Neuronal Differentiation and Growth Control of Neuro-2a Cells After Retroviral Gene Delivery of Connexin43*

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Given the roles proposed for gap junctional intercellular communication in neuronal differentiation and growth control, we examined the effects of connexin43 (Cx43) expression in a neuroblastoma cell line. A vesicular stomatitis virus G protein (VSVG)-pseudotyped retrovirus was engineered to co-express the green fluorescent protein (GFP) and Cx43 in the communication-deficient neuro-2a (N2a) cell line. The 293 GPG packaging cell line was used to produce VSVG-pseudotyped retrovectors coding for GFP, Cx43, or chimeric Cx43-GFP fusion protein. The titer of viral supernatant, as measured by flow cytometry for GFP fluorescence, was approximately $2 \times 10^5$ colony forming units (CFU)/ml and was free of replication-competent retroviruses. After a 7-day treatment with retinoic acid (20 μM), N2a transforms (N2a-Cx43 and N2a-Cx43-GFP) maintained the expression of Cx43 and Cx43-GFP. Expression of both constructs resulted in functional coupling, as evidenced by electrophysiological and dye-injection analysis. Suppression of cell growth correlated with expression of both Cx43 or Cx43-GFP and retinoic acid treatment. Based on morphology and immunocytochemistry for neurofilament, no difference was observed in the differentiation of N2a cells compared with cells expressing Cx43 constructs. In conclusion, constitutive expression of Cx43 in N2a cells does not alter retinoic acid-induced neuronal differentiation but does enhance growth inhibition.

Gap junctions are intercellular plasma membrane channels which provide direct cytoplasmic continuity between adjacent cells as well as coordination of the function of individual cells (1). These channels mediate direct exchange of ions and small molecules (less than 1.2 kDa) including second messengers and electrical current among adjacent cells (2, 3). The fundamental structural unit of the gap junction is the connexin (Cx) subunit. To clarify the role of gap junctional intercellular communication (GJIC) in neural development, the temporal and cellular expression of connexins has been studied. Cx43 is present in neurons in vivo (4) and in vitro (5–7). Several studies have demonstrated that neuronal GJIC decreases during differentiation (8). In addition, we have shown that Cx43-mediated GJIC decreases with neuronal differentiation (7, 9, 10).

Given the multiple roles proposed for Cx43-mediated GJIC in development and differentiation, many studies have been undertaken to alter Cx43 expression both in vitro through transfection (11–13) and in vivo through transgenesis (14, 15). The major limitations to transfection approaches have been efficiency and time required to obtain stable gene expression. The recent advent of retroviral vectors to efficiently deliver genes for stable expression overcomes these limitations (16, 17). Retroviral vectors enable efficient gene transfer and stable gene expression in cells that are not readily susceptible to transfection, such as primary cells, cells in vivo, and neuronal cell lines (18). However, the titer of most current retroviral supernatants is too low (<10^7 particles/ml) to achieve acceptable clinical results (19). To overcome this deficit, more recently developed retrovectors pseudotyped with the vesicular stomatitis virus G (VSVG) protein have become the gene delivery tool of choice (16). VSVG-pseudotyped retrovectors have advantages over the current viral vectors in terms of high titer, complement resistance, particle stability and tumor specificity (19).

In this study, the VSVG-pseudotyped retrovector enabled efficient stable expression of Cx43 and Cx43-GFP. Cell growth was suppressed but neuronal differentiation was unaffected.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The C6 astrocytoma cell line (American Type Culture Collection (ATCC), Manassas, VA; CCL-107) and neuro-2a mouse neuroblastoma cell line (ATCC, CCL-131) were maintained as monolayer cultures in Dulbecco's modified essential medium (DMEM) (Life Technologies, Inc., Burlington, ON) supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Life Technologies, Inc.) at 37 °C, in a humidified atmosphere containing 95% air and 5% CO₂.

The 293 GPG retroviral packaging cell line (20) was a generous gift from Dr. Richard C. Mulligan (Children's Hospital, Boston, MA). 293 GPG cells were maintained as monolayer cultures in 293 medium consisting of DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.3 μg/ml G418 (Mediatech, Herndon, VA), 2.0 μg/ml puromycin (Sigma Chemical Co.), and 1.0 μg/ml tetracycline (Fisher Scientific, Nepean, Ontario, Canada) at 37 °C, in a humidified atmosphere containing 95% air and 5% CO₂.

