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Cell synchronization effect on mammalian cell permeabilization and gene delivery by electric field

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Abstract

Electropermeabilization is a promising nonviral method for gene therapy. However, despite the fact that it is widely used to transfer genes into living cells, the steps that limit DNA transfer remain to be determined. Here, we report the effect of cell synchronization on membrane permeabilization and gene delivery by electric fields. Chinese hamster ovary (CHO) cells were synchronized by aphidicolin or butyrate treatment. Electro-mediated transfection of these cells was evaluated under electric field conditions leading to the same level of membrane permeabilization. Aphidicolin cell synchronization in G2/M phase leads to a slight increase in plasma membrane permeabilization but to a three-fold increase in percentage of transfected cells and to an eight-fold increase in gene expression. This increase in cell transfection is specifically due to the G2/M synchronization process. Indeed, cell synchronization in G1 phase by sodium butyrate has no effect on cell permeabilization and transfection. Our results suggest that the enhanced transfection level in G2/M phase is not simply due to enhanced permeabilization, but reinforce the statement that the melting of the nuclear membrane facilitates direct access of plasmid DNA to the nucleus.

Keywords: Synchronization; Transfection; Aphidicolin; Butyrate; Electroporation; CHO cell

1. Introduction

Nonviral methods of gene delivery have been used in cell transfection for years. These methods are chemical or physical. Among physical methods, electropermeabilization, also named electroporation (see Ref. [1] for review), a method based on the application of electric field pulses to cells, was developed in the early 1980s [2]. Electropermeabilization has been used with increasing popularity for introducing DNA, proteins, metabolites and other small molecules into a large variety of cell types growing both in suspension or attached to surfaces [3–5]. Over the last 10 years, medical applications of this method have been successfully developed, such as antitumoral drug delivery to patients, a method called «electrochemotherapy» [6], and transdermal drug delivery [7]. Electropermeabilization has also been used for in vivo delivery of DNA into the skin, liver, melanoma and skeletal muscle cells [8–11].

Different mechanisms of permeabilization and gene transfer by electric fields have been reported in the literature [12–17]. Electrotransfection has been described as a multistep process. Plasmids have to be present during electropulsation but cross the electropulsed membrane after pulse application [18–21]. Indeed, we proposed a model in which only the localized part of the cell membrane brought to the permeabilized state by the external field is competent for the transfer [21]. It is a complex process, in which an anchoring step connecting the plasmid to the electropermeabilized membrane takes place during the pulse, followed by a post-pulse transfer into the cytoplasm [20]. The plasmid has therefore to be translocated through the membrane inside the cytoplasm. Then, it has to reach the nucleus by crossing the nuclear membrane for its expression. Membrane permeabilization is necessary but not sufficient for an efficient gene transfer.

The nuclear envelope has been thought to be a critical barrier for gene transfection. Indeed, in most cell types a fundamental limitation to gene expression in currently used nonviral systems seems to be the inability of DNA to migrate from cytoplasm into the nucleus [22]. In G2/M cycle phase, nuclear membrane disappeared and gene transfection efficiency has been shown to be improved whatever the gene transfer method used [23–25].

In the case of the electrically mediated gene transfer method, the same result has been obtained. However, no

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study of the potential effect of the cell synchronization process on cell permeabilization has been reported [23,24,26,27]. Indeed, cell electrotransfection is strongly dependent on cell electropermeabilization, which is correlated to the cell diameter [2,21]. To go further into the mechanism, the present study investigates the effect of cell growth phase on gene electrotransfer at three levels: cell size, plasma membrane permeabilization and gene expression.

2. Materials and methods

2.1. Cell culture and synchronization procedures

Chinese hamster ovary (CHO) cells were used. The WTT clone was selected for its ability to grow in suspension or plated on Petri dishes. They were grown as previously described in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% foetal calf serum [28]. Their ability to grow on a support after being maintained in suspension is direct evidence of their viability.

Control cells were plated at a density of 5×10^6 cells per flask (25 cm^2, Nunc). After 24 h of culture, the density was 10×10^6 cells per flask.

For synchronization experiments, cells were plated at a density of 10×10^6 cells per flask 2 h before synchronization. G1 phase synchronization was performed by sodium butyrate (10 mM) treatment added in culture medium during 24 h before experiments [29,30]. G2/M phase synchronization was performed by aphidicolin (1 μg/ml) treatment, added in the culture medium for 20 h. Then, cells were washed twice with PBS and re-fed with fresh medium to stop the G1 phase blockage. Four hours later, cells were synchronized in G2/M phase [27].

Before being processed for cell cycle analysis, cell diameter determination, electropermeabilization and electrotransfection, cells were washed twice with PBS and harvested by trysinization and resuspended in the pulsation buffer.

2.2. Cell cycle analysis

Cells were permeabilized in lysophosphatidylcholine (1 mg/ml) containing pulsation buffer for 10 min. Permeabilized cells were stained by propidium iodide (200 μM) and analyzed for DNA contents by flow cytometry (Becton Dickinson FACScan). The percentage of cells in each cell cycle phase was estimated by using the SFIT model in the cellFIT software. Values represent means ± S.E. ($n = 3$).

