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SHORT COMMUNICATION

Gap junctional intercellular communication and connexin43 expression in human ovarian surface epithelial cells and ovarian carcinomas *in vivo* and *in vitro*

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Gap junctional intercellular communication (GJIC) and the expression of gap junction proteins (connexins) are frequently decreased in neoplastic cells and have been increased by cAMP and retinoids. GJIC and connexin expression were investigated in early passage normal human ovarian surface epithelial (HOSE) cells, human ovarian adenocarcinoma cell lines (CaOV-3, NIH:OVCAR-3, SK-OV-3 and SW626) and surgical specimens of human serous cystadenocarcinomas. We hypothesized that GJIC and connexin expression would be decreased in neoplastic cells and would be increased by cAMP and retinoic acid. Cultured HOSE cells exhibited extensive fluorescent dye-coupling and connexin43 (Cx43) expression; other connexins were not detected. The ovarian adenocarcinoma cell lines had little dye-coupling or connexin expression. Deletions and rearrangements of the Cx43 gene were not detected by Southern blotting in the carcinoma lines. N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate and all-*trans*-retinoic acid inhibited cell proliferation, but did not enhance GJIC or Cx43 expression. Surface epithelial cells of benign ovaries expressed Cx43, but this expression was barely detectable in ovarian serous cystadenocarcinomas. Thus, normal HOSE cells had extensive GJIC and Cx43 expression whereas ovarian carcinoma cells had less and cAMP and retinoic acid did not change these, although both agents inhibited cell growth.

Several mechanisms regulate cellular growth and involve secreted factors such as growth factors and inhibitors as well as cell contact with the extracellular matrix and other cells (1). Defects in any of these controls may contribute to neoplastic transformation. Gap junctional intercellular communication (GJIC) is one mechanism of growth control that involves cell–cell contact (1,2). Gap junctions enable the direct exchange of small molecules and ions (<1000 Da) between neighboring cells through gap junction channels. Growth regulation via GJIC may occur by the direct exchange of growth modulators such as Ca^{2+} and cAMP between cells that in turn may modulate the expression or function of cell cycle proteins (1,3). Gap junctions are formed from protein subunits known as connexins; there are at least 13 mammalian connexins (4). Gap junction function, size and number, as well as connexin expression, are decreased in most neoplastic cells (1,2,5). The enhancement of GJIC by connexin gene transfection or by treatment with pharmacological agents such as retinoids and cAMP agonists has restored a more normal phenotype in neoplastic cells (3,6–15).

Over 90% of human ovarian adenocarcinomas arise from the ovarian surface epithelium (16). Although GJIC and connexin expression have been characterized in many types of normal cells and their neoplastic counterparts, little data exist for human ovarian surface epithelial (HOSE) cells and ovarian carcinomas. Gap junctions have been identified in hamster, rat and ovine ovarian surface epithelium (17–19). The major gap junction protein expressed by rat ovarian surface epithelial cells was connexin43 (Cx43) (18) whereas connexin26 (Cx26) was the major connexin expressed by ovine ovarian surface epithelium and Cx43 was not detected in these cells (19). Cultured rat ovarian surface epithelial cells exhibited extensive dye-coupling with neighboring cells and neoplastic derivatives of these cells had much less dye-coupling (20). Two human ovarian carcinoma cell lines, A2780 and COLO-316, exhibited different levels of GJIC, but they were not compared with normal HOSE cells nor was connexin expression examined (21). Therefore, we have investigated gap junction function and connexin expression in normal HOSE cells, human ovarian carcinoma cell lines and surgical specimens of human ovarian serous cystadenocarcinomas. We also investigated whether the cell-permeable cAMP analog (N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate, DBcAMP) and all-*trans*-retinoic acid (ATRA) affected the growth of ovarian adenocarcinoma cells and how this was related to GJIC and connexin expression.

