.

In this study, we demonstrate that hCG inhibits the proliferation of MCF-7 cells and elevates cellular differentiated state via hCG receptors/cyclic adenosine monophosphate (cAMP) signal pathway. These data thus provide important and novel insights into the function of hCG in regulating breast cancer proliferation and differentiation signaling.

Materials and Methods

Cell Lines

Human breast epithelial cell line HBL-100, human breast cancer cell lines MCF-7 and MDA-MB-231, human choriocarcinoma JAR cells (hCG-positive), and human umbilical vein endothelial cells (HUVEC, hCG-negative) were obtained from American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator.

Cell Culture With Phosphorothioate Antisense and Sense LH/hCG Receptors and hCG-β oligodeoxynucleotides

MCF-7 cells were cultured for 24 h and then replaced the medium containing 6 μM antisense or sense LH/hCGR and hCG-β oligodeoxynucleotides (ODNs). The sequences are as follows: LH/hCGR (antisense oligodeoxynucleotide [A-ODN]: 5′-GCCGAGAACCGCTGCTTCATG-3′; and sense oligodeoxynucleotide [S-ODN]: 5′-CATGAAGCAGCGGTTCTCGGC-3′) designed from LH/hCGR cDNA sequence covering ATG translation initiation codon and synthesized in life technologies; and hCG-β (A-ODN: 5′-GAACATCTCCATCCTTGGTGC-3′; and S-ODN: 5′-GCA CCAAGGATGGAGATGTTC-3′) designed from hCG-β cDNA sequence covering ATG translation initiation codon and synthesized in life technologies.

Examination of Cell Proliferation With MTT Assay

The effect of hCG on the cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma, USA). The cells were treated with hCG (1IU, 2IU, 5IU, 10IU, and 15IU) at 4, 5, 6, and 7 days. Then the absorbance of each well was measured using a Synergy™ 4 (Bioteck, USA) with a wavelength of 570 nm, with the reference wavelength set at 630 nm. Absorbance was directly proportional to the number of survival cells.

Semiquantitative Reversed Transcript PCR

Reversed transcript PCR (RT-PCR) analysis was carried out as described previously (20). RT-PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV transillumination. Data were shown as relative expression level after being normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primer sequences are as follows: GAPDH: Forward-ATTCAACGGCACAGTCAAGG, Reverse-GCAGAAGGGGCGGAGATGA; CK-18: Forward-CCGCT ACGCCCTACAGA, Reverse-CCACTTTGCCATCCACTA; perlecan: Forward-ATGCCTCCCGCAGATTC, Reverse-TGCCACGCTCGTCA

CAG; E-cadherin: Forward-ACCACCTCCACAGCCACCGT, Reverse-GTCCAGTTGGCACTCGCCCC; β-casein: Forward-GCA AGGGAGACCATAGAA, Reverse-CTGTGGCTGGAAAGAGGG; cell nuclear antigen (PCNA): Forward-CTGTAGCGGCGTTGTTGC, Reverse-TCGTTGATGAGGTCCTTG; proliferation-related Ki-67 antigen (Ki-67): Forward-CAACTATCCTCGTCTGTCC, Reverse-GGTCCCTAAAGATGTGCT; alpha-subunits hCG (CGA): Forward-CCTCCCAAAGTGCTA, Reverse-CCATTACTGTGACCCT; beta-subunits hCG (CGB): Forward-GCTGCTGCTGTTGCTG, Reverse-TGGTGTTGACGGTGATG; LH/hCGR: Forward-CATAA CCACCATACCAGG, Reverse-AGTCAGTGTCGTCCCATT; and ERα: Forward-TTATGGAGTCTGGTCCTGTG, Reverse-TGTTCTT CTTAGAGCGTTTGA.

Western Blotting

Western blotting was performed as described previously (21). The total proteins of treated or transfected cells were immunoblotted with rabbit anti-β-casein (1:500 dilution; Santa Cruz, USA), CK-18 (1:1,000 dilution; Abcam, UK), E-cadherin (1:500 dilution; Santa Cruz, USA), ERα (1:1,000 dilution; Abcam, UK), hCG (1:1,000 dilution; Abcam, UK), goat-anti LH/hCGR (1:500 dilution; Santa Cruz, USA), mouse anti-Ki-67 (1:1,000 dilution; Abcam, UK), and mouse anti-PCNA (1:1,000 dilution; Abcam, UK) antibodies overnight at 4°C and then incubated with IR Dye™-800-conjugated anti-rabbit secondary antibodies and IR Dye™-680-conjugated anti-mouse or anti-goat secondary antibodies (Li-COR, USA) for 90 min at room temperature. The specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Company, USA). β-Actin expression was used as an internal control to show equal loading of the protein samples.

