

Cancer Research



Reversion of the Neoplastic Phenotype of Human Glioblastoma Cells by Connexin 43 (cx43)

Ruo-Pan Huang, Yan Fan, Mohammad Z. Hossain, et al.

Cancer Res 1998;58:5089-5096.

Updated Version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/58/22/5089>

Citing Articles This article has been cited by 39 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/58/22/5089#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Reversion of the Neoplastic Phenotype of Human Glioblastoma Cells by Connexin 43 (cx43)¹

Ruo-Pan Huang,² Yan Fan, Mohammad Z. Hossain, Ao Peng, Zi-Li Zeng, and Alton L. Boynton

Molecular Medicine, Northwest Hospital, Seattle, Washington 98125 [R.-P. H., Y. F., M. Z. H., A. P., Z.-L. Z., A. L. B.], and Shantou University Medical College, Shantou City, Guangdong Province, People's Republic of China, 51503 [Z.-L. Z.]

ABSTRACT

Connexins (cx), structural components of gap junction, are believed to play a role in the regulation of cell proliferation and suppression of the neoplastic phenotype. We used human brain glioblastoma tumor cells as a model system to test this hypothesis. Western blot and reverse transcription-PCR analysis indicate that the expression levels of the gap junction protein connexin 43 (cx43) are profoundly decreased in several human brain tumor cell lines examined. Transfection of human cx43 into human glioblastoma cell lines U251 and T98G profoundly reduces cell proliferation in monolayer culture, in soft agar, and in athymic nude mice. Surprisingly, these effects are not associated with the establishment of gap junction communication in cx43 transfected cells. We conclude that the loss of cx43 expression may play a role in the development of human gliomas and that cx43 acts as a tumor suppressor gene to human glioblastoma.

INTRODUCTION

Adjacent cells can directly share ions and small molecules of size less than 1000 Da through intercellular channels present in the morphological structures known as the gap junctions (1). Gap junctions can be found in almost all mammalian tissues except circulating blood cells and adult skeletal muscle. cx³ proteins are the major, if not only, component of gap junctions. Thus far, at least 12 members of the cx protein family have been identified ranging in size from 26 to 56 kDa. These cxs are differentially expressed in a variety of tissues; and this is generally believed to reflect cell-specific regulation of gap junctional coupling and functional demands for gap junctions in different cell types. cx43 is the most widely expressed cx, particularly in brain and heart.

GJC is believed to be involved in the regulation of cell homeostasis, proliferation, and differentiation. Accumulated evidence indicates that cx proteins may function as tumor suppressor genes. Many tumor-promoting agents, oncogenes, and growth factors inhibit GJC (1, 2). In contrast, antineoplastic agents, such as retinoids, vitamin D, and carotenoids up-regulate GJC (2, 3). Restoration of GJC by transfection of cx proteins has been demonstrated to reverse the transformed phenotype of cancer cells, including rat C6 glioma (cx43; Refs. 4, 5), human mammary carcinoma (cx26 and cx43; Ref. 6), human hepatoma cells (cx32; Ref. 7), transformed dog kidney epithelial cell line (cx43; Ref. 8), and rhabdomyosarcoma (cx43; Ref. 9). The mechanisms responsible for the tumor suppression by cx43 are unknown. Expression of cx proteins restored differentiation potential in human mammary carcinoma cells (cx26 and cx43; Ref. 6) and induced myogenic differentiation in rhabdomyosarcoma cells (cx43; Ref. 9). Transfection of the cx43 gene also enhanced genetic stability in HeLa

cells (10). cx43 may also be involved in the regulation of cell cycle progression (8).

Brain tumors are one of the leading causes of death among young children and adults. Glioblastomas are the most common primary brain tumors and are among the deadliest of tumors: most of the 20,000 people diagnosed each year in the United States with this form of brain cancer die within 2 years. To explore the possible role of cx43 in brain tumors, we first examined the expression levels of cx43 in brain tumor cells and also transfected cx43 into human glioblastoma cells. Our results show that expression of cx43 is decreased in human brain tumor cell lines. Transfection of the cx43 gene into human glioblastoma cells reverses the transformed phenotype of these tumor cells. Furthermore, it is likely that the tumor suppression by cx43 is not directly related to GJC.

MATERIALS AND METHODS

Cell Culture. U251, T98G, U-87 MG, U373MG, and IMR-32 were originally obtained from ATCC and maintained in DMEM containing 10% FCS. Rat astrocyte primary cells were a gift from Dr. A. Nishiyama at Cleveland Clinic Foundation (Cleveland, OH). Human fetal astrocytes, glioma (Grade IV), glioblastoma, astrocytoma III, medulloblastoma, neuroblastoma were obtained from Clonetics (San Diego, CA) and cultured according to manufacturer's instruction. T51B are rat liver oval epithelial cells and were grown in Eagle's basal medium containing 10% bovine calf serum.

Reverse Transcription-PCR. Total RNA was isolated from cultured cells by the guanidine isothiocyanate RNazolB method (Cinna/Biotech Laboratories, Houston, TX). Five μ g of total RNA was used for cDNA synthesis using random hexamer primer (Boehringer Mannheim, Germany). PCR amplification was carried out by using all of the reverse-transcribed RNA. The PCR reaction mixture contained 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris (pH 8.0), 10 mM dNTP, 10 μ M of each primer, and 0.5 unit of *Taq* polymerase (Boehringer Mannheim) in the final volume of 50 μ l. The PCR profile was 94°C for 40 s, 52°C for 50 s, and 72°C for 60 s for 25 cycles, followed by 75°C for 5 min. After PCR, the input RNA was removed by RNase digestion. The amplified DNA was then precipitated and separated on 1.8% agarose gel containing ethidium bromide. The sense primer was 5'-CATGGGTGACTG-GAG-3' starting at nt 226. The antisense primer was 5'-AGGACCGA-GAAGCGCA-3' starting at nt 465. Both primers were synthesized using Oligo 1000-M DNA synthesizer (Bechman, Fullerton, CA). The expected amplified fragment for human connexin 43 was 236 bp. As an internal control, β -actin primers were used as described previously (11) to detect 245 bp of β -actin product.

Plasmid Construction and Transfection. The expression vector encoding full-length cx43 was made by the insertion of cx43 DNA fragment from HCGJ-2 (provided by Dr. G. Fishman, Albert Einstein College of Medicine, Bronx, NY) into mammalian expression vector pFCS (provided by Dr. P. Brulet, Pasteur Institute, Paris, France). pFCS was opened at the unique *Sac*II and *Eco*RV sites and cx43 was removed from HCGJ-2 by *Sac*II and *Hinc*II digestion. The final construction contained the *neo* gene driven by the Rous sarcoma virus promoter and the cx43 gene driven by the human cytomegalovirus promoter. After transfection using the calcium phosphate precipitation procedure, cultures were selected with 400 μ g/ml G418, and clones were isolated using cloning rings.