Retroviral Construction and Synthesis—The AP2 retroviral vector (19) was used in these studies. Two genes including an inserted cDNA and the enhanced green fluorescent protein reporter gene can be expressed from this single bicistronic, nonsplicing murine plasmid ret-
The cDNA fragment coding for IRES and EGFP was removed from the plasmid backbone by single endonuclease digestion with NotI, and the resulting fragment was religated together as outlined below. The resulting plasmid was designated NAP2 (Fig. 1B).

The Cx43 cDNA (1.45 kilobases) (43) (kindly provided by Dr. E. Beyer, Washington University, School of Medicine, St. Louis, MO) and the Cx43-GFP chimeric cDNA (2.25 kilobases) was removed from the pEGFPN1Cx43 plasmid (44) was inserted into the NAP2 vector. The resulting plasmids were designated NAP2Cx43 and NAP2Cx43-GFP (Fig. 1, C and D).

Production of VSVG-pseudotyped Retrovirus—To produce pseudotype retroviral vectors, 293 GPG cells were plated at 2 × 10^6 cells per 60-mm dish the night before transfection in 293 media. They were then transiently transfected with AP2, NAP2Cx43, and NAP2Cx43-GFP plasmid retrovectors by the LipofectAMINE PLUS™ (Life Technologies, Inc.) procedure according to the manufacturer's instructions. Serum-free DMEM medium with tetracycline (1 μg/ml) was used for all of the transfection processes. After a 6-h incubation of cells in the incubator, an additional 2 ml of the 293 medium was added for transfection. The following morning, the medium was replaced with fresh 293 medium. On the next day, the 293 medium was removed from cells and replaced with 3 ml of DMEM with 10% fetal calf serum. On the next day, the supernatants were collected about every 12–24 h for 1 week. All culture supernatants were filtered through 0.45-μm syringe-mounted filters (Gelman Sciences, Ann Arbor, MI). The filtered culture supernatants were used as infectious viral stock for subsequent experiments. Aliquots of 1.0 ml were also frozen at −80 °C for later use.

Titration of Retroviral Supernatant—Flow cytometric analysis was performed to determine the titer of the viral supernatant as measured by GFP fluorescence. In brief, 2 × 10^5 C6 cells/well were plated in 6-well tissue culture plates the night before infecting the cells with the retrovirus. The next day, cells from 3 wells were trypsinized and counted to determine the average number of cells per well at the time of exposure to retrovirus. Serial dilutions (1:10) of the viral supernatant in a final volume of 1 ml of DMEM, 10% fetal bovine serum were prepared and added to each well. The following day, 2.0 ml of medium were added, and cells were incubated for 2 more days. Three days after viral infection, the transduced C6 cells were trypsinized and resuspended in 2 ml of DMEM for flow cytometric analysis assay. Analysis was performed on a FACScan™ (Becton Dickinson). Live C6 cells were gated based on scatter/side scatter profile and analyzed for GFP fluorescence to determine the percentage of GFP-positive cells. Data acquisition and analysis were performed using CELLQuest™ software (Becton Dickinson). The titer (colony-forming units (CFU) per ml) was calculated as: (% GFP-positive cells × cell number at initial viral exposure)/viral volume (ml) applied when transduction was not saturated (19).

Replication Competent Retrovirus (RCR) Assay—C6 cells infected with GFP were passed in culture for 6 weeks (15 passages) to allow for spread of RCR that might be present. 3 ml of culture supernatant from these C6 cells was then used to infect 10^6 naïve C6 cells, and 48 h later, the newly infected naïve cells were evaluated by detecting GFP-fluorescent cells.