Immunofluorescence Staining

Immunocytochemistry assays were performed as described previously (22). The cells after treatment were fixed in 4% paraformaldehyde for 15 min and then blocked with normal goat serum for 20 min at room temperature. Then, rabbit antibodies of β-casein (1:100 dilution; Santa Cruz, USA), CK-18 (1:200 dilution; Abcam, UK), and E-cadherin (1:100 dilution; Santa Cruz, USA) were added and incubated in a humid chamber overnight. After washing with PBS thrice, cells were incubated with appropriate secondary antibodies (fluorescein isothiocyanate goat anti-rabbit IgG; Santa Cruz, USA) for 30 min at 37°C. After washing with PBS, the samples were observed under laser scanning confocal microscope (Olympus, Japan). DAPI stain (blue) highlights the total nuclei.

Luciferase Reporter Assays

Luciferase reporter assays were performed as described previously (23). After 24-h transfection, luciferase activity was measured by using a luciferase reporter assay system (Promega, USA) on a Synergy™ 4 (Bioteck, USA). Transfection efficiencies were normalized by total protein concentrations of each luciferase assay preparations. All experiments were performed at least three times with different preparations of plasmids and primary cells, producing qualitatively similar results. Data in each experiment are presented as the mean ± standard deviation of triplicates from a representative experiment.

Measurement of cAMP Levels

The cAMP levels in cell culture medium were quantified following the instructions provided in the cAMP ELISA Kit (Sigma, USA). After knocking down hCG autocrine or LH/hCGR expression, MCF-7 cells were treated with 10IU hCG for 2 h. Then, the ELISA assay was performed to test the cAMP level. The absorbance of each well was measured using a Synergy™ 4 (Bioteck, USA) with a wavelength of 450 nm. Absorbance was directly proportional to the content of cAMP in cell culture medium. The accurate concentration of cAMP is calculated by using standard curve.

Statistical Analysis

Data were expressed as mean ± SE and accompanied by the number of experiments performed independently and analyzed by *t*-test. Differences at $P < 0.05$ were considered statistically significant.

Results

Effect of hCG on Cell Growth Is Associated With LH/hCGR Expression Level in MCF-7, MDA-MB-231, and HBL-100 Cells

We first investigated whether hCG can inhibit the growth in three breast cell lines including MCF-7 (ERα-positive breast cancer cell line), MDA-MB-231 (ERα-negative breast cancer cell line), and HBL-100 (ERα-negative breast epithelial cell line) cells. Our results showed that hCG inhibited cell growth of MCF-7 and HBL-100 cells, which sensitively responded to hCG. MCF-7 cells showed first inhibition of growth at 4 days of culture. However, MDA-MB-231 cells did not show any change in growth at any time during culture with 10 IU hCG when compared with the controls (Fig. 1A).

Then, we used Western blotting to determine whether MCF-7, MDA-MB-231, or HBL-100 cells expressed LH/hCGRs. As shown in Fig. 1B, the protein levels were higher in MCF-7 cells than other two cell lines. Thus, our data indicate that the differences of LH/hCGRs expression may be positively correlated with hCG-induced growth inhibition in MCF-7, MDA-MB-231, and HBL-100 cells.

Expression of Ectopic hCG in Breast Cancer

As breast cancer cells contain different levels of LH/hCGRs, the function mechanism of hormone–hormone receptors led us to consider whether breast cancer cells have the hormone autocrine way to control cell differentiation and proliferation. At present, ectopic hCG (ehCG) has been observed in many malignant tumors and cancer cell lines, which is related to the initiation, progression, malignization, and metastasis of tumors (24,25).

To further clarify whether ehCG expresses in breast cancer cells, we detected the expression of ehCG by RT-PCR and Western blotting in breast cancer cell lines MCF-7 and MDA-