Western Blot. Western blot assay was conducted as described in Huang *et al.* (12). Cell extracts containing equal amounts of protein were analyzed by 10% SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore, Corp., Bedford, MA). cx43 protein was detected by

Received 4/29/98; accepted 9/17/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by NIH Grants CA39745 and CA57064 (both to A. L. B.).

² To whom requests for reprints should be addressed, at Molecular Medicine, Northwest Hospital, 120 Northgate Plaza, Suite 230, Seattle, WA 98125. Phone: (206) 368-3063; Fax: (206) 368-3009; E-mail: rphuang@nwlink.com or rhuang@nwhsa.org.

³ The abbreviations used are: cx, connexin; GJC, gap junction communication; ATCC, American Type Culture Collection; LY, Lucifer yellow dye; FACS, fluorescence-activated cell sorting.

anti-cx43 antiserum and enhanced chemiluminescence system (Amersham Corp., Amersham, United Kingdom)

Northern Blot. Total RNA was isolated from cultured cells by the RNAzol method according to the manufacturer's instruction (Biotech, Houston, Texas). Northern blotting was performed as described previously (13). Briefly, 30 μ g of total RNA was denatured by formaldehyde and fractionated on a 1.0% agarose gel containing 1.0 M formaldehyde. The RNA was transferred to a nitrocellulose filter by blotting. After UV cross-linking, the filter was hybridized with different [³²P]dCTP (New England Nuclear, Boston, MA) labeled probes at 42°C for overnight, washed three times for 10 min with an excess amount of 2 \times SSC-0.1% SDS at 50°C and twice for 30 min with 0.1%SSC-0.1%SDS at 50°C. Human cx43 cDNA was used as probe in hybridization.

Cell Proliferation Assay. Cells (10,000/well) were seeded in each well of a 24-well plate and were allowed to grow for varying times. The trypsinized cells were counted in duplicate for each cell line using a Coulter counter. The experiments were repeated three times. The cell number was then plotted against culture times.

FACS Analysis. The cell cycle distribution of log phase cx43 transfected cells and control cells was determined by flow cytometry as described by Huang *et al.* (14). Briefly, 4-day-old cells were collected, fixed in 70% ethanol, and stained with propidium iodide. The DNA contents in cells were then analyzed in a FACS (Model FACSCalibur, Decton Dickinson).

Soft Agar Assay. Soft agar assay was performed as described previously (15, 16). Briefly, cx43 transfected cells and control cells were assayed by seeding 5,000 cells or 10,000 cells in 0.26% agar medium into 60-mm plates previously lined with 0.65% agar medium. The plates (in duplicate and repeated three times) were cultured for 3–4 weeks and then stained with *p*-iodotetrazolium violet overnight before photography and counting. Colony size [mteq]15,625 μ m (2) was scored as positive.

Tumorigenicity Assay. Tumorigenicity assay was performed as described previously (15, 16). Trypsinized cells (1×10^7) derived from each of the clones were injected into two s.c. sites in the flanks of BALB/c nu/nu mice. Animals were inspected twice a week for the appearance of visible tumors. Mice were killed at day 55 after injection. Tumors were carefully removed by blunt dissection and weighed. Tumorigenicity was measured as tumor weight per injection site. The animal experiments were performed at Bio-Support (Redmond, WA).

Measurement of GJC. GJC was assayed by transfer of the fluorescent LY after single-cell microinjection as described previously (2). The cells were observed under a fluorescence inverted microscope 10 min after microinjection, and the number of neighboring cells labeled with fluorescent dye was recorded.

Immunofluorescent Staining. Immunofluorescent staining of cx43 in cx43 transfected cells and control transfected cells were conducted as described previously (17). Briefly, cells were grown on glass coverslips to confluence, fixed with 3% formaldehyde, permeabilized with 2% Triton, and reduced with 1% sodium borohydride. The coverslips were incubated with anti-cx43 antiserum or normal rabbit antiserum as negative control and subsequently reacted with FITC-conjugated antirabbit IgG. The staining was visualized and photographed with a Zeiss photomicroscope II.

RESULTS

Analysis of cx43 Expression in Human Brain Tumor Cell Lines.

We first examined cx43 expression in various human glioblastoma (Fig. 1A, Lanes 2–5) and neuroblastoma cell lines (Fig. 1A, Lane 6) obtained from ATCC. As shown in Fig. 1A, except for U-87MG, the several brain tumor cell lines studied expressed very little or no cx43. In contrast, normal rat primary astrocytes showed high amounts of cx43 protein. We next examined the level of cx43 in a set of human brain tumor cell lines obtained from Clontech. As shown in Fig. 1B, normal fetal astrocytes expressed readily detectable amounts of cx43, whereas human brain tumor cells expressed very small amounts of cx43 protein. Interestingly, human fetal astrocytes primarily expressed the unphosphorylated form of cx43, but rat astrocytes expressed both the unphosphorylated form (P1) and phosphorylated forms (P2 and P3) equally.

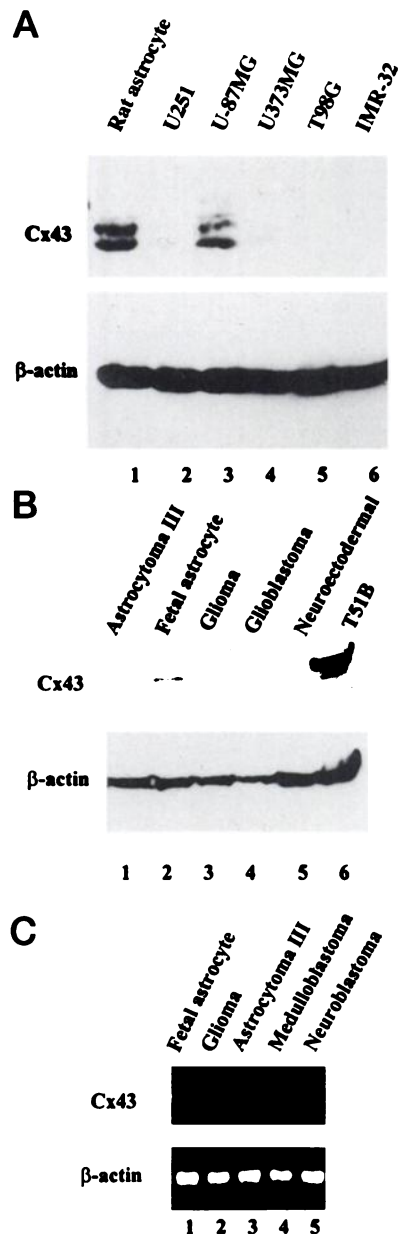


Fig. 1. Expression of cx43 is reduced in human brain tumor cell lines. A, cx43 levels in a set of human brain tumor cell lines obtained from ATCC. Lane 1 is rat normal primary astrocytes. Lanes 2–4 are human glioblastoma cell lines. Lane 5 is human neuroblastoma cell line. B, expression of cx43 in a set of human brain tumor cell lines obtained from Clontech (San Diego, CA). Fetal normal astrocytes express the unphosphorylated form of cx43 (Lane 2). Lane 6 is T51B rat liver epithelial cell lines showing the expression of multiple phosphorylated forms of cx43. Other tumor cell lines express a very low amount of cx43. C, reverse transcription-PCR analysis of cx43 mRNA in human normal fetal astrocytes compared with four different brain tumor cell lines in which the expression of cx43 was profoundly reduced. All lanes were equally loaded, as shown in the lower panels of A, B, and C by the levels of β -actin protein in A and B and β -actin mRNA in C.