Normal HOSE cells were obtained from ovaries that had been surgically removed for benign, non-ovarian disease at the Toledo Hospital or the Medical College Hospital. HOSE cells were isolated and cultured in a 1:1 mixture of Medium 199:MCDB-105 (Sigma Chemical Co., St Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and 50 μg/ml gentamicin sulfate (Sigma) as described (22) and were subcultured by trypsinization. Experiments were performed using cells at passages 2–4. Each HOSE cell preparation was identified numerically. These procedures were approved by the Medical College of Ohio and Toledo Hospital Institutional Review Boards. Cytokeratin immunostaining was performed to verify the epithelial nature of cultured HOSE cells (22). Cells were subcultured on glass coverslips, fixed in cold 5% acetic acid/95% methanol for 15 min, rehydrated in phosphate-buffered saline (PBS) and stained for cytokeratin using rabbit pan-anti-cytokeratin and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies (Sigma). The coverslips were mounted using Prolong™ Antifade reagent (Molecular Probes, Eugene, OR). Cells were viewed and photographed with a

Abbreviations: ATRA, all-*trans*-retinoic acid; Cx26, connexin26; Cx32, connexin32; Cx37, connexin37; Cx40, connexin40; Cx43, connexin43; DBcAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; GJIC, gap junctional intercellular communication; HOSE, human ovarian surface epithelium; PBS, phosphate-buffered saline.

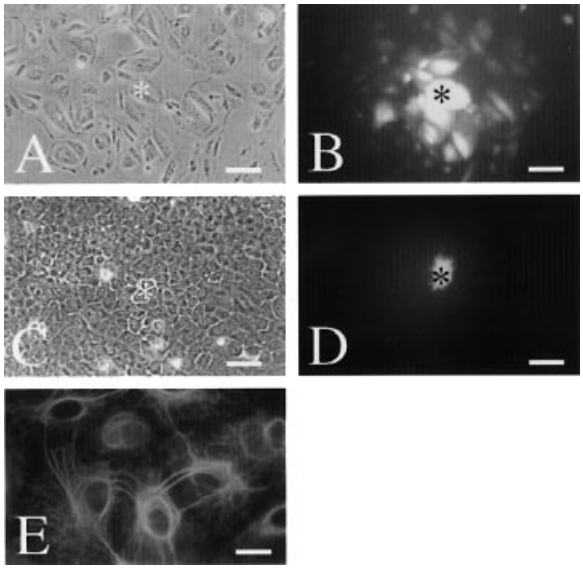

Fig. 1. Morphology and dye-coupling of HOSE 7 (**A** and **B**) and SK-OV-3 cells (**C** and **D**) and of immunofluorescence staining of cytokeratin in HOSE 7 cells (**E**). Bar 50 μm (A–D) or 10 μm (E). The asterisks indicate the microinjected, dye-filled cells.

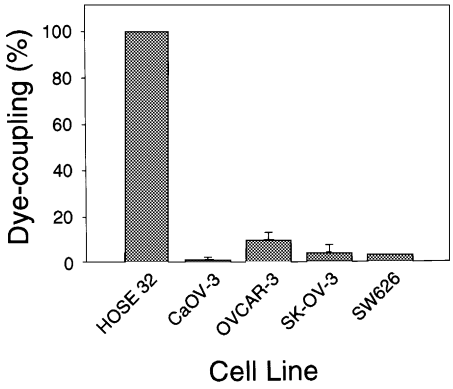

Fig. 2. Dye-coupling percentages in human ovarian surface epithelial cells (HOSE 32) and human ovarian carcinoma cell lines (CaOV-3, NIH:OVCAR-3, SK-OV-3 and SW626). Each bar represents the percentage of first-order neighboring cells that took up dye from microinjected, dye-filled cells (n = 168–203 cells from three to four cultures; means ± SD).

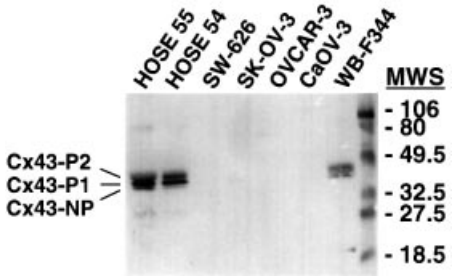

Fig. 3. Western blot analysis of Cx43 protein in human ovarian epithelial cells (HOSE 55 and HOSE 54) and human ovarian carcinoma cell lines (SW-626, SK-OV-3, NIH:OVCAR-3 and CaOV-3). WB-F344 rat liver epithelial cells were included as a positive control. The gel was loaded with 20 μg of protein. Three forms of Cx43 are identifiable: non-phosphorylated Cx43-NP and phosphorylated Cx43-P1 and Cx43-P2.

Nikon Diaphot epifluorescence microscope using the fluorescein filter.