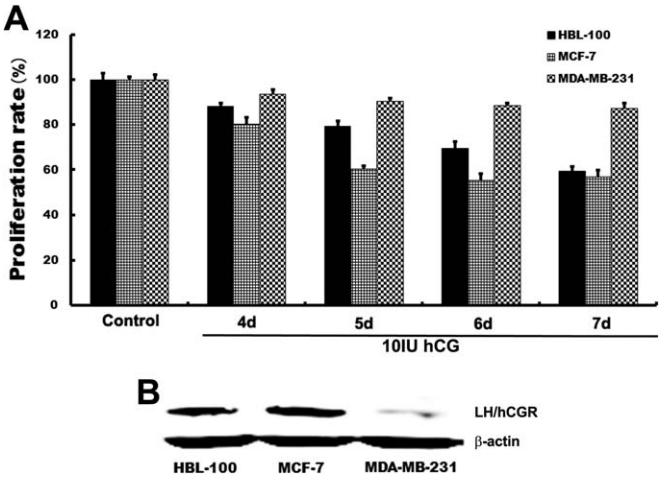

FIG 1 *Effect of hCG on cell growth is associated with LH/hCGR expression level in HBL-100, MCF-7, and MDA-MB-231 cells. A: HBL-100, MCF-7, and MDA-MB-231 cells were treated with hCG (10IU) for 4, 5, 6, and 7 days, and then MTT assay was performed to test proliferation rate. B: The expression of LH/hCGR was tested in several cell lines HBL-100, MCF-7, and MDA-MB-231 by Western blot.*

MB-231. JAR and HUVEC were used to act as hCG-positive and hCG-negative control, respectively. Interestingly, our results show that the expression of ehCG can be detected in both MCF-7 and MDA-MB-231 cells. MCF-7 cell line displayed higher expression level of hCG than MDA-MB-231 cell line (Figs. 2A and 2B). These data show that ehCG plays a major role in controlling MCF-7 cell differentiation and proliferation.

hCG Inhibited the Proliferation of MCF-7 Cells

It is obvious that hCG can affect the growth of MCF-7 cells, not MDA-MB-231 cells. Moreover, MCF-7 cells contain much higher level of LH/hCGRs and endogenous ehCG than MDA-MB-231 cells. Together with previous reports (3–6,26,27),

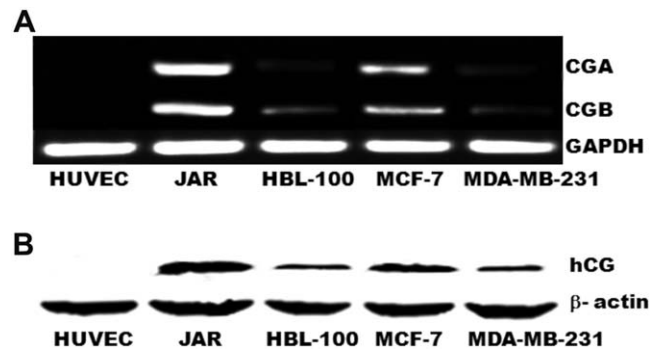

FIG 2 *The expression of ectopic hCG in breast cancer cells. A and B: RT-PCR and Western blot were performed to test the expression of ectopic hCG in several cell lines including HUVEC, JAR, HBL-100, MCF-7, and MDA-MB-231 cells. HUVECs were used as hCG-negative control, and JAR cells were used as hCG-positive control.*

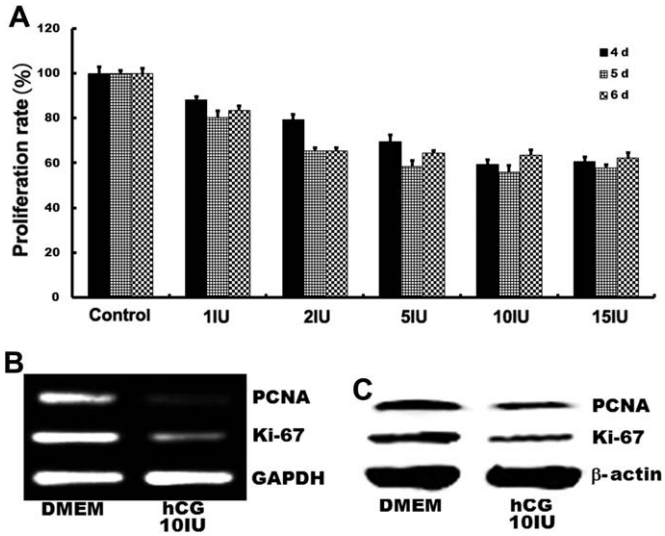

FIG 3 *hCG inhibited the proliferation of MCF-7 cells. A: MCF-7 cells were treated with hCG (1IU, 2IU, 5IU, 10IU, and 15IU) for 4, 5, and 6 days, and then MTT assay was performed to test proliferation rate. B: After MCF-7 cells were treated with 10IU hCG for 5 days, total RNAs were isolated, and then the expression of PCNA and Ki-67 was examined by RT-PCR. C: After MCF-7 cells were treated with 10IU hCG for 5 days, the protein expression of PCNA and Ki-67 was detected by Western blot analysis.*

these results lead us to think whether hCG play an important role in regulating MCF-7 cell proliferation and differentiation.

