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Inhibitors

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The Cx43 gene, which codes for a widely expressed gap junction in the breast, is down-regulated in most tumors and is up-regulated in non-transformed cells by certain chemopreventive agents. To determine its actions we forced expression in MDA-345 cells using a tetracycline-inducible promoter. Cells induced to express Cx43 assembled functional gap junctions as assessed by dye-transfer, however their behavior in monolayer culture was not changed. Induction did not alter: cell saturation density, cell-cycle distribution and capacity to invade Matrigel. However their capacity to form large colonies in suspension in soft agar was decreased by over 80%. Analysis by RT-PCR of cells recovered from suspension revealed that in addition to Cx43, expression of cyclin D1 was strongly inhibited while the cyclin-dependent kinase inhibitor p27 was strongly enhanced. Further, Cx43 induction strongly enhance TRAIL expression and reduced bcl-2 expression. No change in expression of these genes were seen in cells induced in monolayer culture. These results indicate that Cx43 expression arrests growth in G1 phase of the cells cycle and sensitizes them to apoptosis, only in cells maintained in suspension. The mechanism by which Cx43 induces these effects remains to be determined.				
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Introduction:

We propose to chemically identify the junctionally transmitted signals which we hypothesize to be responsible for the observed growth inhibition of breast tumor cells when in junctional communication with growth inhibited normal cells. Our previous studies with murine cells have shown that when recently derived, neoplastically transformed fibroblasts were placed in junctional communication with growth inhibited fibroblasts, the transformed fibroblasts were arrested in G1 of the cell cycle. This cell cycle arrest strongly correlated with the degree of junctional communication (1). We lately extended these studies to human epithelial tumor cells in culture and demonstrated that the inducible expression of connexin 43, a gap junction family member expressed in normal epithelial cells but lacking in the carcinoma cells, connexin expression resulted in strong attenuation of the neoplastic phenotype. This was detected as a reduction in anchorage independent growth and a reduction in the ability to grow as xenografts in the nude mouse (2). Other investigators have also demonstrated reductions in connexin assembly or expression in neoplastic cells (reviewed in (3)). In several cases, this reduction in expression has been associated with an increase in DNA methylation, a method of gene silencing commonly employed by tumor cells to silence tumor suppressor genes (4). We have hypothesized that growth inhibitory signals can be transferred through gap junctions (5). Because of the physical constraints of the channel formed by a gap junction, these inhibitory signals must be mediated by molecules or ions which are water-soluble and of a size below approximately 1000 daltons (6). Restoration of gap junction function could thus lead to the decreased proliferation of carcinogen-initiated cells thereby reducing their progression to fully transformed cells. In this context it may be significant that two classes of cancer preventive agents, the retinoids and carotenoids cause upregulated expression of connexin 43 in cells of epithelial and fibroblastic origin (7,8). Moreover, the chemical identification of the putative junctional transfer signal could offer new avenues for cancer therapy and perhaps prevention.

1/. Research goals.

Technical objectives:

1. To develop in vitro methods for the transfer of the putative growth-inhibitor signal from quiescent cells and its transfer to breast cancer cells.
- 2.

Task 1a. Develop tet-inducible breast cancer cells lines.

This goal has been achieved, several lines have been constructed which express Cx43 at levels comparable to that seen in normal epithelial tissues. This is achieved by withdrawal of doxycycline from the cell culture medium. The scheme for production of these cells is shown in Fig 1.