Protein Isolation and Western Blot Analysis—Both undifferentiated and differentiated N2a wild-type and transformant cell cultures were washed twice with PBS and scraped off the plates in lysis buffer (0.05 M Tris, pH 6.8, 0.1% SDS) with a rubber policeman. The protein concentration of the cell lysate was determined in each case using the Bio-Rad protein assay kit. Protein samples from an equal number of cells (2.5 × 10^5 each) were subjected to 10% SDS-polyacrylamide gel electrophoresis. The gel was transferred to nitrocellulose membranes

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**Fig. 1. Schematic representations of plasmid retrovectors.** A, AP2 plasmid retrovector is a bicistronic murine retroviral vector allowing the insertion of a cDNA sequence in multiple cloning sites upstream of the IRES and the EGFP cassette. B, NAP2 plasmid retrovector resulted from cutting the IRES and EGFP fragment of the AP2 plasmid retrovector. C, the NAP2-Cx43 plasmid retrovector resulted from insertion of Cx43 cDNA into the NotI-ClaI cloning sites of NAP2. D, the NAP2-Cx43-GFP retrovector resulted from insertion of Cx43-GFP cDNA into the BglII-NotI sites.
Roles of Cx43 in Neuronal Differentiation and Growth Control

Effects of Retroviral Infection of C6 Glioma Cells—C6 cells were chosen for titering retrovector supernatant as C6 cells divide rapidly and are easily infected. Using GFP as a reporter protein allowed the rapid titration of retroviral supernatant. The supernatant containing AP2 retrovector was sequentially collected, filtered, and serially diluted in a final volume of 1 ml and added to 3.28 × 10⁵ C6 cells/well in a 6-well plate. Three days after a single application of retrovector, the infected C6 cells were analyzed by flow cytometric analysis to determine the percentage of cells expressing the GFP reporter protein (Fig. 2). The titer extrapolated from these experiments was about 2.0 × 10⁴ CFU/ml, calculated as described under “Experimental Procedures.” This titer is at least 1,000 times higher than that of NIH 3T3-based retroviral packaging cell lines (22).

Results

Effectiveness of Retroviral Infection of C6 Glioma Cells—C6 cells were chosen for titering retrovector supernatant as C6 cells divide rapidly and are easily infected. Using GFP as a reporter protein allowed the rapid titration of retroviral supernatant. The supernatant containing AP2 retrovector was sequentially collected, filtered, and serially diluted in a final volume of 1 ml and added to 3.28 × 10⁵ C6 cells/well in a 6-well plate. Three days after a single application of retrovector, the infected C6 cells were analyzed by flow cytometric analysis to determine the percentage of cells expressing the GFP reporter protein (Fig. 2). The titer extrapolated from these experiments was about 2.0 × 10⁴ CFU/ml, calculated as described under “Experimental Procedures.” This titer is at least 1,000 times higher than that of NIH 3T3-based retroviral packaging cell lines (22).

Stability of Retroviral Gene Delivery and Replication Competent Retrovirus Assay—C6 cells infected with viral supernatant containing AP2 retrovectors demonstrated strong GFP fluorescence for at least 15 passages over a 6-week period (Fig. 3). Naturally or because of recombination events, viruses that contain all of the cis-acting viral elements and genes coding for necessary viral structural proteins are “replication-competent” (18). To test for RCR, 3 ml of culture supernatant from these C6 cells was then used to infect 10⁶ naïve C6 cells. 48 h later the newly infected naïve cells were evaluated by detecting GFP-fluorescent cells. Retroviruses produced from 293 GPg packaging cells were free of detectable RCR upon long term cultivation as demonstrated by the failure of GFP fluorescence to be transferred from virally transduced C6 cells to naïve C6 cells (data not shown).

Expression of Cx43 and Cx43-GFP in Differentiated N2a Cells—Communication-deficient N2a cells were infected with retrovector encoding for Cx43 and Cx43-GFP to determine their ability to express full-length Cx43 and Cx43-GFP chimeric protein after differentiation. Wild-type N2a and N2a-GFP cells were used as controls. The synthesis of Cx43 and Cx43-GFP protein was investigated by Western blot analysis of total cell lysates from wild-type N2a cells, N2a-GFP, N2a-Cx43, and N2a-Cx43-GFP transforms under differentiated conditions. Differentiated N2a-Cx43 and N2a-Cx43-GFP cells expressed Cx43 protein (42–44 kDa) and Cx43-GFP protein (72–74 kDa) (Fig. 4). As expected Cx43 was not detected in wild-type N2a cells or N2a-GFP expressing cells. The C6–13 cells which were transfected with Cx43 were used as a positive control, and contain multiple bands of immunoreactivity, which represents phosphorylated and unphosphorylated species of Cx43 (23). The low amount of phosphorylated species in N2a cells infected with wild-type Cx43 or Cx43-GFP cDNAs may reflect poor post-translational processing of Cx43 in this cell line.
Localization of Cx43 and Cx43-GFP in Undifferentiated and Differentiated N2a Cells—To determine the localization of Cx43 in N2a-Cx43, N2a-Cx43-GFP, N2a-GFP, or wild-type N2a cells, differentiated or undifferentiated cells were either immunolabeled with anti-Cx43 antibodies or directly examined for GFP fluorescence (Fig. 5).