First, to identify the exact role of hCG in regulating MCF-7 cell proliferation, MCF-7 cells were treated with 1IU, 2IU, 5IU, 10IU, and 15IU of hCG for 4, 5, and 6 days, and then MTT assay was performed. As shown in Fig. 3A, the viability of MCF-7 cells exposed to hCG was significantly decreased in a dose-dependent manner at 5 days. Then, the mRNA and protein level of proliferation markers PCNA and Ki-67 were examined by RT-PCR and Western blotting. The levels of PCNA and Ki-67 were substantially suppressed in hCG-treated MCF-7 cells (Figs. 3B and 3C). These results indicate that hCG inhibits the proliferation of MCF-7 cells.

Effect of hCG on the Expression Level of β-Casein, CK-18, Perlecan, and E-Cadherin

To demonstrate the effect of hCG in regulating the differentiation of MCF-7 cells, next we investigated the expression of CK-18, E-cadherin, and β-casein, three known differentiation markers of epithelial cells. The upregulation of CK-18, E-cadherin, and β-casein expression was observed in MCF-7 cells treated with hCG by immunofluorescence staining (Figs. 4A–4C). Furthermore, the expression of differentiation markers CK-18, E-cadherin, β-casein, and perlecan was tested in hCG-treated or untreated cells by RT-PCR, and the expression of differentiation markers CK-18, E-cadherin, and β-casein was tested by Western blotting. As shown in Figs. 4D and 4E, the expression of CK-18, E-cadherin, β-casein, and perlecan was