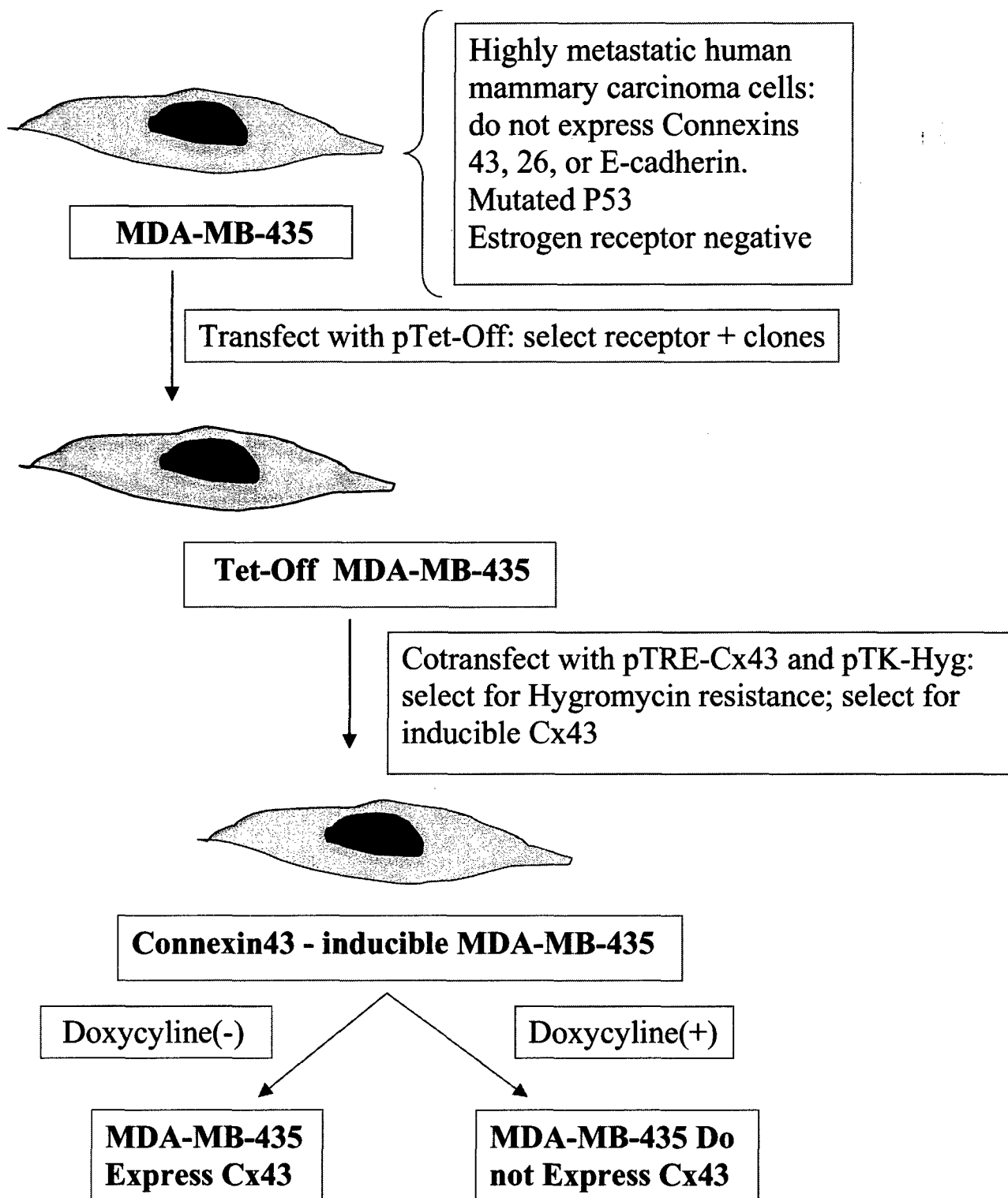


Figure 1. Scheme for creation of Connexin43-inducible MDA-MB-435 cells

Cells express Cx43 on induction and this protein is assembled into plaques in regions of cell/cell contact (Fig 2a) and is of the correct molecular size as shown by Western blotting (Fig 2b).

We had demonstrated that the parental cells were negative for expression of Cx43 and Cx26, the two connexins normally expressed in the breast epithelium, accordingly non-induced cells were junctionally negative as assessed by dye transfer experiments. However after induction of Cx43 expression, an extensive network of communicating cells was observed (Fig 3b).