To investigate whether this lack of cx43 expression was mediated by transcriptional regulation, mRNA levels were assayed by semi-quantitative PCR analysis. As shown in Fig. 1C, cx43 mRNA was only detected in normal fetal astrocyte but not in the brain tumor cell lines examined. These results suggest that the expression of cx43 is frequently lost in human brain tumor cell lines and the decreased cx43 expression occurs at the transcriptional level.

Establishment of Stable Human Glioblastoma Cell Lines Expressing cx43. To determine whether loss of expression of cx43 in human brain tumor cells plays a role in the transformed state of human

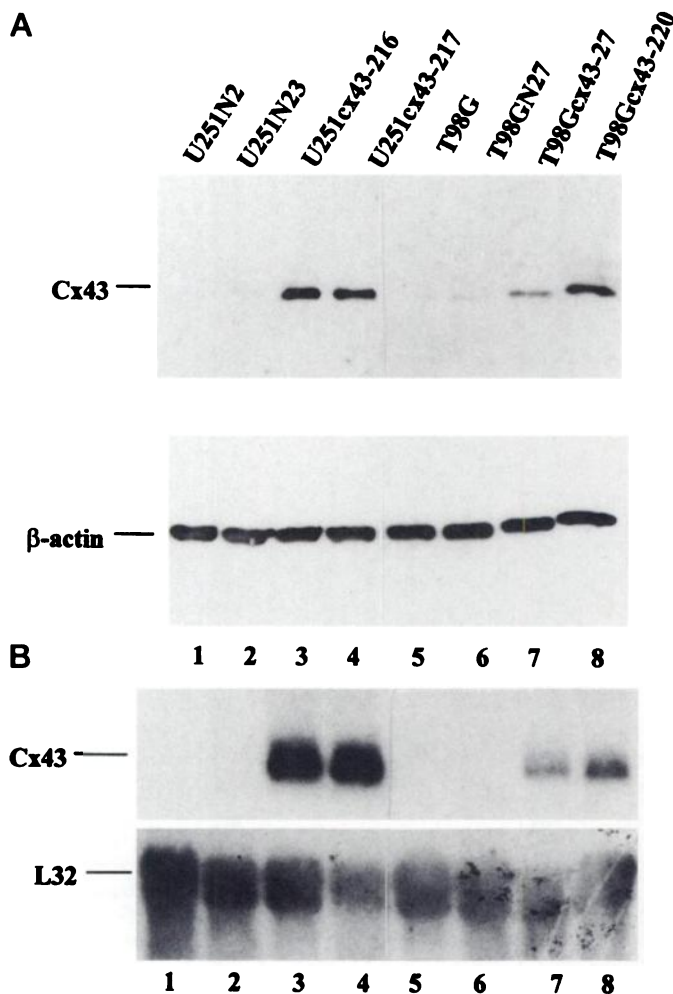


Fig. 2. Western (A) and Northern (B) blot analyses of *cx43* expression in *cx43* transfected clones and control clones. In both A and B, Lanes 3, 4, 7, and 8 are *cx43* transfected clones. Lanes 1, 2, and 6 are control transfected clones. Lane 5 is parent T98G cells. The equal loading of each lane is demonstrated by reprobing the same blot with β -actin in Western blot analysis and L32, a rRNA, in Northern blot analysis.

glioblastoma, we transfected *cx43* expression vector or control vector (without *cx43* insertion) into human glioblastoma cells U251 and T98G. U251 and T98G are well-characterized human glioblastoma cell lines and are able to grow in soft agar, but only U251 forms tumors in nude mice.

Several different stable transfected cell lines were selected by Western blot. As shown in Fig. 2A, U251*cx43*-216 and U251*cx43*-217 are two clones expressing high amounts of *cx43* in U251 cells. U251 N2 and U251 N23 are two clones transfected with control vector without insertion of *cx43*. T98G*cx43*-27 and T98G*cx43*-220 are two clones transfected with *cx43* expression vector in T98G cells, both of which expressed high levels of *cx43*, whereas in T98G N28, a control transfected clone and in the parental T98G, *cx43* levels were very low. The *cx43* expression levels were further confirmed by Northern blotting analysis (Fig. 2B).

Expression of *cx43* Reverses the Transformed Phenotype of Human Glioblastoma Cells. After the establishment of *cx43* transfected cell lines, we then tested whether expression of *cx43* suppresses tumor cell growth. The first simple experiment was to measure cell proliferation rates of different clones by counting cell number. As shown in Fig. 3, *cx43* transfected-cell lines grew much slower than control cell lines. The average doubling time (Table 1) for *cx43* transfectants was longer. In U251 cells, the population doubling time

for control transfected clones U251N2 and U251N23 was 34.4 and 33.5 h, respectively, whereas *cx43* transfected clones U251*cx43*-216 and U251*cx43*-217 have a population doubling time of 43.1 and 43.9 h, respectively. In T98G cells, the *cx43* transfectants T98G*cx43*-27 and T98G*cx43*-220 required 53.2 and 48.6 h, respectively, to double their population, whereas the parent cells, T98G, and control transfected cells, T98GN28, only required 33.4 and 33.9 h, respectively, to double their cell number.

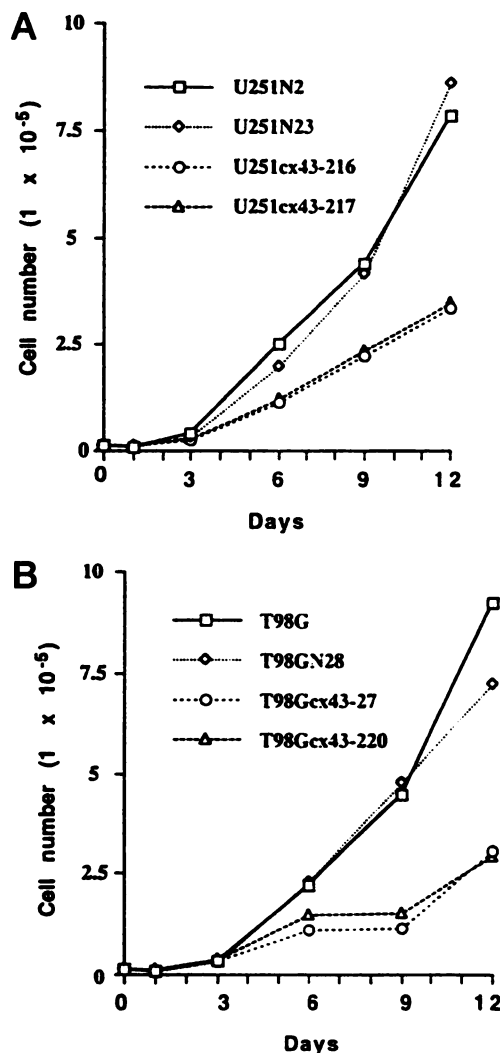


Fig. 3. The inhibition of monolayer growth of human glioblastoma U251 (A) and T98G (B) cells by transfection of *cx43*. The control cells and *cx43* transfected cells were seeded in 24-well tissue culture plates and counted in duplicate on the indicated days as described in the "Materials and Methods" section. Reported are means from three separate experiments.