In both undifferentiated and differentiated N2a-Cx43 and N2a-Cx43-GFP cells, Cx43 was localized to areas of cell-cell contact as well as in punctate structures within the cytoplasm (Fig. 5, A, B, E, and F). As control, GFP fluorescence was located throughout the cytoplasm in N2a-GFP cells (Fig. 5, C and G) and no fluorescence was detected in wild-type N2a cells immunolabeled for Cx43 (Fig. 5, D and H). In all cases, the undifferentiated N2a and N2a transformants were not neurofilament positive (data not shown).

An induction of neurofilament protein (200 kDa, phosphorylated and non-phosphorylated) was apparent in processes of wild-type N2a cells and all three N2a transformants (N2a-GFP, N2a-Cx43 and N2a-Cx43-GFP) after 20 μM RA treatment for 7 days (Fig. 5, I, J, K, and L). On average, 90% of the cells were neurofilament positive after exposure to 20 μM RA for 7 days. There was no apparent difference in neurofilament staining between wild-type N2a, N2a-Cx43 and N2a-Cx43-GFP cells.

Gap Junctional Intercellular Electrical Coupling Is Up-regulated When Cx43 or Cx43-GFP Is Expressed in N2a Cells—To examine the dye-coupling capacity of Cx43 or Cx43-GFP-mediated GJIC, both undifferentiated and differentiated N2a wild-type and transformants were microinjected with 10 mM carboxyfluorescein (CF). Undifferentiated and differentiated N2a-Cx43-GFP cells (Fig. 6, A and B, asterisk), N2a-Cx43 cells (Fig.
E and F, asterisk) and N2a wild type (Fig. 6, I and J, asterisk) were injected with CF. CF spreads rapidly to adjacent cells in both differentiated and undifferentiated N2a-Cx43-GFP and N2a-Cx43 cells within a minute (Fig. 6, A, B, E, and F). The gap junctional plaques formed by Cx43-GFP were seen as punctate fluorescent spots between adjacent cells (Fig. 6, A and B, arrows). N2a-Cx43 and N2a-Cx43-GFP cells effectively transferred CF to neighboring cells in more than 70% of cases under undifferentiated conditions and in greater than 50% after neuronal differentiation (Table I).

Furthermore, CF was extensively transferred to at least the 6th order of neighboring undifferentiated Cx43 or Cx43-GFP expressing cells (Fig. 6, A and E), although this was reduced to the 2nd to 3rd order when the same cells were differentiated with retinoic acid (Fig. 6, B and F). As a control, communication-deficient wild-type N2a cells exhibited no dye coupling before or after differentiation (Fig. 6, I and J).

**DISCUSSION**

By virtue of its intrinsic fluorescence, GFP is readily detected in living cultured cells, and the efficiency of transduction can be rapidly and directly determined. GFP fluorescence provides a visual assessment for rapidly determining the viral titer by flow cytometric analysis, which is consistent with a direct test of viral-mediated transfer of drug resistance to host cells (24).

In this study, the titer of the AP2 retrovector determined by flow cytometric analysis of GFP fluorescence was about 2.0 \( \times 10^7 \) CFU/ml which is similar to other studies (19). The efficient gene transduction of high titer viral supernatant eliminates the need to generate stable transformed cell lines, which requires months of selection and characterization. Therefore, the AP2 retroviral gene delivery system is well suited for quickly examining expression of exogenous gene(s) in vitro.