In conclusion, we have successfully created a several lines of breast cancer cells which can be induced to express Cx43, and assemble the protein into functional gap junctions.

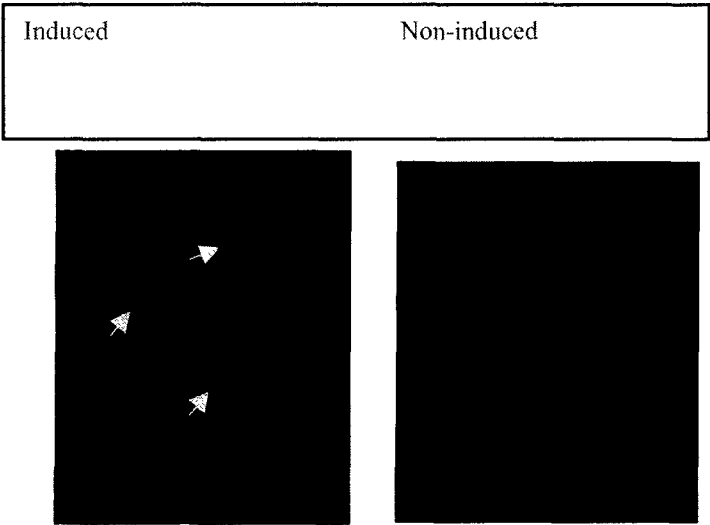
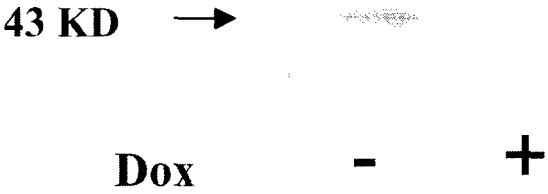


Figure 2. Induction of MDA cells by withdrawal of doxycycline, results in assembly of junctional plaques revealed by staining with a Cx43 specific antibody, Fig 2a. and synthesis of the appropriately sized protein, Fig 2b.
 Fig 2a.. Note junctional plaques (arrows).

Fig 2b Western blott of induced (left) and non-induced (right) MDA cells. Arrow indicates 43KD MW immune-reactive band.



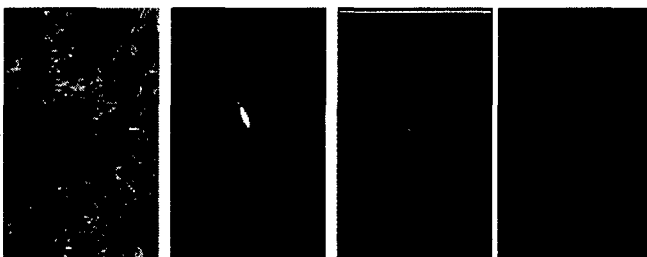
**Passage of Lucifer Yellow in Connexin43 Induced
MDA-MB-435 in Microinjection assay Demonstrated
Communication Competence**

Cx43 Induced MDA-MB-435 Cells



Phase: 0 minute 0.5 minutes 5 minutes 10 minutes

Cx43 un-induced MDA-MB-435 Cells



Phase: 0 minute 0.5 minutes 5 minutes 10 minutes

Figure 3. Induced Cx43 is assembled into functional gap junctions as revealed by dye injection studies. Top panel; induced cells; Bottom panel; non-induced cells. Note extensive and rapid spread of dye from the injected cell, center, to surrounding cells only in the induced cultures.

Task 1b, Developments of in vitro protocols for the delivery of the growth inhibitory signal from quiescent cells to junctionally competent breast cancer cells.