Table 1 Population doubling time and saturation density

The population doubling time was determined from the regression line in cell proliferation assay.

Cells	Population doubling time (log phase)	Saturation density (cells/cm ²)	Regression coefficient (from day 1 to day 12)
U251N2	34.4	2.8 × 10 ⁵	0.98
U251N23	33.5	3.5 × 10 ⁵	0.96
U251 <i>cx43</i> -216	43.1	1.0 × 10 ⁵	0.99
U251 <i>cx43</i> -217	43.9	1.1 × 10 ⁵	0.99
T98G	33.4	3.1 × 10 ⁵	0.96
T98GN28	33.9	2.9 × 10 ⁵	0.99
T98G <i>cx43</i> -27	53.2	1.0 × 10 ⁵	0.92
T98G <i>cx43</i> -220	48.6	1.0 × 10 ⁵	0.97

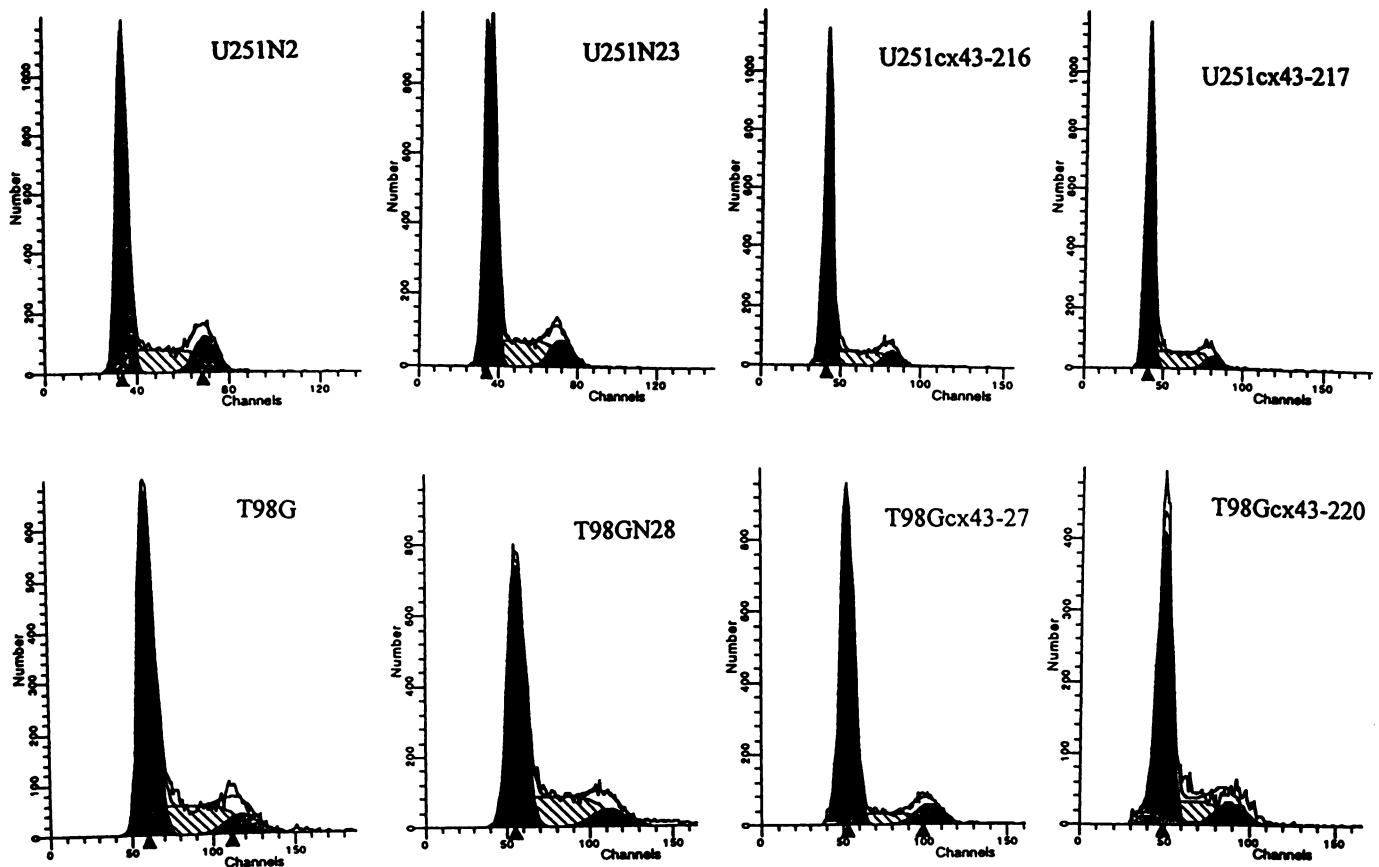


Fig. 4. Cell cycle distribution of log phase control cells and cx43 transfected cells. Cells were plated and sampled on day 4 for FACScan analysis of cell cycle distribution of G₁, S phase, and G₂-M as determined by DNA content.

cx43 transfected cells also reached lower saturation densities than their controls. The average saturation density for cx43 transfected cells was about $1.0 \times 10^5/\text{cm}^2$, whereas control transfected cells have an average saturation density $\sim 3.0 \times 10^5/\text{cm}^2$.

The decreased cell proliferation rates in cx43 expressing cell lines could be caused by either an increase of a noncycling population of cells or a slower transit time through their cell cycle. To distinguish between these two possibilities, we analyzed the cell cycle distribution of log phase cells by FACS analysis. Representative FACS analysis is shown in Fig. 4, and these experimental results are summarized in Table 2. The most profound difference between cx43 transfected cells and control cells is that cx43 transfected cells have a prolonged G₁ phase.

The morphology of cx43 transfected cells is significantly different from control cells (Fig. 5). The cx43 transfected cells are flatter and larger. On the other hand, control transfected cells were more fibroblast-like in morphology and, when confluent, became smaller and denser, finally proliferating on top of each other. These results clearly demonstrate that cx43 expression can reverse the sensitivity of cells to density-dependent factors and alter cellular morphology.

The formation of colonies in soft agar is one of several common phenotypes to assess cellular transformation. Control transfected cells grew well in anchorage-independent culture, forming numerous large loose clusters of ~ 200 – 800 cells in soft agar, whereas clones expressing cx43 grew poorly with almost no colony formation in soft agar (Fig. 6 and Table 3).