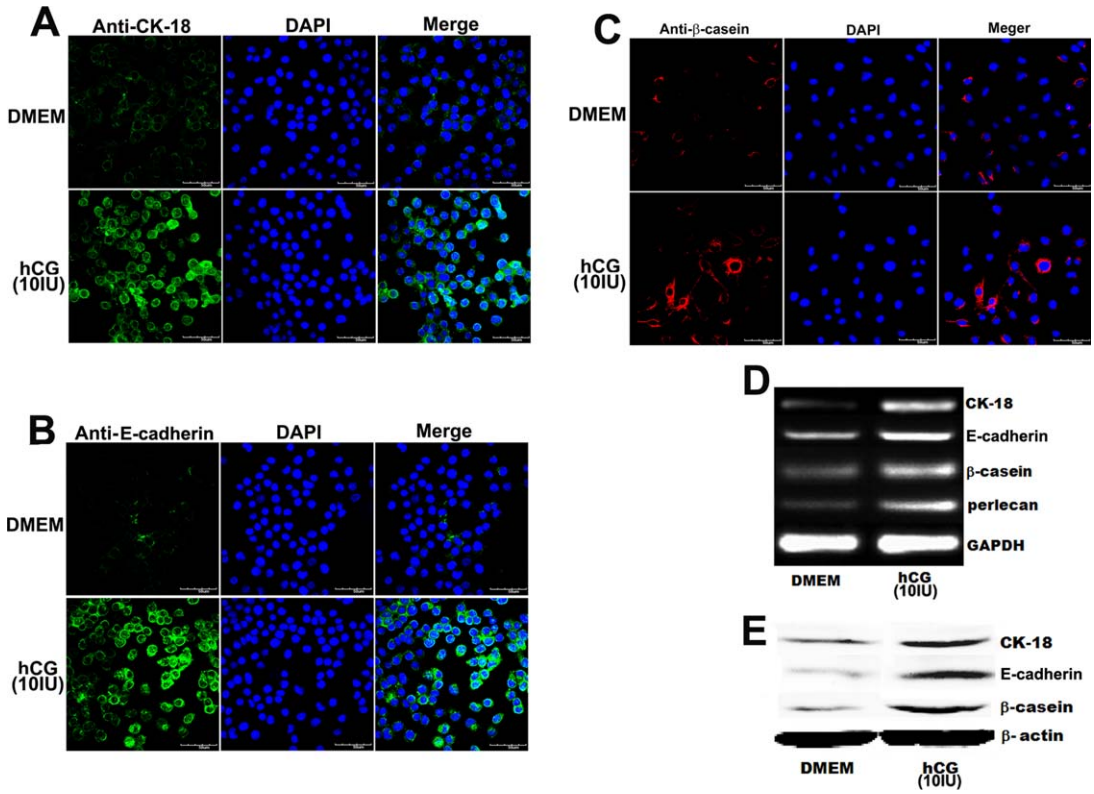

FIG 4

hCG promoted the differentiation of MCF-7 cells. A–C: Representative images of immunostained MCF-7 cells treated with 10IU hCG. Cells treated with DMEM are served as control. The left panels (red or green) show anti-E-cadherin, CK-18, and β-casein antibody reactivity to demonstrate gross morphology. The middle panels (blue) show the DAPI staining for nuclei. The right panels show double immunostaining for E-cadherin, CK-18 or β-casein, and nuclei. Scale = 50 μm. D: After MCF-7 cells were treated with 10IU hCG for 5 days, total RNAs were isolated, and then the expression of E-cadherin, CK-18, β-casein, and perlecan was examined by RT-PCR. E: After MCF-7 cells were treated with 10IU hCG for 5 days, the protein expression of CK-18, E-cadherin, and β-casein was detected by Western blot analysis. Cells treated with DMEM are served as control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

distinctly increased in hCG-treated cells. These observations suggest that hCG elevates the state of MCF-7 cell differentiation.

hCG-Induced MCF-7 Cell Differentiation Partially Depends on LH/hCG Receptor

hCG binds to LH/hCGRs and activates different signal transduction pathways. The MCF-7 cells contain higher level of LH/hCGRs than MDA-MB 231 and normal human breast epithelial HMEC cells (16). To further confirm the roles of hCG and LH/hCGRs in the differentiation of MCF-7 cells, we used LH/hCGR ODN to block endogenous LH/hCGRs synthesis and used antisense hCG-β to inhibit hCG secretion in MCF-7 cells and then tested the changes of differentiation marker expression.

First, the expression of LH/hCGRs was downregulated in MCF-7 cells by treating with phosphorothioate ODNs synthesized from LH/hCGR sequence (Figs. 5A and 5B). As expected, the treatment of MCF-7 cells with antisense but not sense LH/hCGR ODNs and then 10IU hCG for 5 days resulted in a decrease in the mRNA and protein level of differentiation markers when compared with the control cells (Figs. 5C and 5D). Our results showed that inhibition of LH/hCGRs function by treatment with antisense LH/hCGR ODNs resulted in a suppression of differentiation marker expression.

Next, to test the effect of ehCG on the differentiation of MCF-7 cells, we blocked the autocrine way of hCG in MCF-7 cells by treating with phosphorothioate ODNs synthesized from hCG-β sequence and then detected the differentiated state of MCF-7 cells. As expected, the treatment of MCF-7 cells with antisense hCG-β ODNs resulted in a decrease of hCG mRNA and protein level (Figs. 5E and 5F). After inhibiting hCG secretion with antisense hCG-β ODNs, the expression of differentiation markers were obviously suppressed (Figs. 5G and 5H). Taken together, our data demonstrate that blocking the LH/hCGRs synthesis or hCG secretion can decrease cellular differentiated state.

hCG Can Suppress the Expression of ERα

As hCG is an important regulator during the differentiation of MCF-7 cells and ERα is a major ER subtype in the mammary epithelium and plays a critical role in mammary gland biology as well as in breast cancer progression, we wanted to determine whether hCG could affect steady ERα mRNA levels. We first tested the expression of ERα after treating a series of