Growth in monolayer. To determine if the MDA breast cancer cell line produced any response to endogenous growth regulatory signals when allowed to junctionally communicate after connexin 43 induction, we plated cells at high density and monitored proliferation rates and cell cycle parameters under conditions of induction or when maintained with doxycycline. As previously reported cells became junctionally competent as judged by dye injection studies, however we could detect no change in cellular proliferation rates. We have previously reported that in one cell line, there was an increase in cells in the G2 portion of the cell cycle. However, we have been unable to reproduce these findings in subsequent studies of this line and other inducible lines, and we conclude, as was suggested by the reviewer of our prior progress report, that this conclusion was the result of abnormally low numbers of cells in the control, non-induced situation. It thus appears that the MDA breast carcinoma cells, are unable to either generate or respond to junctionally transmitted signal molecules. We reported a similar finding in the HeLa human cervical cancer cell line, which also failed to alter proliferation rates when forced into communication by induction of connexin 43 expression (2,7).

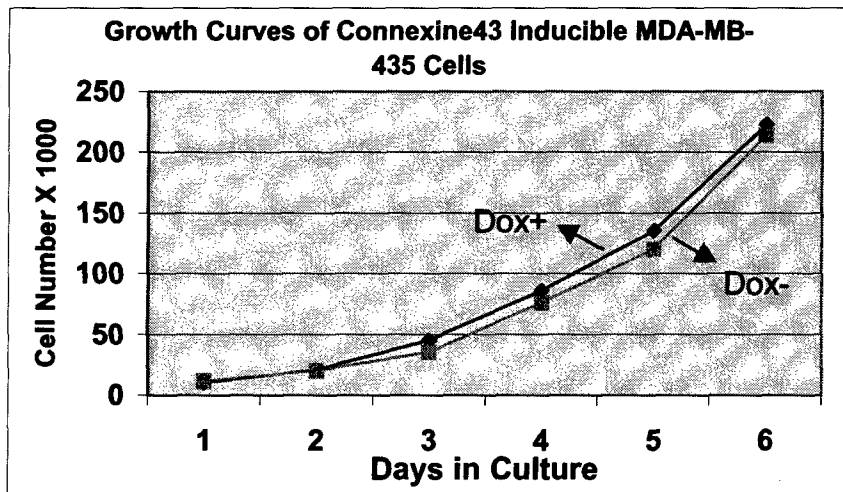


Figure 4. Growth of induced (Dox-) and non-induced cells (Dox +) in monolayer culture.

Growth in co-culture with quiescent normal cells. Even if the MDA cells can no longer produce growth inhibitory signals, they may well be able to respond to such signals if delivered through gap junctions. We have previously reported on the ability of cAMP modifying agents to increase heterologous gap junctional communication (9); an effect presumed to be due to the requirement for phosphorylation on the C-terminal region of connexin 43. We have thus cultured these MDA cells with a variety of growth inhibited non-transformed cells in the presence and absence of cAMP modulating drugs.

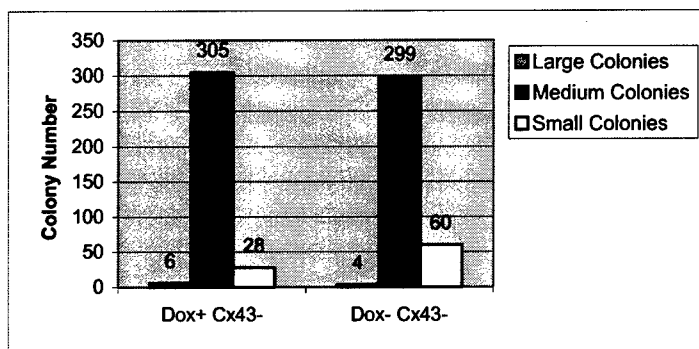
We have co-cultured these MDA cells with: growth inhibited mouse 10T1/2 cells, human MCF-10a cells which represent an immortalized yet growth controlled mammary epithelial cell line, with NRK cells and with normal human fibroblasts. In all cases the MDA cells failed to make adequate contact with these growth controlled cells and formed discrete clusters of proliferating cells. Numerous techniques have been attempted to increase the interactions between these various cell types including; growth on Matrigel coated dishes and growth on collagen coated dishes. In no case did these strategies increase the extent of growth inhibition of the MDA cells, and did not result in growth inhibition by any of the other cell types mentioned above. It should