The human ovarian adenocarcinoma cell lines CaOV-3, NIH:OVCAR-3, SK-OV-3 and SW626 (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's minimal essential medium (Sigma) supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin sulfate. The WB-F344 rat liver epithelial cell line (23) was cultured as described (24). C3H10T1/2 murine fibroblasts (American Type Culture Collection) were cultured in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin sulfate.

GJIC was quantified in HOSE cells and ovarian carcinoma cell lines by microinjection of fluorescent Lucifer Yellow CH dye and enumeration of first-order neighboring cells to which dye spread (dye-coupling assay) (24). In the figures, the percentage of dye-coupled cells is shown.

Cultured HOSE cells exhibited cobblestone, epithelial-like morphologies (Figure 1A) and proliferated slowly in culture. Positive immunostaining for cytokeratin was seen in these cells, indicating their epithelial nature (Figure 1E). These cells were passaged when 90–100% confluent and never exceeded five passages before senescence. Of 72 HOSE cell isolations, 18 (25%) gave rise to successful cultures which could be passaged at least twice and which were not contaminated with non-epithelial cells. HOSE cells also exhibited extensive GJIC (dye-coupling) (Figure 1). Essentially all HOSE cells were coupled to neighboring cells (Figure 2). Ovarian carcinoma cell lines, in contrast, exhibited little GJIC (Figures 1 and 2).

Connexin expression was examined in these cells by northern blot, western blot and immunocytochemistry. Northern and western blot analyses of Cx26, connexin32 (Cx32), connexin37 (Cx37) and connexin40 (Cx40) and Cx37 connexin expression in the cultured cells were performed as described (24). Connexin immunostaining of the cells was performed using mouse monoclonal anti-Cx43, anti-Cx32 and anti-Cx26 antibodies (Zymed Immunologicals, South San Francisco, CA) as described (24). HOSE cells expressed a Cx43 transcript ~3.0 kb in size, but no transcripts for Cx26, Cx32, Cx37 or Cx40 (data not shown). The Cx43 transcript was not detected in the ovarian carcinoma cell lines (data not shown). Western analyses indicated that HOSE cell isolates 54 and 55 expressed phosphorylated (Cx43-P1 and Cx43-P2) and non-phosphorylated (Cx43-NP) Cx43 protein, as evidenced by the multiple bands which migrated at ~42–46 kDa (Figure 3; 24,25). In the ovarian carcinoma cell lines, only Cx43-NP was barely detectable (SK-OV-3) or was not detected (OVCAR-3, CaOV-3 and SW-626) (Figure 3). WB-F344 cells were included as a positive control and expressed abundant Cx43. Strong Cx43 staining was also observed in HOSE cells and was evident as spots localized between and over the cells (Figure 4A). The location of this staining was difficult to interpret because distinct cell borders were not obvious. Little Cx43 staining was seen in the ovarian adenocarcinoma cell lines (Figure 4B–E).

Southern blot analyses were performed to evaluate the integrity of the Cx43 gene in the cultured cells. High molecular weight DNA was prepared (26) and 15 μg samples were digested completely with *Eco*RI or *Hin*dIII, separated by electrophoresis in 1% agarose gels and vacuum blotted to charged nylon membranes (Hybond N^+; Amersham Corp., Palo Alto, CA). The membranes were probed using ^{32}P-labeled probes prepared by random primer labeling from a full-length rat Cx43 cDNA, pG2B (27). Hybridization and washing conditions were the same as described for northern blotting (24). The Southern blot analyses revealed that the Cx43 gene was not deleted or rearranged in the carcinoma cell lines. Two major restriction fragments were detected in HOSE and