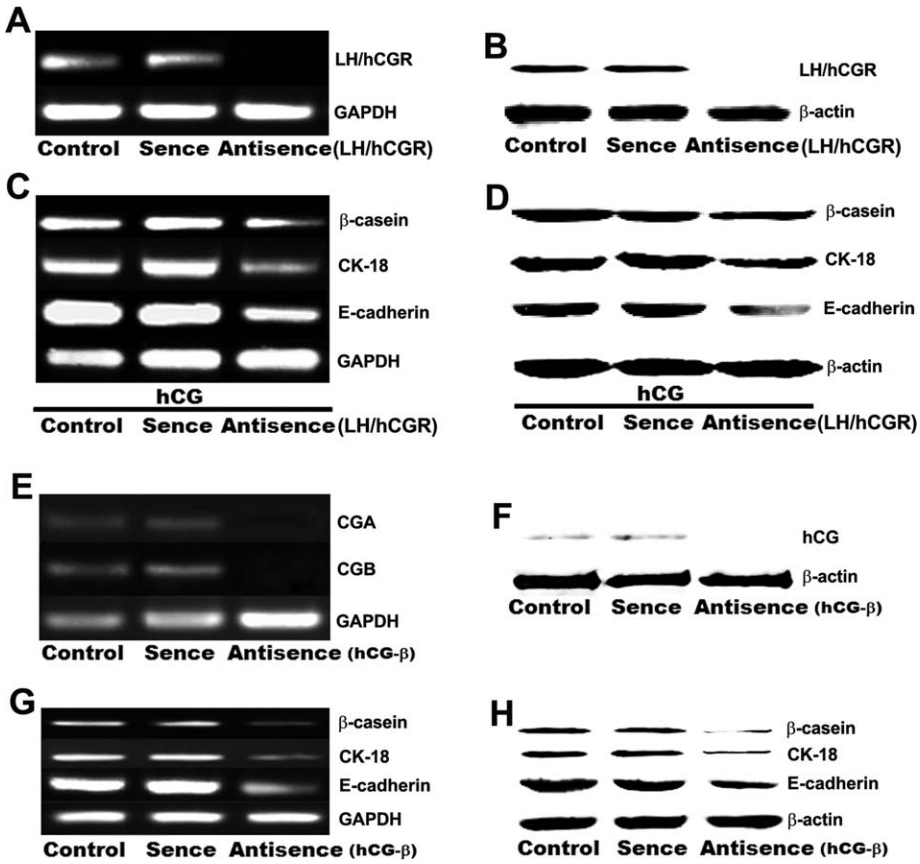

FIG 5

Knockdown of LH/hCG receptors affect hCG-induced MCF-7 cell differentiation. A and B: MCF-7 cells were treated with 6 μM antisense and sense LH/hCGR ODNs for 24 h. Then, the mRNA and protein expression of LH/hCGRs was examined by RT-PCR and Western blotting. C and D: MCF-7 cells were treated with 6 μM antisense and sense LH/hCGR ODNs for 24 h and then treated with 10IU hCG for 5 days. The expression of β-casein, CK-18, and E-cadherin was examined by RT-PCR and Western blotting. E and F: MCF-7 cells were treated with 6 μM antisense and sense hCG-β-ODNs for 24 h, and then the mRNA level of CGA and CGB was examined by RT-PCR, and the protein level of hCG was examined by Western blotting. G and H: MCF-7 cells were treated with 6 μM hCG-β sense and antisense ODNs for 24 h. Then, the expression of β-casein and CK-18 was examined by RT-PCR and Western blotting.

concentration hCG by real-time PCR and RT-PCR. The results showed that culturing MCF-7 cells with highly purified hCG resulted in a significant decrease in ERα mRNA levels at 5 days when compared with the corresponding control (Figs. 6A and 6B).

To determine whether hCG treatment concomitantly decreased ERα protein levels, we cultured MCF-7 cells in the presence or absence of hCG and then performed Western blotting. As shown in Fig. 6C, MCF-7 cells contained ERα protein that significantly decreased after culture for 5 days with different doses of hCG when compared with the corresponding control. Our results show that the treatment of MCF-7 cells with highly purified hCG resulted in a modest dose-dependent decrease in the expression of endogenous ERα. As estradiol stimulates the growth of MCF-7 cells and hCG treatment decreases ERα levels, it may seem that hCG inhibits cell growth by partly decreasing the ERα levels.

hCG Activates the cAMP Signal by LH/hCGRs

The cAMP signal is a known second messenger that can be activated by hCG/hCG receptors (4,26,27). Here, we investigate whether hCG increases cAMP synthesis in MCF-7 cells. As shown in Figs. 7A, inhibition of hCG secretion by hCG-β antisense ODNs inhibited the level of cAMP when compared with the control. The sense ODN-treated cells had similar cAMP level as non-ODN-treated cells. To further determine whether hCG activated the cAMP signal by LH/hCGRs, MCF-7 cells were treated with antisense LH/hCGR ODNs or sense ODNs for 24 h and incubated with 10IU hCG, and then ELISA assay was performed to test cAMP level. Our data demonstrate that hCG treatment can increase the cAMP level (Figs. 7B). Expectedly, the cells treated with LH/hCGR antisense ODNs but not sense ODNs block the hCG-induced increase of cAMP level (Figs. 7B). These results show that hCG may promote breast cancer MCF-7 cell differentiation by combining the LH/hCGRs to activate the cAMP signal.

Discussion

The accumulated data demonstrate that hCG may have a direct role in breast cancer protection and that hCG treatment