represent a cell line which has been extensively cultured and was derived from a highly metastatic human tumor. It seems likely that this extensive history of proliferation in the host and in cell culture conditions has led to the loss of many of the pathways that are required for efficient cell cycle inhibition. What is surprising is that these cells form extensive homologous contacts but fail to form junctions with other cells types which themselves communicate and express Cx43.

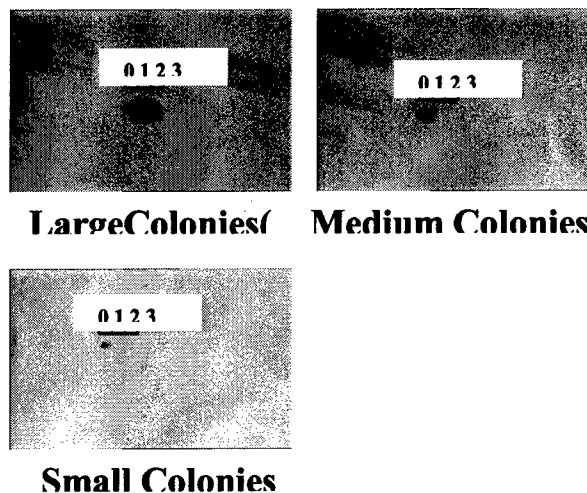
Anchorage independent growth. We have now firmly established that induction of connexin 43 results in a greatly attenuated ability of these MDA cells to grow in an anchorage independent manner. As shown in Fig. 5, induction of connexin 43 by withdrawal of doxycycline causes a profound decrease in the ability of cells to form spheroids suspended in agarose. After induction, there was a total loss of formation of large colonies, and reduction from 514 medium-size colonies in the control situation to only 129 colonies in the induced situation. In terms of total colonies, the figure of 572 in controls was reduced to 372 in the induced situation. As shown in the top left panel of Fig 5, when the parental MDA cells, containing just the dox-receptor but no Cx43 construct, were plated in semi solid agarose the addition or subtraction of doxycycline had no effect on colony formation, clearly indicating that this is a connexin 43-specific event. The ability to grow in soft agar, i.e. anchorage independent growth, is a widely used indicator of neoplastic potential, and thus the suppression of growth caused by connexin 43 induction is significant in terms of the potential neoplastic properties of these cells.

Connexin43 Expression Reduces Soft Agar Colony Formation in Connexin43-induced MDA-MB-435 Cells