Many connexins are differentially expressed during neuronal differentiation. During development, neuroblasts are highly coupled, with a loss or reduction of GJIC during termi-
nal differentiation (8, 25). A similar decrease in the connexin expression level and GJIC has been reported during in vitro differentiation of two neuronal stem cells, i.e., the P19 mouse embryonal carcinoma cell line (9) and the NT2 human teratocarcinoma cell line (6, 7).

In our study, N2a neuroblastoma cells provide an ideal in vitro model to examine the putative role of Cx43-mediated GJIC in neuronal differentiation and growth control because they are of neuronal origin, do not express any known connexins (26), and exhibit no detectable gap junctional intercellular coupling. Retroviral gene transduction, Western blot analysis, and immunocytochemistry revealed that N2a cells were able to maintain the expression of Cx43 and Cx43-GFP protein after neuronal differentiation. Cx43 and Cx43-GFP were localized as punctate staining in the cytoplasm and in the membrane between apposed cells. About 90% of the N2a cells overexpressing Cx43 and Cx43-GFP could be differentiated into mature neurons, similar to wild-type cells. These results suggest that Cx43-mediated GJIC does not alter neuronal differentiation of N2a cells. It is possible that in their undifferentiated state, N2a cells have already gone through a possible GJIC-dependent differentiation stage because undifferentiated N2a cells express a certain level of the neuronal marker microtubule-associated protein 2 (MAP2) (27).

Gap junctions are known to provide a channel for the intercellular flow of essential electrical signals and small molecules (28). The current flow through gap junctions is important in the rapid and synchronous activation of excitable tissues (29). In our studies, all N2a-Cx43 and N2a-Cx43-GFP cells were electrically coupled both before and after neuronal differentiation. However, the cells expressing Cx43-GFP exhibited lower levels of electrical coupling than cells expressing Cx43. Because the carboxyl-terminal region of Cx43 is presumably involved in the regulation of channel gating (1), fusion of GFP to this portion of the connexin may interfere with channel function. Using dual whole-cell patch clamp recording, Bukauskas et al. (30) have reported some alterations in transjunctional voltage gating for N2A cells transfected with Cx43 or Cx43-GFP but offered no explanation for this difference. We have also noted that N2A cells transfected with Cx43 or Cx43-GFP are less dye coupled after differentiation. This result is consistent with the in vivo studies, which have indicated that electrotonic junctions are widespread between mammalian neurons in many areas, including neocortex, hippocampus, inferior olive, locus coeruleus, hypothalamus, striatum, and retina (3, 31, 32).

From our dye-coupling analysis, undifferentiated N2a-Cx43

![Figure 5. Cx43 and Cx43-GFP expression in N2a wild-type and transformants before and after neuronal differentiation.](image-url)
and N2a-Cx43 GFP cells were found to be coupled on average to 6th order neighbors. After differentiation, however, the coupling decreased on average to 2nd or 3rd order. One possibility is that the differentiated cells bear long processes, and their spatial arrangement differs from undifferentiated cells. Therefore, the dye must pass a similar distance but involves fewer cells. It is also possible that the functional dye-coupling capacity of Cx43 and Cx43z GFP does decrease with neuronal differentiation. Our studies demonstrate for the first time highly coupled differentiated neuronal cells, which provide a suitable model to further investigate the electrotonic junction between mature neurons both in vitro and in vivo.

One of the major characteristics of neoplastic cells is their uncontrolled rapid growth. Extensive aspects of tumor growth have been studied, including the implication that loss or lack of gap junctional communication is associated with tumor development (33–37). The introduction and overexpression of connexin cDNAs in tumor cell lines by transfection has demonstrated a reduced growth rate (38–40). C6 cells transfected with Cx43 cDNA exhibited a decrease in cell growth both in vitro and in vivo (11–13, 41). In contrast to C6-Cx43 cells which grow slower than the parental C6 cells, the N2a-Cx43 and N2a-Cx43z GFP transformants demonstrated no significant decrease in growth rate in vitro when undifferentiated. It appears that the establishment of GJIC in communication-deficient

### FIG. 6.
Dye-coupling assay of N2a wild-type and transformants both before and after differentiation. The undifferentiated N2a-Cx43 and N2a-Cx43-GFP cells were coupled on average to 6th order neighbors (A and E). After differentiation, however, the coupling decreased on average to 2nd to 3rd order (B and F). CF spreads rapidly from injected cells (A, B, E, and F; asterisk) to adjacent cells when compared with phase contrast images (C, D, G, and H). The direct visualization of Cx43-GFP during microinjection is shown in A and B (arrows). N2a wild type and N2a-GFP exhibited no dye coupling to adjacent cells either before or after differentiation (I and J) when compared with phase contrast images (K and L). Magnification is × 850.