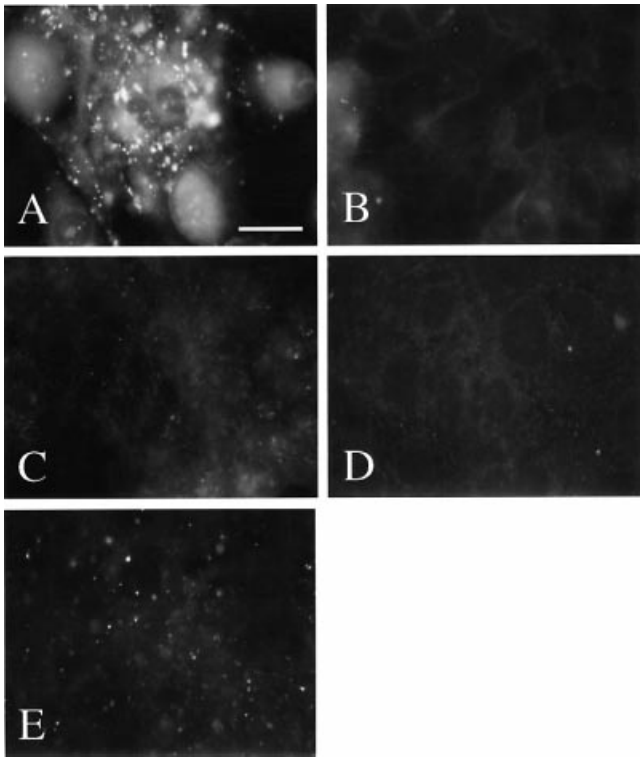

Fig. 4. Fluorescence images of Cx43 immunostaining in human ovarian surface epithelial cells [HOSE 3 (**A**)] and human ovarian carcinoma cell lines [CaOV-3 (**B**); NIH:OVCAR-3 (**C**); SW-626 (**D**); SK-OV-3 (**E**)]. Bar 25 μm.

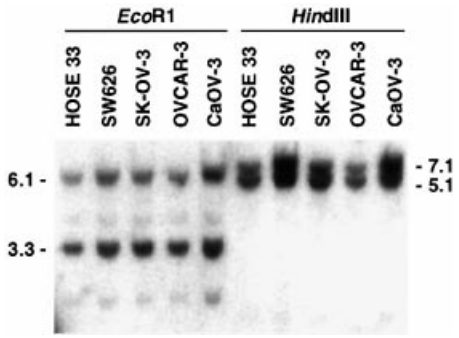

Fig. 5. Southern analysis of the Cx43 gene in human ovarian epithelial cells (HOSE 33) and ovarian carcinoma cell lines (SW-626, SK-OV-3, NIH:OVCAR-3 and CaOV-3). Genomic DNA (20 μg) was digested with *Eco*RI or *Hin*dIII, separated by electrophoresis on an agarose gel, blotted to a nylon membrane and hybridized with radiolabeled full-length Cx43 DNA probe.

carcinoma cell DNA after digestion with *Eco*RI (6.1 and 3.3 kb) or *Hin*dIII (7.1 and 5.1 kb) and hybridization with a probe prepared from a full-length Cx43 cDNA (Figure 5). These fragment sizes are similar to those obtained with other human tissues (28,29).

Human ovarian serous cystadenocarcinomas were also examined for Cx43 expression by immunohistochemistry. Tumor tissues were purchased from the Cooperative Human Tissue Network/Gynecologic Division (Columbus, OH) through the Gynecologic Oncology Group or were obtained from surgical specimens at the Medical College Hospital. Cryostat sections (4 μm) were mounted on aminopropyltriethoxysilane-treated glass slides, fixed in ice-cold acetone, rehydrated in PBS and immunostained for Cx43, Cx32 and Cx26 using rabbit

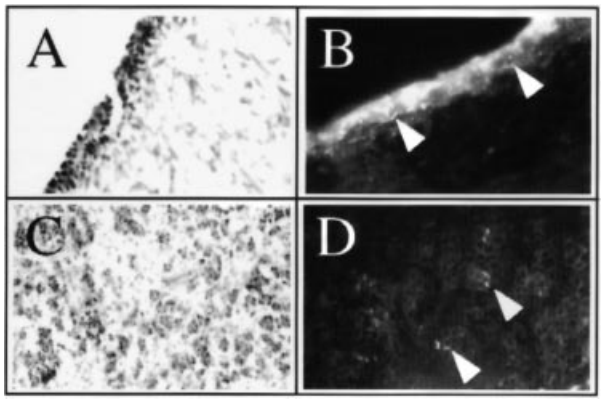

Fig. 6. Immunostaining of Cx43 in human ovarian surface epithelium and human ovarian serous cystadenocarcinoma. (**A**) Bright field image of hematoxylin and eosin stained cryostat section from a benign ovary. (**B**) Fluorescence image of Cx43 stained cryostat section from a benign ovary; the arrowheads mark the punctate Cx43-positive staining. (**C**) Bright field image of hematoxylin and eosin stained cryostat section from a serous cystadenocarcinoma (sample 2901); note the nests of tumor cells surrounded by stromal tissue. (**D**) Fluorescence image of Cx43 stained cryostat section from a serous cystadenocarcinoma (sample 2901); note the paucity of Cx43-positive staining in neoplastic cells (B).