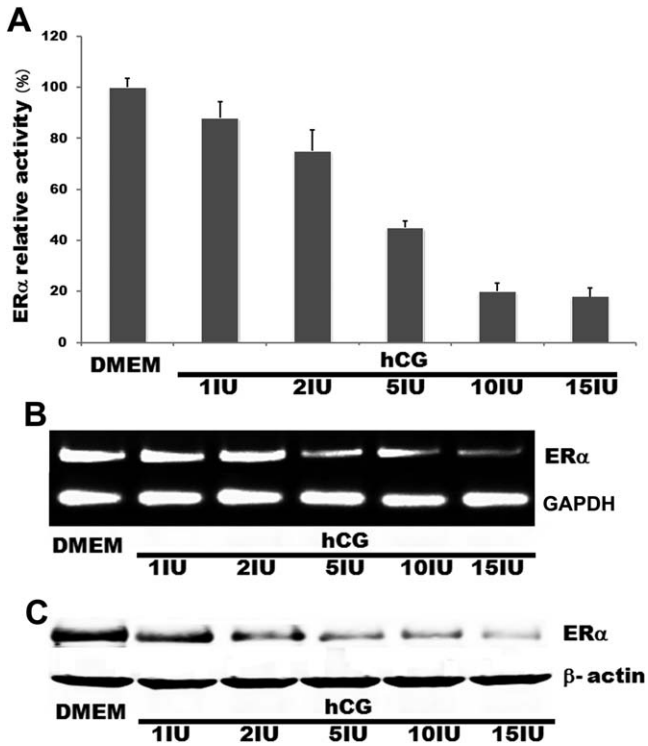

FIG 6 *hCG affected the expression of ERα. A and B: MCF-7 cells were treated with 1IU, 2IU, 5IU, 10IU, and 15IU of hCG for 5 days, and then total RNAs were isolated, and the expression of ERα was examined by qRT-PCR and RT-PCR. C: After treatment with 1IU, 2IU, 5IU, 10IU, and 15IU hCG in MCF-7 cells for 5 days, the protein expression of ERα was detected by Western blot analysis. Cells treated with DMEM are served as control.*

can inhibit breast cell growth and proliferation (5). Consistent with the possibility that the protective effect of hCG is due to induce cell differentiation, hCG induced permanent genomic changes, shifted the stem cell 1 to a stem cell 2, and caused mammary gland differentiation (28–31). hCG induced full differentiation of the mammary gland, which was morphologically manifested as a reduction in the number of terminal end buds (TEBs) (30).

Mammary gland and breast cancer cell differentiation is associated with specific measurable features, such as enhanced cytokeratins and milk proteins, defined as cell differentiation change (12–15). Cytokeratins, characteristic intermediate filament of epithelial cells, especially CK-18, have been shown to act as a luminal epithelial marker (14). A luminal cytokeratin, CK-18, is an epithelial-specific cytokeratin (13). Indeed, downregulation of CK-18 has recently been shown to be associated with the progression of human breast cancer (14). Both of these cytokeratins are of the luminal type, and their expressions in the breast luminal duct may determine the grade and invasiveness of the cancer (12). The caseins are encoded by a family of milk protein genes (32). Mammary epithelial cells comprise the myoepithelial and milk-secreting cells of the mammary gland. Our results show that hCG induces the expression of a broad range of luminal differentiation markers, including milk proteins such as caseins (β-casein) and perlecan (33). Indeed, hCG seems to be a fine candidate in the chemoprevention of breast cancer and may represent a promising therapeutic strategy.

In this study, we found that hCG can inhibit growth of MCF-7 cells and promote cell differentiation by enhancing the expression of differentiation markers β-casein, perlecan, CK-18, and E-cadherin and by suppressing the expression of proliferation markers PCNA and Ki-67. Based on our findings, we propose that hCG not only induces mammary stem cell differentiation but also mammary gland tumor cell differentiation. These findings support the premise that hCG could be responsible for the pregnancy-induced protection against breast cancer.

LH/hCGRs are reported to express in breast tissue and cells from a number of species (16,26,27). Human breast cell