### FIG. 7.
In vitro growth analysis in the presence of RA (20 μM). N2a wild-type and derived transformants from confluent cultures were seeded at 2.0 × 10⁵ per 6-well culture plate. 3, 5, and 12 days after plating in the presence of RA, triplicate plates were dissociated into individual cell suspensions and counted. The statistical significance (p < 0.05, 0.01, and 0.001) are represented by 1, 2, or 3 asterisks, respectively. n = 6; bars, mean ± S.E.

### TABLE I
Summary of dye injection assay

| Cells                  | No. of injections | % coupled | No. of orders |
|------------------------|-------------------|-----------|--------------|
| Undifferentiated wild-type N2a | 20                 | 0         | 0            |
| Undifferentiated N2a-Cx43      | 65                 | 75        | 6            |
| Undifferentiated N2a-Cx43z GFP | 70                 | 72        | 6            |
| Differentiated wild-type N2a   | 25                 | 0         | 0            |
| Differentiated N2a-Cx43      | 68                 | 55        | 3            |
| Differentiated N2a-Cx43z GFP | 72                 | 52        | 2            |

* The number of injected cells that resulted in CF transfer is expressed as percent.
* The number of orders indicates the number of cells from the injected cell to the farthest cell containing detectable CF.
tumor cells does not always lead to growth inhibition. It has been demonstrated that certain connexin genes exert a growth control effect whereas others do not (38). In addition, gap junctions composed of different connexins demonstrate some aspects of the “bystander effect” associated with Herpes simplex virus thymidine kinase expression and combined pro-drug (i.e. ganciclovir) treatment, we propose that retroviral delivery of connexin genes may be therapeutically relevant.

This study constitutes the first report of an in vitro generation of highly coupled mature neurons obtained using retroviral delivery of connexin constructs. Recent therapeutic applications have employed retroviral delivery of the Herpes simplex virus thymidine kinase gene to brain tumors, with limited success (19). Given the potential tumor suppressor action of Cx43 itself.

This finding suggests that growth inhibition in N2a transformants may be because of the RA treatment and related transjunctional molecule(s) rather than Cx43 itself.