polyclonal antibodies (Zymed Immunochemicals) (30). The acquisition of these tissues was fully approved by the Medical College of Ohio Institutional Review Board. In benign ovaries, the surface epithelium stained positively for Cx43; distinct, punctate, fluorescent spots as well as diffuse cytoplasmic staining of the surface cells were evident (Figure 6A and B). In contrast, ovarian adenocarcinomas had little Cx43 staining (Figure 6C and D). Staining for Cx26 and Cx32 was not detected in any specimen. Similar results were seen with 11 benign and 10 serous cystadenocarcinoma ovarian specimens.

The effects of DBcAMP and ATRA on GJIC and growth of ovarian adenocarcinoma cells were determined. Cells were plated into 24-well culture plates (2×10^4 cells/well), allowed to attach overnight, refed and treated daily with DBcAMP (1 mM) or ATRA (0.01–10 μM) and trypsinized and counted using a hemacytometer after 3 and 6 days treatment. DBcAMP was dissolved in the culture medium and control cultures were not treated. ATRA was dissolved in ethanol then added to the culture medium; control cultures were treated with ethanol (1 μl/ml of medium). DBcAMP (1 mM) significantly reduced the growth of the four carcinoma cell lines (Figure 7A), whereas ATRA (0.01–10 μM) decreased the growth of OVCAR-3 and SK-OV-3 cells, but not CaOV-3 and SW626 cells (Figure 7B). This differential effect of ATRA on ovarian carcinoma cell growth has been attributed to the expression of retinoic acid receptors in sensitive cells (31,32). Neither agent increased dye-coupling in sensitive or insensitive cells (data not shown). DBcAMP enhanced Cx43 expression in SK-OV-3 cells, but not in the other carcinoma cell lines (Figure 8A) and ATRA did not affect Cx43 expression in the ovarian carcinoma cells (Figure 8B). Both agents increased Cx43 expression in C3H10T1/2 fibroblasts (positive control); the effect of ATRA was greater than that of DBcAMP.

We have found that HOSE cells exhibited extensive GJIC and expressed Cx43 *in vivo* and *in vitro* and that GJIC and Cx43 expression were nearly absent in human ovarian adenocarcinoma cell lines and surgical specimens of ovarian serous cystadenocarcinoma. Little information exists on gap junctions and connexin expression in ovarian surface epithelial