The most stringent approach to test tumor suppressor function of cx43 is tumorigenicity assays. Tumorigenicity assays were carried out

by the injection of 1×10^7 log phase cells into the shoulder regions of nude mice. The results, summarized in Table 4, indicate that tumor formation by clones that overexpress cx43 is significantly reduced when measured by tumor formation efficiency and the average tumor weight compared with non-cx43 expressing cells. All of the injected sites from control transfected U251 cells developed tumors in nude mice, whereas only $\sim 25\%$ of injected sites from cx43 expressing cells (U251cx43-216 and U251cx43-217) developed tumor. Statistical analysis (unpaired *t* test) demonstrated a highly significant probability (*P*s, 0.03–0.008) for comparison of each cx43-expressing clone with its non-cx43-expressing control. T98G cells did not form any tumor in nude mice, consistent with previous reports.

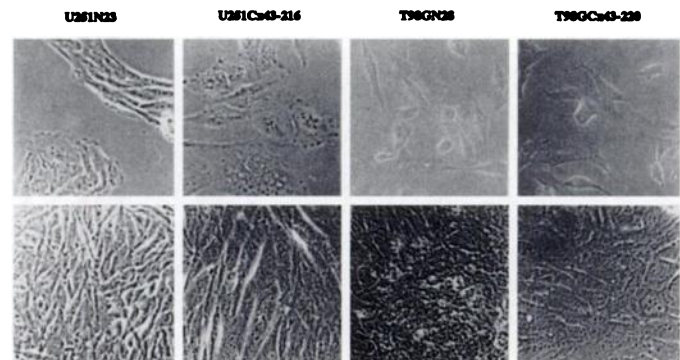


Fig. 5. Comparison of the morphology of control transfected and cx43 transfected clones at high- and low-cell density by phase-contrast images. The flatter and more ordered cellular configuration was seen in cx43 transfected cells.

Table 2 Cell cycle distribution and cell cycle phase duration times

The log phase cells at day 3 after seeding were analyzed by FACScan for G₁, S phase, and G₂-M phase. The distribution of cell cycle phase is derived from three individual experiments. The duration of G₁, S phase, and G₂-M phase time was calculated based on the fraction of cells in each specific cell cycle phase and the doubling time determined as shown in Fig. 4 and Table 1.

	Phase	%	Hours
U251N2	G ₁	61.72 ± 1.90	21.23
	S	25.37 ± 2.38	8.73
	G ₂ -M	12.91 ± 0.48	4.44
U251N23	G ₁	64.71 ± 1.26	21.68
	S	23.71 ± 0.61	7.94
	G ₂ -M	11.58 ± 1.22	3.88
U251cx43-216	G ₁	71.90 ± 4.55	30.98
	S	18.47 ± 2.78	7.96
	G ₂ -M	9.64 ± 3.06	4.15
U251cx43-217	G ₁	68.16 ± 4.70	29.92
	S	22.59 ± 4.62	9.92
	G ₂ -M	9.26 ± 3.27	4.06
T98G	G ₁	66.56 ± 0.37	22.23
	S	24.84 ± 0.95	8.30
	G ₂ -M	8.61 ± 0.68	2.88
T98GN28	G ₁	61.69 ± 1.80	20.9
	S	29.04 ± 2.64	9.84
	G ₂ -M	8.38 ± 1.98	3.15
T98Gcx43-27	G ₁	78.22 ± 0.67	41.61
	S	14.42 ± 2.29	7.67
	G ₂ -M	7.37 ± 2.94	3.92
T98Gcx43-220	G ₁	71.91 ± 5.16	34.95
	S	18.29 ± 2.05	8.89
	G ₂ -M	9.80 ± 4.94	4.76

Tumor Suppression Function of cx43 Is Independent of GJC.

The results presented above clearly demonstrated that expression of cx43 reverses several transformed phenotypes of human glioblastoma cells. We next determined whether the tumor suppressor function of cx43 is related to GJC. To answer this question in our system, we measured GJC by injection of the fluorescent LY into single cells in a monolayer culture and counted the number of fluorescent neighboring cells 10 min later. As shown in Table 5, the cx43 expressing cells did not significantly increase GJC ability in 7-day-old cultured cells. Immunocytochemistry showed that only a few gap junction plaques could be found. Most of the cx43 protein was distributed in the nucleus and cytoplasm (Fig. 7). However, when we used the same experimental procedures to stain cx43 in T51B rat liver epithelial cells, which abundantly communicate, cx43 was exclusively distributed in gap junction plaques. It is believed that the phosphorylated form of cx43 is responsible for GJC. Therefore, the cx43 in cx43 transfected cell lines may be in an unphosphorylated form. Indeed, the cx43 transfected U251 and T98G cells expressed only unphosphorylated cx43 (Fig. 8). Therefore, it is likely that the tumor suppressor function of cx43 is unrelated to its GJC abilities.

It should be emphasized that these results did not simply reflect the clonal variation. Four other clones expressing cx43 also displayed the slower proliferation rates in monolayer culture and the inability to form colonies in soft agar in our survey experiments.

Table 3 Colony formation efficiency in soft agar (%)

Colony size $\geq 15,625 \mu\text{m}^2$ was scored as positive. The colony efficiency was determined as percentage of positive/(positive + negative). In experiment 1, cells were seeded at 5,000 cells/60-mm dish; in experiment 2, cells were seeded at 10,000 cells/60-mm dish. Similar results were obtained with higher seeding density.

Cells	Experiment 1	Experiment 2
U251N2	50.0 ± 3.5	61.9 ± 4.5
U251N23	88.0 ± 4.2	76.5 ± 2.1
U251cx43-216	0.0 ± 0.0	3.4 ± 4.7
U251cx43-217	0.0 ± 0.0	1.0 ± 0.0
T98G	42.5 ± 0.7	31.5 ± 2.0
T98GN28	57.0 ± 4.2	41.7 ± 5.0
T98Gcx43-27	12.0 ± 1.4	8.7 ± 0.6
T98Gcx43-220	11.8 ± 1.1	3.0 ± 0.0

DISCUSSION

Connexins are a family of genes that are responsible for establishing junctional communication between cells and some of which have tumor suppressor function. Transfection of cx genes into various types of tumor cells leads to suppression of their growth *in vitro* and *in vivo*. To study the role of cx43 in human brain gliomas, one of leading causes of death among young children and adults, we examined the expression of cx43 in human brain tumor cell lines by Western blot and semiquantitative PCR assays. Our results show that expression of cx43 is frequently decreased in human brain tumor cell lines. Gene transfection experiments provide strong evidence that the restoration of cx43 expression in human glioblastoma cells reduces tumor cell proliferation *in vitro* and *in vivo*, supporting the notion that cx43 may act as a tumor suppressor gene in human glioblastoma. This is the first report of cx43 expression in human brain tumor cells and the suppression of human glioblastoma cell growth by cx43. The study suggests that cx43 is a good candidate for gene therapy in human glioblastoma.