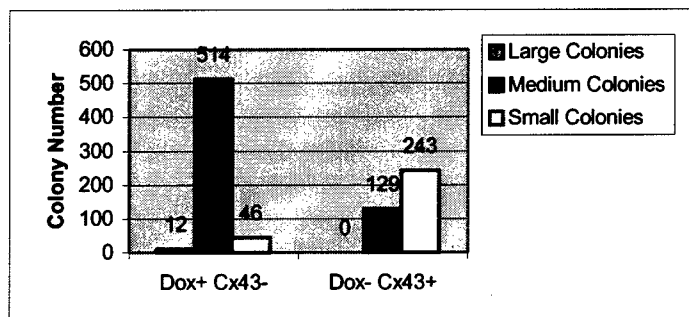
Parental MDA-MB-435 Cells



Phase Image of Colonies in Soft agar Assay



Connexin43 inducible MDA-MB-



Immunofluorescence Staining of Colonies in Soft agar Assay

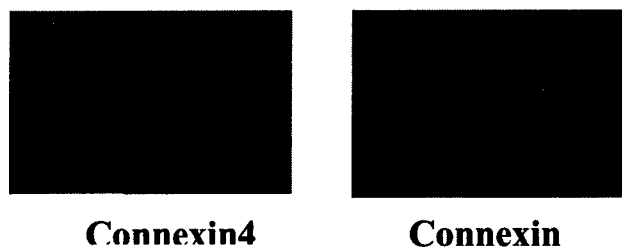


Figure 5. Influence of Cx43 induction on the growth of MDA cells in suspension in soft agar. Top left panel; parental MDA cells containing only the tet receptor; bottom left panel, cells engineered to express CX43 on withdrawal of Dox. Also shown are images of colonies in soft agar and immunofluorescent staining with a Cx4343 antibody of induced and non-induced colonies to show Cx43 expression. Note the complete loss of large colonies and major reduction in medium colonies in induced cells.

Task 1c. Detection of the junctionally transferred signal in cancer cells by measurement of cell cycle related parameters.

While growth in semi solid agarose offers us an excellent assay with which to determine effects of connexin 43 expression on growth control, it creates a major problem in the detection of cell cycle events. First is the issue of contamination of cells by agar; second is the issue of obtaining sufficient cells for analysis. We have attempted to digest the agar with a specific enzyme, we have attempted to melt the agar and centrifuge while warm, we have attempted to dilute the agar and centrifuge cells from the matrix. In all cases material from the agar remains associated with the cells and does not allow FACS analysis. We have changed the source of the agar but these problems remain. Other approaches, as discussed below, were also not successful.

Task 1d/ Detecting of molecular events produced by junctionally transferred signals.

The presence of agar in the assays also presents a formidable challenge in obtaining meaningful data which would identify molecular events induced in the cells as a consequence of connexin 43 induction. The difficulties here relate to problems in extracting cells in sufficient numbers, and uncontaminated by agarose, for us to perform of RT/PCR analysis of gene expression. These difficulties are twofold: first connexin 43 induction dramatically reduces the numbers of cells available for assay; second we have discovered that agarose is particularly difficult to remove from cells and contaminates nucleic acid extracts. To our knowledge, molecular assays have not before been reported in cells cultured in this manner. In an attempt to circumvent the problems associated with agarose contamination, we have grown cells in suspension in conventional media and denied their ability to attach to the culture dish by coating it with poly HEMA. However, in this situation, when cells did not proliferate to form spheroids, as they did in agarose, but remained as single cells without stable cell/cell contacts, no reduction in proliferation was observed. While this observation strongly supports the role of junctionally-mediated intercellular communication afforded by connexin expression, it did not solve our problem.

We have thus decided to utilize the most direct route to obtain cells for analysis and by culturing cells in larger volumes of agarose at much higher densities than employed in the colony counting assays described in Fig 5 above, we now have been successful in obtaining sufficient RNA for analysis by RT-PCR.

Because it is possible that the reduced colony size in induced cultures is due either to inhibition of proliferation or to increased apoptosis, we examined the expression of genes known to be involved in G1 arrest and in programmed cell death.

As shown in Fig 6, there is a major induction of the cyclin dependant kinase inhibitor p27 and a parallel decrease in cyclinD1. Both events strongly implicate G1 arrest as a cause of the decreased colony size. The presence of Cx43 transcripts confirms that these cells were indeed induced under the conditions of suspension culture. Moreover, this message has a half life of about 2-3 hours in all cells so far examined, indicating that cells are viable.