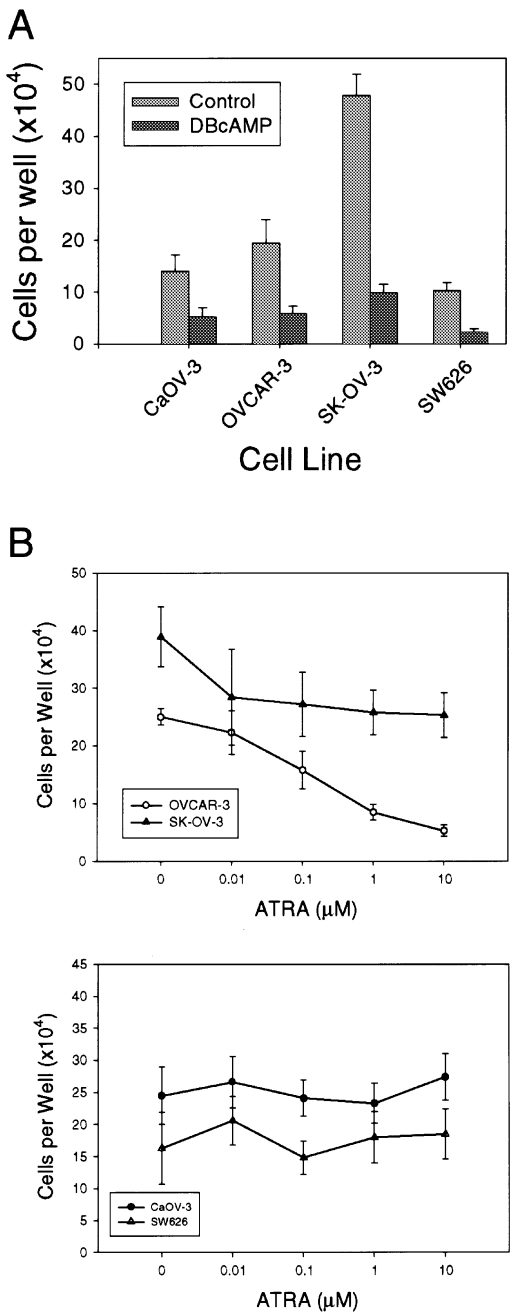

Fig. 7. Effects of DBcAMP (**A**) and ATRA (**B**) on the growth of human ovarian carcinoma cells (CaOV-3, NIH:OVCAR-3, SK-OV-3 and SW-626). Cells were plated into 24-well dishes and treated with DBcAMP (1 mM) or ATRA (0.01–10 μM) then trypsinized and counted 3 (DBcAMP) or 7 days (ATRA) later (means ± SD of eight wells).

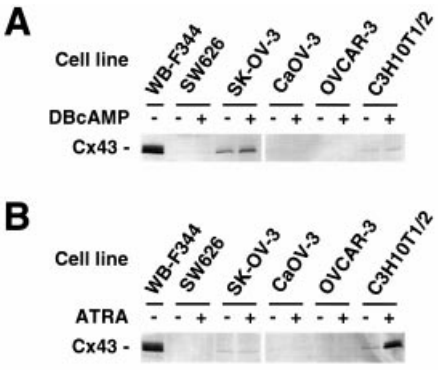

Fig. 8. Western blot analyses of Cx43 protein content in human ovarian carcinoma cells (CaOV-3, NIH:OVCAR-3, SK-OV-3 and SW-626) and murine fibroblasts (C3H10T1/2) after treatment with DBcAMP or ATRA. Cells were treated with 1 mM DBcAMP for 3 days (**A**, +), 10 μM ATRA for 7 days (**B**, +) or not treated (control, –). WB-F344 rat liver epithelial cells were included as a positive control for Cx43 (20 μg protein/lane).

cells and neoplasms (17–21). To our knowledge, this is the first report that HOSE cells express Cx43 and that this expression is highly reduced in human serous cystadenocarcinomas. We do not know, however, whether this change occurs early or late in the development of these neoplasms or whether other types of human ovarian cancers exhibit reduced GJIC and connexin expression. In a preliminary study, we examined three human serous cystadenomas for Cx43 expression by immunostaining and found that this expression was reduced (data not shown). It is unclear why ovarian carcinoma cells express less Cx43 than HOSE cells. Northern and western blotting indicated that the neoplastic cell lines had less Cx43 mRNA and protein than HOSE cells. This decrease could be due to Cx43 gene loss, rearrangement or methylation or to transcriptional or post-transcriptional defects. Southern blot analyses suggested that the Cx43 gene was not deleted or rearranged in these cells.

The decreased GJIC and connexin expression we have observed in human ovarian carcinoma cells has also been documented in other types of human neoplasms (5) and may be important in the ability of neoplastic cells to express a transformed phenotype. Many groups have shown that transfection of neoplastic cells with connexin genes restored GJIC and connexin expression, decreased growth *in vitro*, increased differentiated functions and reduced tumorigenicity (3,6–13). Furthermore, reduction of connexin expression in normal cells or tissues by antisense techniques or connexin gene disruption ('gene knockout') has enhanced cell growth and tumor formation (33–36). Thus, the altered GJIC and Cx43 expression we have documented in ovarian carcinoma cells likely contributes to their neoplastic phenotype. It is also reasonable to speculate that the enhancement of GJIC/Cx43 expression in these cells will reverse this phenotype.

As a first approach to identifying agents that might increase GJIC of ovarian carcinoma cells and that ultimately might be useful therapeutically, we tested DBcAMP and ATRA. Both agents increased GJIC and Cx43 expression in other types of normal and neoplastic cells (14,15). We found that DBcAMP inhibited the growth of the four ovarian carcinoma cell lines, whereas ATRA only reduced the growth of receptor-positive NIH:OVCAR-3 and SK-OV-3 cells (31,32). Neither agent, however, affected GJIC or greatly altered Cx43 expression. These results indicate that cAMP analogs and retinoids reduce ovarian carcinoma growth, but that this is not related to GJIC. Therefore, other methods will be required to increase GJIC in these cells.

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