The aberrant or reduced expression of various cx molecules has been reported in numerous cancer cells such as human prostate cancer cells (cx43; Refs. 18, 19), human breast cancer cells (cx43; Refs. 20, 21), human bladder cancer cells (cx26; Ref. 22), and rat hepatocarcinoma cells (cx32; Ref. 23). In the present study, we show, moreover, that expression of cx43 is also reduced in human brain tumor cells. The down-regulation of cx43 expression in human brain tumor cells is likely to take place at the transcriptional level. Such transcriptional down-regulation of cx43 expression is also observed in human prostate cancer cells. The mechanism responsible for the down-regulation of cx43 expression is currently unknown, but it may be attributed to one or more of the following reasons: (a) deletion of the cx43 promoter; (b) point mutation in the promoter; (c) methylation of inactivation; (d) loss of transactivation factor; and (e) presence of an inhibitory factor. The identification of mechanisms responsible for the loss of cx43 expression in human glioblastoma cells may provide a molecular basis for the development of suitable drugs to activate cx43 expression in human glioblastoma cells.

Tumor suppressor genes fall into two main categories: (a) class I consists of those with DNA mutations, such as p53 (24), WT-1 (25),

Table 4 Tumorigenicity assay of cx43 transfected cells

Tumor weight was measured as final weight in mg. Average tumor weight was expressed as total tumor weight from all injected mice divided by the number of total injected mice in each group. P was determined by t test. The data were derived from two separate experiments. In experiment 1, three animals were injected with each clone. In experiment 2, six animals were injected with U251N23 and U251cx43-216, respectively.

Cells	Efficiency (tumors/injection)	Average tumor weight ± SD (mg)	P		
			U251N2	U251N23	U251cx43-216
U251N2	3/3	27.2 ± 17.0			
U251N23	9/9	112.9 ± 3.1	0.0759		
U251cx43-216	2/9	1.7 ± 3.1	0.0008	0.0019	
U251cx43-217	1/3	2.1 ± 3.7	0.0334	0.0065	0.4157

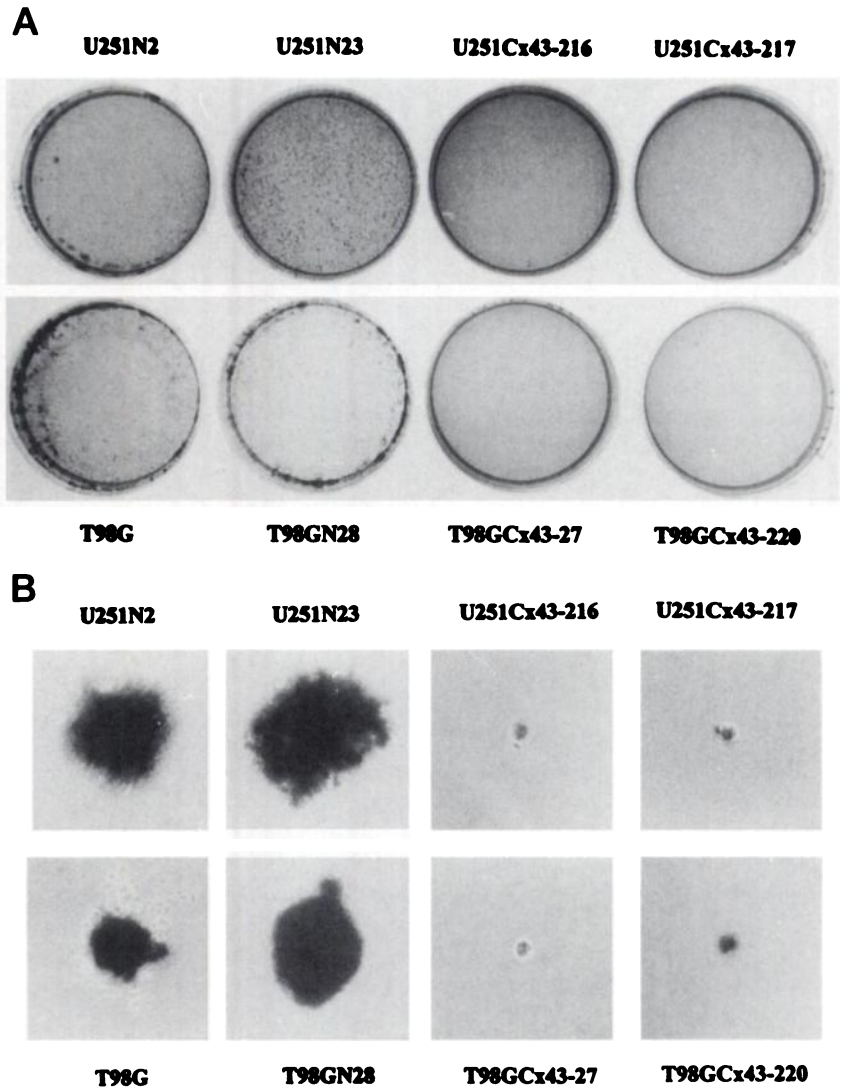


Fig. 6. *cx43* expression reduces the soft agar colony-forming ability of human glioblastoma cells. The *cx43* expressing clones were assayed for anchorage-independent growth by soft agar as shown in total colonies (A) and in the representative individual colony (B). The appearance of large clones was readily detected in control transfected cells; very few colonies were present in *cx43* transfected cells. The experiments were repeated three times with similar results.

BRCA1 (26), and *p16* (27); (b) class II comprises those with reduced expression in tumor cells, including maspin (28), integrin $\alpha 6$ (29), and *Egr-1* (13, 15). The data presented here support the idea that *cx43* acts like a tumor suppressor gene of the class II type. In fact, in spite of multiple effort, thus far, no *cx* mutations have been found in human tumor tissues. From the viewpoint of cancer therapy, class II genes may be a more attractive and suitable target for therapy inasmuch as

one can invent drugs that will reactivate the expression of the genes rather than replace defective DNA.

The Role of *cx43* and GJC in the Control of Cell Growth. We report here that stable expression of *cx43* suppresses human glioblastoma proliferation *in vivo* and *in vitro*. However, the tumor suppressor function of *cx43* in this model system is not correlated by GJC. This conclusion is supported by the following observations: (a) there is no significant difference in dye transfer between *cx43* transfected cells and control cells; (b) *cx43* transfected cells predominantly express the unphosphorylated form of *cx43*, whereas posttranslational phosphorylation of *cx43*—a supposedly critical requirement for the assembly of gap junction—was absent in *cx43* transfected cells; and (c) most of *cx43* locates in the nucleus and cytoplasm as evidenced by immunofluorescent staining. The results observed in this study are not caused by this specific plasmid. We transfected the same plasmid into other cell lines and observed significantly enhanced GJC in those *cx43* transfected cell lines. The communication deficiency in *cx43* transfected cells in this study remains to be defined but could be due to decreased expression of cell adhesion molecules (30), increased glycosylation (31), or defects in specific protein kinases responsible for *cx43* phosphorylation. In the present study, we used LY transfer to measure GJC, which may not accurately reflect the ability of intra-

Table 5 Gap junction intercellular communication in *cx43* transfected cells

Cells	No. of fluorescent neighboring cells ^a
U251N2	2.16 ± 2.99 (67)
U251N23	2.67 ± 3.40 (54)
U251 <i>cx43</i> -216	4.87 ± 6.14 (58)
U251 <i>cx43</i> -217	5.21 ± 6.60 (59)
T98G	0.95 ± 1.75 (43)
T98GN28	1.58 ± 3.05 (58)
T98G <i>cx43</i> -27	1.60 ± 2.50 (30)
T98G <i>cx43</i> -220	2.70 ± 2.59 (30)
T51B ^b	68.39 ± 10.98 (31)

^a Gap junction intercellular communication was determined by the number of fluorescent neighboring cells 10 min after injection of LY into single cells. The values are the average of at least 30 injections as indicated in parentheses.