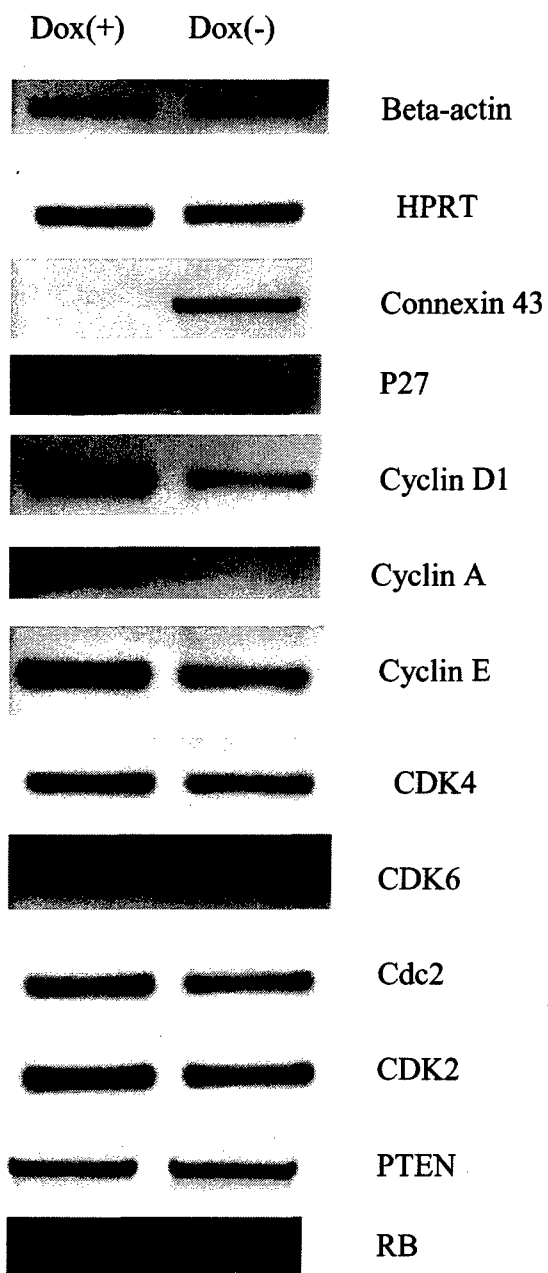
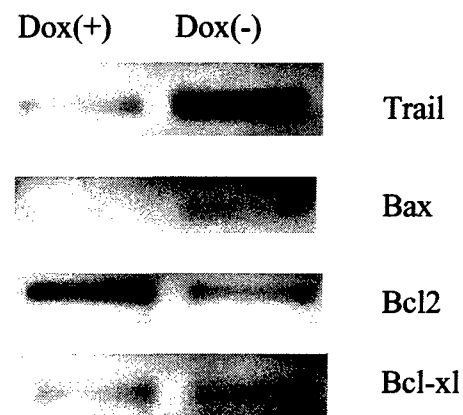


Figure 6. RT-PCR of cells extracted from soft agar assays. Input cDNA was normalized for GAPDH expression. Left lanes; non-induced; right lanes, induced.



mRNA	Ratio Ind/Non
HPRT	1.05
Actin	2.3
Cx43	∞
p27	∞
Cyclin D1	0.08
Cyclin E	0.43
Bcl-2	0.002
TRAIL	113

While these data strongly indicate G1 cell cycle arrest as a cause of the reduced colony sizes in Cx43 induced cells, examination of the panel of apoptotic genes suggests that cells are also subjected to stimuli that in normal cells would induce apoptosis. Cx43 induction resulted in a dramatic increase in expression of the death-inducing ligand TRAIL, and concurrently caused an equally dramatic decrease in Bcl-2 expression which would serve to sensitize cells to apoptosis (10). We at present do not know if these cells are resistant to TRAIL-induced apoptosis as are many tumor cells. However the data strongly supports the conclusion that both mechanisms are contributing to the decreased growth of Cx43 expression cells in suspension culture. It is known that these MDA tumor cells are p53 null, but can undergo apoptosis via p53 independent mechanisms. As discussed above, the presence of short-lived transcripts such as mRNA for Cx43, implies that some cells remain viable even in the face of these pro-apoptotic signals.

Technical Objective 2. Chemical characterization of the molecules responsible for induction of growth control in breast cancer cells lines.