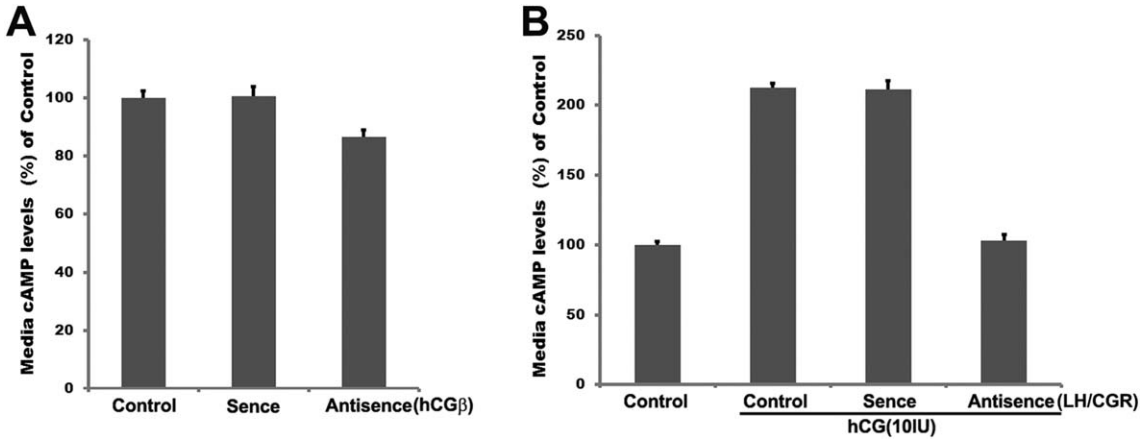

FIG 7 *hCG activates the cAMP/PKA signal pathway depending on LH/hCGRs. A: MCF-7 cells were incubated for 24 h in the presence of 6 μM antisense or sense hCG-β ODNs. Then, the medium cAMP levels were measured. B: MCF-7 cells were incubated for 24 h at 37°C in the presence of 6 μM antisense or sense LH/hCGR ODNs and then treated with 10IU hCG for another 2 h. Then, the medium cAMP levels were measured.*

lines contain LH/hCGRs that are functionally coupled to the downregulation of ER levels and the growth inhibition of MCF-7 and HBL-100 cells (30). Our results demonstrate that MCF-7 cells contain higher expression of LH/hCGRs than MDA-MB-231 and HBL-100, and thus breast LH/hCGRs may play a role in growth and differentiation of breast cancer cells. The knockout of LH receptors resulted in a gross underdevelopment of mammary glands (28). The recent evidence that women with LH/hCGR-positive tumors had a longer metastasis-free survival supports the premise from the current findings that the hCG/hCGR-induced protection against breast cancer may be involved in preventing tumor cell invasion (16). Rao et al. (4) reported that LH/hCG receptor activation is required for the antiproliferative effects of hCGs in MCF-7 cells. Our results showed that when LH/hCGR synthesis was inhibited, the effects of hCG on MCF-7 cell differentiation were abrogated. All the findings suggest that breast LH/hCGRs might play a role in the growth and differentiation of breast cancer cells.

hCG can stimulate the ovaries to produce estrogen and progesterone (34). Kocdor et al. (31) reported that hCG prevented the transformed phenotypes induced by E2 in human breast epithelial cells. The 17β-estradiol (E2) treatment increased the proliferation of breast cancer cells, and hCG prevented the action of E2 acting as antagonist (31). E2 regulates breast cancer cell proliferation by activating ERα and its downstream genes. It has been reported that hCG can induce the synthesis of inhibin, a tumor suppressor factor that downregulates the level of expression of the ERα by methylation of CpG islands (27). It is well known that E2 regulates the ER-dependent genes to affect breast cancer cell proliferation by activating ER receptors, whereas hCG controls the expression of LH/hCGR-dependent genes to influence the differentiation of the breast cancer cells by activating LH/hCGRs expression.

Our data show that culturing MCF-7 cells with highly purified hCG resulted in a dose-dependent significant decrease of ERα mRNA and protein levels when compared with controls. The cross-talk between these two signaling pathways that regulate the differentiation and proliferation of the breast cancer cells needs to be further studied.

Hormonal stimulation of cAMP and the cAMP-dependent protein kinase A (PKA) regulates cell growth by multiple mechanisms. A hallmark of cAMP is its ability to stimulate cell growth in many cell types while inhibiting cell growth in others (35). cAMP elevation affects growth arrest and differentiation in a wide variety of breast cell lines. It has been reported that cAMP/PKA signal pathway activation can reduce the proliferation of MCF-7 cells and play a crucial role in inhibiting the breast cancer progress (36). After treatment of MCF-7TH cell with 8-Cl-cAMP, a site-selective analog of cAMP, upregulation of CK-18 expression, and an increase in cytoplasmic filaments were observed, suggesting a potential differentiation induced by 8-Cl-cAMP (37). Our data show that hCG treatment can increase the level of cAMP. Expectedly, blocking endogenous LH/hCGRs synthesis with antisense LH/hCGRs ODNs inhibits the activity of cAMP and then affects hCG-dependent differentiation of MCF-7 cells. Moreover, consistent with our results, Rao et al. (4) also reported that hCG treatment increased cAMP levels in MCF-7 cells. These findings suggest that LH/hCGRs are required for hCG to activate the cAMP signal. Our studies indicate a possibility that under some circumstances, hCG may control the breast cancer progression by combining the LH/hCGRs to activate the cAMP/PKA signaling pathway.

In conclusion, hCG can influence the differentiation of mammary gland tumor and is critical for breast cancer cell survival and differentiation. A molecular understanding of hCG-mediated breast cancer proliferation and differentiation could reveal further insights into a differentiation therapy of breast cancer.

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