^b T51B are rat liver epithelial cells with good communication ability and used here as positive control.

cellular substances to pass through gap junctions. Nevertheless, although numerous lines of evidence support the proposed functions of GJC in the regulation of growth control, the key questions regarding the role of cx43 and GJC in the control of cell growth still remain unanswered. Several lines of evidence suggest that the function of cx43, including cx43 in cell proliferation, is due to the action of cx43 itself rather than being related to GJC. An additional argument against the need for GJC to suppress tumor formation comes from soft agar assays. Single cells are plated in soft agar and do not communicate with neighboring cells; thus, the passage of information through gap junction is not required for the suppression of cell proliferation in soft agar. In addition to the membrane localization, cx43 is also localized in the nucleus and can directly bind to DNA, which suggests that cx43 may function in the nucleus separately from its well-known GJC function (32). In our transfected cells, cx43 primarily localized in the nucleus and cytoplasm. Cx43 that transfected into transformed dog kidney epithelial cells was also strongly positive in the nucleus (8). Expression of cx43 in HeLa cells significantly reduced chemically induced genetic changes. This function of cx43 is not directly related to GJC inasmuch as the inhibition of GJC by α -glycyrrhetic acid did not affect cx43 function (10). Immortalized cells from embryonic cx43 knockout mice (cx43^{-/-}) proliferate at faster rates and grow to higher saturation densities. Reintroducing cx43 gene into the cx43^{-/-} cells reduced the cell proliferation rates and saturation densities without significant restoration of GJC (from less than 1 cell of cx43 null clones to 3–4 cells of cx43 transfected clones; Ref. 33). Additional evidence comes from studies using three mutated cx26 genes (C60F, P87L, and R143W), which were transfected into HeLa cells that already expressed exogenous cx26. Interestingly, transfection of the P87L and R143W mutants enhanced the tumorigenicity of HeLa cx26 cells without any change in GJC capacity. In contrast, transfection of the C60F mutant reduced the GJC of cx26-expressing HeLa cells without affecting their growth *in vivo* (34). Our results,

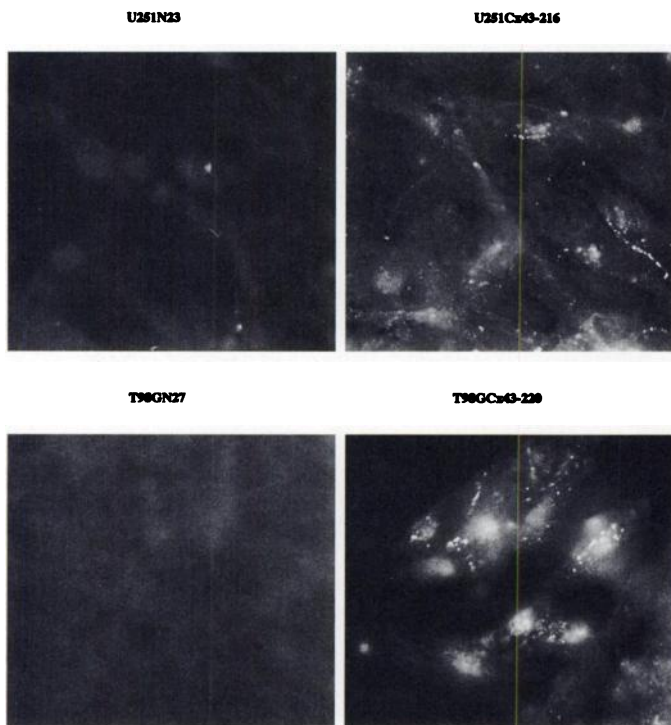


Fig. 7. Immunofluorescence staining of cx43 in control cell lines (U251N23 and T98GN28) and cx43 FACS transfected cell lines (U251cx43-216 and T98Gcx43-220). Cells were cultured on glass coverslips and incubated with a cx43-specific antiserum. FITC-conjugated goat-antirabbit IgG was used to detect the sublocalization of cx43.

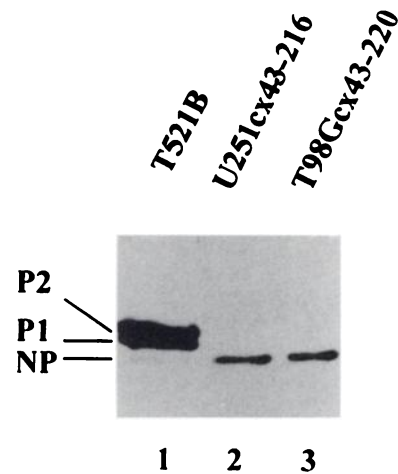


Fig. 8. cx43 transfected cell lines predominantly expressed the unphosphorylated form of cx43. Equal amounts of protein were loaded and separated by 10% SDS-PAGE. The cx43 protein was visualized by anti-cx43 serum and the enhanced chemiluminescence system.

together with other observations, suggest that the tumor suppressor function of cx may be uncoupled from GJC. These studies also raise the intriguing question concerning the molecular mechanism of cx43 in the suppression of tumor cell growth.

In conclusion, this study raises several questions that deserve further investigation: (a) the inactivation of cx43 expression appears to be involved in the development of human gliomas; (b) the model system described here can be used to further study the molecular mechanisms and define the specific functional domains of cx43 responsible for the tumor suppression; and (c) the restoration of cx43 expression may represent a significant approach to the normalization of human glioblastoma cell growth.

ACKNOWLEDGMENTS

We thank Dr. Glenn I. Fishman (Albert Einstein College of Medicine, Bronx, NY) for human cx43 cDNA.