Because of the technical difficulties encountered with the in vitro assays in obtaining sufficient cells for analysis, and the implications that these MDA cells will only respond to junctionally transmitted signals when in suspension in soft agar, we have been able to approach these objectives.

Key research accomplishments:

- * Production of MDA breast carcinoma cells containing Cx43 driven by an inducible promoter.
- * Demonstration that induction of Cx43 results in junctionally competent cells
- * Demonstration that in contrast to effects in non-transformed cells, junctional communication does not result in altered growth control in monolayer culture, or altered ability to migrate and invade Matrigel.
- * Demonstration of strong inhibition of soft agar growth, an indicator of neoplastic potential, when MDA breast carcinoma cells are induced to express connexin 43.
- * Demonstration of strong down-regulation of Cyclin D and Bcl-2 and up-regulation of p27 and TRAIL expression in CX43 –induced cells.

Conclusions:

Connexin 43 acts as a tumor suppressor gene in breast carcinoma cells decreasing growth in soft agar. This reduction in growth is accompanied by decreased expression of genes involved in cell cycle stimulation (cyclin D) and protection from apoptosis (bcl-2) and increased expression of genes inhibiting cell cycle progression (p27) and sensitizing cells to apoptosis.

Reportable outcomes:

- 1/. Presentation of results at the International Gap Junction Symposium Honolulu, HI, 2001. "Restoration of connexin43 mediated communication restores aspects of normal growth control in human mammary epithelial cells", p53 of the Proceedings.
- 2/. Development of 5 inducible Cx43 MDA breast cells lines.
- 3/. Results comprise PH.D thesis of Xiao-Li Chen a graduate student in the Cell and Molecular Science program at the University of Hawaii (graduation expected 2002).
- 4/. Manuscript is in preparation detailing the results of these funded studies.
- 5/.

Conclusions:

This research has firmly established Cx43 as a gene with the potential to suppress the neoplastic phenotype. Because of the use of an inducible system, any ambiguities resulting from clonal heterogeneity are eliminated. The ability of connexin expression to suppress only anchorage independent growth is surprising in view of our previous work using recently established transformed lines and may reflect the long history of the MDA cells in culture. It may be that these cells have lost the ability to respond to

junctionally transmitted signals. In future work it would be interesting to evaluate results in recently transformed mammary cells. However such studies are technically difficult for many reasons.

Of mayor interest is the finding that connexin expression modulates the expression of genes involved in both cell cycle arrest and in sensitizing cells to apoptosis, making connexin expression an exciting target for therapy.

If methods could be found to induce Cx43 expression in tumors, it may be expected that those cells not becoming growth arrested would undergo programmed cell death. We have shown that retinoic acid and many dietary carotenoids will cause over-expression of this gene in human keratinocytes (11), but it is not known if this would work in carcinoma cells. An alternative would be to identify and target the reason for non-expression of Cx43 in carcinoma cells. As we have shown in HeLa cells, DNA methylation appears to be one mechanism used by cancer cells to silence Cx43. While use of methylation inhibitors such as 5-aza-cytidine may be too toxic for use clinically, agents which modify histone acetylation may be capable of overcoming silencing and be less toxic. This we have shown in HeLa cells (12), but do not know the methylation status of this gene in MDA cells. However, Cx26 which is also expressed in breast epithelium, has been shown to be silenced by this mechanism (13,14). The most promising cells in which to enhance junctional communication are pre-neoplastic cells; these can be expected to retain intact many growth control pathways and to be highly sensitive to growth inhibition. Unfortunately, a clinical trial with a synthetic retinoid, 4-HPR, which in a model 10T1/2 system we showed to strongly enhanced junctional communication (15), failed to reduce the incidence of breast cancers in a high-risk population (16). Thus either target cells did not respond as did cultured cells, or induced communication was insufficient to influence aberrant cell division.

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REPLY TO
ATTENTION OF

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MCMR-RMI-S (70-1y)

15 May 03

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VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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