REFERENCES

1. Bruzzone, R., White, T. W., and Paul, D. L. (1996) Eur. J. Biochem. 238, 1−27
2. Bruzzone, R., and Ressot, C. (1997) Eur. J. Neurosci. 9, 1−6
3. Dermietzel, R., and Spray, D. C. (1993) Trends Neurosci. 16, 186−192
4. Nadarajah, B., Thomaidou, D., Evans, W. H., and Parnavelas, J. G. (1996) J. Comp. Neural. 376, 326−342
5. Bozental, R., Meehler, M. F., Morales, M., Andrade-Rozental, A. F., Kesseler, J. A., and Spray, D. C. (1995) Dev. Biol. 167, 350−362
6. Belliveau, D. J., Becherberger, J. F., Rogers, K. A., and Naus, C. C. (1997) Dev. Genet. 21, 197−200
7. Bani-Yaghoub, M., Becherberger, J. F., and Naus, C. C. (1997) J. Neurosci. Res. 49, 19−31
8. Kandler, K., and Katz, L. C. (1995) Curr. Opin. Neurobiol. 5, 98−105
9. Bani-Yaghoub, M., Underhill, T. M., and Naus, C. C. (1999) Dev. Genet. 24, 69−81
10. Bani-Yaghoub, M., Becherberger, J. F., Underhill, T. M., and Naus, C. C. (1999) Exp. Neural. 166, 16−32
11. Naus, C. C., Zhu, D., Todd, S. D., and Kidder, G. M. (1992) Cell. Mol. Neurobiol. 12, 163−175
12. Zhu, D., Cavenee, S., Kidder, G. M., and Naus, C. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1883−1887
13. Zhu, D., Kidder, G. M., Cavenee, S., and Naus, C. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10218−10221
14. Jurea, S. C., Barr, K. J., Enders, G. C., and Kidder, G. M. (1999) Biol. Reprod. 60, 1263−1270
15. Reaume, A. G., De Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., Jurea, S. C., Kidder, G. M., and Rossant, J. (1995) Science 267, 1834−1835
16. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 8033−8037
17. Aboudy-Guterman, K. S., Pechan, P. A., Rainov, N. G., Sena-Esteves, M., Jacobo, J., Snyder, E. Y., Wild, F., Schrane, E., Tobler, K., Breskefeld, X. O., and Fraefel, C. (1997) Neuropept 5, 8801−8808
18. Frederick, M. A., Roger, B., and Robert, E. K. (1993) Current Protocols in Molecular Biology, pp. 9.9−9.14, John Wiley & Sons, Inc., New York
19. Galipeau, J., Li, H., Paquin, A., Sicilia, F., Karpatis, G., and Nalbantoglu, J. (1999) Cancer Res. 59, 2384−2394
20. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8392−8396
21. Laird, D. W., and Revel, J. P. (1990) J. Cell Sci. 97, Part 1, 109−117
22. Peer, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8392−8396
23. Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990) J. Cell Biol. 111, 2077−2088
24. Bagley, J., Aboudy-Guterman, K., Breakefield, X., and Iacomini, J. (1998) Transplantation 65, 1233−1246
25. Cepeda, C., Walsh, J. P., Peacock, W., Buchwald, N. A., and Levine, M. S. (1999) Cereb. Cortex 3, 95−107
26. Veentstra, R. D., Wang, H. Z., Westphale, E. M., and Beyer, E. C. (1992) Circ. Res. 71, 1277−1283
27. Wang, L. J., Colelia, R., Yorke, G., and Roisen, F. J. (1996) Exp. Neurol. 139, 11−11
28. Spray, D. C., Harris, A. L., and Bennett, M. V. (1981) J. Gen. Physiol. 77, 77−93
29. Spray, D. C., and Bennett, M. V. (1985) Annu. Rev. Physiol. 47, 281−303
30. Bukauskas, F. F., Jordan, K., Bukauskiene, A., Bennett, M. V., Lampe, P. D., Laird, D. W., and Verselis, V. K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2556−2561
31. Dudek, F. P., Andrew, R. D., MacVicar, B. A., Snow, R. W., and Taylor, C. P. (1983) In Basic Mechanisms of Neuronal Hyperexcitability (Jasper, H., and van Gelder, N., eds) pp. 31−73, A. R. Liss, New York
32. Cook, J. E., and Becker, D. L. (1985) Microsc. Res. Tech. 31, 408−419
33. Loewenstein, W. R., and Kanno, Y. (1966) Nature 209, 1248−1249
34. Mesnil, M., and Yamasaki, H. (1993) Mol. Carcinogenesis 7, 14−17
35. Holder, J. W., Elmore, E., and Barrett, J. C. (1993) Cancer Res. 53, 3475−3485
36. Ruch, R. J. (1994) Ann. Clin. Lab. Sci. 24, 216−231
37. Yamasaki, H., and Naus, C. C. (1996) Carcinogenesis 17, 1199−1213
38. Mesnil, M., Krutovskikh, V., Piccoli, C., Elfgang, C., Traub, O., Willecke, K., and Yamasaki, H. (1985) Cancer Res. 45, 629−639
39. Rose, B., Mehta, P. P., and Loewenstein, W. R. (1993) Carcinogenesis 14, 1073−1075
40. Eghbali, B., Kessler, J. A., Reid, L. M., Roy, C., and Spray, D. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10701−10705
41. Naus, C. C., Elisevich, K., Zhu, D., Belliveau, D. J., and Del Maestro, R. F. (1992) Cancer Res. 52, 4208−4213
42. Elfgang, C., Eckert, B., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F., and Willecke, K. (1995) J. Cell Biol. 129, 805−817
43. Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1987) J. Cell Biol. 106, 2621−2629
44. Jordan, K., Solan, J. L., Dominguez, M., Sia, M., Hand, A., Lamp, P. D., and Laird, D. W. (1999) Mol. Biol. Cell 10, 2033−2050