REFERENCES

1. Yamasaki, H., and Naus, C. C. G. Role of connexin genes in growth control. *Carcinogenesis (Lond.)*, *17*: 1199–1213, 1996.
2. Lau, A. F., Kanemitsu, M. Y., Kurata, W. E., Danesh, S., and Boynton, A. L. Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Mol. Biol. Cell*, *3*: 865–874, 1992.
3. Schmidt, J. N., Traenckner, E. B., Meier, B., and Baeuerle, P. A. Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF- κ B. *J. Biol. Chem.*, *270*: 27136–27142, 1995.
4. Zhu, D., Caveney, S., Kidder, G. M., and Naus, C. C. Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc. Natl. Acad. Sci. USA*, *88*: 1883–1887, 1991.
5. Naus, C. C., Elisevich, K., Zhou, D., Beliveau, D. J., and Del Maestro, R. F. *In vivo* growth of C6 glioma cells transfected with connexin43 cDNA. *Cancer Res.*, *52*: 4208–4213, 1992.
6. Hirschi, K. K., Xu, C. E., Tsukamoto, T., and Sager, R. Gap junction gene cx26 and cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth Differ.*, *7*: 861–870, 1996.
7. Eghbali, B., Kessler, J. A., Reid, L. M., Roy, C., and Spray, D. C. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with cx32 cDNA retards growth *in vivo*. *Proc. Natl. Acad. Sci. USA*, *88*: 10701–10705, 1991.
8. Chen, S.-C., Pelletier, D. B., Ao, P., and Boynton, A. L. Connexin43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin-dependent kinases. *Cell Growth Differ.*, *6*: 681–690, 1995.
9. Proulx, A. A., Lin, Z.-X., and Naus, C. C. G. Transfection of rhabdomyosarcoma cells with connexin43 induced myogenic differentiation. *Cell Growth Differ.*, *8*: 533–540, 1997.
10. Zhu, W.-B., Mironov, N., and Yamasaki, H. Increased genetic stability of HeLa cells after connexin 43 gene transfection. *Cancer Res.*, *57*: 2148–2150, 1997.
11. Kenney, N. J., Huang, R.-P., Johnson, G., Wu, J. X., Okamura, D., Metheny, W., Gordon, E., Gulick, W., Plowman, G., Smith, G. H., Salomon, D. S., and Adamson,

- E. D. Expression of amphiregulin (AR) and cripto (CR-1) in the developing murine mammary gland. *Mol. Reprod. Dev.*, 41: 277–286, 1995.
12. Huang, R. P., Wu, J. X., Fan, Y., and Adamson, E. D. UV activates growth factor receptors via reactive oxygen intermediates. *J. Cell Biol.*, 133: 211–220, 1996.
 13. Huang, R. P., Fan, Y., De Belle, I., Niemeier, C., Gottardis, M. M., Mercola, D., and Adamson, E. D. Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. *Int. J. Cancer*, 72: 102–109, 1997.
 14. Huang, R. P., Fan, Y., deBelle, I., Ni, Z., Matheny, W., and Adamson, E. D. Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation. *Cell Death Differ.*, 5: 96–106, 1998.
 15. Huang, R. P., Liu, C., Fan, Y., Mercola, D., and Adamson, E. D. Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. *Cancer Res.*, 55: 5054–5062, 1995.
 16. Huang, R. P., Darland, T., Okamura, D., Mercola, D., and Adamson, E. D. Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. *Oncogene*, 9: 1367–1377, 1994.
 17. Huang, R. P., Ngo, L., Okamura, D., Tucker, M., and Adamson, E. D. V-sis induces Egr-1 expression by a pathway mediated by c-Ha-Ras. *J. Cell. Biochem.*, 56: 469–479, 1994.
 18. Tsai, H., Werber, L., Davia, M. O., Edelman, M., Tanaka, K. E., Melman, A., Christ, G. J., and Gelieber, J. Reduced connexin43 expression in high grade, human prostatic adenocarcinoma cells. *Biochem. Biophys. Res. Commun.*, 227: 64–69, 1996.
 19. Wilgenbus, K. K., Kirkpatrick, C. J., Kneuchel, R., and Traub, O. Expression of cx26, cx32 and cx43 gap junction proteins in normal and neoplastic human tissues. *Int. J. Cancer*, 51: 522–529, 1992.
 20. Lee, S.-W., Paul, D. L., Keyomarsi, K., and Sager, R. Transcriptional down-regulation of gap junction proteins blocks junctional communication in human mammary tumor cell lines. *J. Cell Biol.*, 118: 1213–1221, 1992.
 21. Tomasetto, C., Neveu, M. J., Daley, J., Horan, P. K., and Sager, R. Specificity of gap junction communication among human mammary cells and connexin transfections in culture. *J. Cell Biol.*, 122: 157–167, 1993.
 22. Grossman, H. B., Liebert, M., Lee, I. W., and Lee, S. W. Decreased connexin expression and intercellular communication in human bladder cancer cells. *Cancer Res.*, 54: 3062–3065, 1994.
 23. Tsuda, H., Asamoto, M., Baba, H., Iwahori, Y., Matsumoto, K., Iwase, T., Nishida, Y., Nagao, S., Hakoi, K., and Yamaguchi, S. E. Cell proliferation and advancement of hepatocarcinogenesis in the rat associated with a decrease in connexin 32 expression. *Carcinogenesis (Lond.)*, 16: 101–105, 1995.
 24. Finlay, C. A., Hinds, P. W., and Levine, A. J. The *p53* protooncogene can act as a suppressor of transformation. *Cell*, 57: 1083–1093, 1989.
 25. Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., Jones, C., and Housman, D. E. Isolation and characterization of a Zinc finger polypeptide gene at the human chromosome 11 Wilm's tumor locus. *Cell*, 60: 509–520, 1990.
 26. Miki, Y. E. a strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science (Washington DC)*, 266: 66–69, 1994.
 27. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q.-Y., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. *Science (Washington DC)*, 264: 436–440, 1994.
 28. Zou, A., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science (Washington DC)*, 263: 526–529, 1994.
 29. Sager, R., Anisowicz, A., Neveu, M., Liang, F., and Sotiropoulou, G. Identification by differential display of $\alpha 6$ integrin as a candidate tumor suppressor gene. *FASEB J.*, 7: 964–970, 1993.
 30. Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. Differential phosphorylation of the gap junction protein cx43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.*, 111: 2077–2088, 1990.
 31. Wang, Y., Metha, P. P., and Rose, B. Inhibition of glycosylation induces open connexin43 cell-cell channels and phosphorylation and Triton X-100 insolubility of connexin43. *J. Biol. Chem.* 270: 26581–26585, 1995.
 32. de Feijter, A. W., Matesic, D. F., Ruch, R. J., Guan, X. J., Chang, C. C., and Trosko, J. E. Localization and function of the connexin 43 gap-junction protein in normal and various oncogene-expressing rat liver epithelial cells. *Mol. Carcinog.*, 16: 203–212, 1996.
 33. Martyn, K. D., Kurata, W. E., Warn-Cramer, B. J., Burt, J. M., TenBroek, E., and Lau, A. F. Immortalized connexin43 knockout cell lines display a subset of biological properties associated with the transformed phenotype. *Cell Growth Differ.*, 8: 1015–1027, 1997.
 34. Dufloy-Dancer, A., Mesnil, M., and Yamasaki, H. Dominant-negative abrogation of connexin-mediated cell growth control by mutant connexin genes. *Oncogene*, 15: 2151–2158, 1997.