2.3. Plasmid

A 4.7 kb plasmid (pEGFP-C1 from Clontech, Palo Alto, CA) carrying the gene of the green fluorescent protein controlled by the CMV promoter was used. It was prepared from transfected *Escherichia coli* cells by using the Maxiprep DNA purification system according to manufacturer's instructions (Qiagen, Chatsworth, CA).

2.4. Electropulsation apparatus

Electropulsation was operated by using a CNRS cell electropulsator (Jouan, St. Herblain, France), which delivered square-wave electric pulses. An oscilloscope (Enertec, St. Etienne, France) monitored pulse shape. Two stainless-steel flat parallel electrodes, connected to the voltage generator, gave a uniform electric field. The distance between the electrodes was 5 mm.

2.5. Electropermeabilization procedures

Penetration of propidium iodide (100 μM) in low ionic strength pulsation buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) was used to monitor permeabilization. Cells were centrifuged for 5 min at $120 \times g$ and resuspended in the pulsing buffer. One hundred microliters of the cell suspension (i.e. 10^6 cells) were poured between the electrodes. Ten pulses lasting 5 ms at a frequency of 1 Hz were applied at a given electric field intensity at room temperature. After pulsation, cells were left at 30 °C for 10 min for membrane resealing. Cells were analyzed by flow cytometry (Becton Dickinson FACScan) to determine both the percentage of fluorescent cells (i.e. the percentage of permeabilized cells) and the level of fluorescence associated with this permeabilization (i.e. the efficiency of permeabilization). The percentage of permeabilization is determined based on the total number of cells submitted to treatment.

2.6. Electrotransfection procedures

Cells were resuspended in pEGFP-C1 plasmid containing pulsation buffer. For each assay, 100 μl of cell suspension were used corresponding to 10^6 cells mixed with 4 μg plasmid. The number of copies per cell was 7.9×10^5. This preparation was incubated for 5 min at 4 °C before pulsation. Ten pulses lasting 5 ms at a frequency of 1 Hz were applied at a given electric field intensity at room temperature. Then, cells were incubated for 10 min at 30 °C [31]. They were cultured in Petri dishes with 2 ml of culture medium for 24 h at 37 °C in a 5% CO_2 incubator. Only plated cells (i.e. viable) were taken into account in the assay. Cells were harvested by trypsinization and analyzed by flow cytometry to evaluate both the percentage of fluorescent cells (i.e. percentage of GFP transfected cells) and the mean level of fluorescence associated with this transfection (i.e. the efficiency of transfection). The percentage of transfection was therefore determined relative to the viable cells.

2.7. Cell diameter determination

An acquisition card (DC20, MIRO, Germany) captured frames from a video camera (SONY, Japan) connected to an

inverted microscope with an X 63 objective (Leica DM IRB, Germany). Cell diameter was obtained by directly measuring the size of cells in suspension on the PC monitor. The precision of the measurement is 0.1 μm. More than 30 cells were assayed per condition. Cell diameter is given as mean ± S.E.

2.8. Statistical analysis of the data

Each histogram is the mean ± S.E. of three separate experiments done in duplicate.

3. Results and discussion

3.1. Cell synchronization

The percentage of cells in G2/M phase was 20% in control cells and reached 82% after aphidicolin treatment. The percentage of cells synchronized in G1 phase was 62% in control cell and 82% with butyrate treatment (Fig. 1). These observations were in agreement with those reported by others [32–34].

3.2. Cell permeabilization

Cell permeabilization by electric fields depends on the cell size [35]. Any increase in cell size could be associated with a more efficient permeabilization for a given electric field intensity. It has indeed been shown that the electric field modulated the membrane potential difference [2]. The transmembrane potential difference induced by the electric field, ΔV_M, is a complex function of the specific conductivities $g(\lambda)$ of the membrane, the pulsing buffer and the cytoplasm, the membrane thickness and the cell size. Thus,

$$\Delta V_M = fg(\lambda) rE\cos\theta \quad (1)$$

in which θ designates the angle between the direction of the normal to the membrane at the considered point on the cell surface and the field direction, E the field intensity, r the radius of the cell and f is a shape factor. ΔV_M is not uniform on the cell surface. It is maximum at the positions of the cell facing the electrodes. When the resulting transmembrane potential difference ΔV (i.e. the sum between the resting value of cell membrane ΔV_o and the electroinduced value ΔV_M) reaches threshold values closed to 250 mV, membranes become permeable [36,37]. Electric membrane properties of aphidicolin synchronized cells have been studied by Sukhorukov et al. [34]. They showed that electric properties, i.e. $g(\lambda)$ function, were similar for asynchronous and synchronous cell membranes. Slight ΔV_o changes during cell cycle have been reported in the case of Chinese hamster lung cells [38]. It varied from −20 mV in G1 phase to −29 mV in S and G2 phases. During mitotic division, it was equal to −22 mV. Thus, these two parameters should not dramatically influence ΔV. Moreover, ΔV is a function of cell geometry: cell shape and size. As cells in suspension were used in this study, their shape was considered to be a sphere and f remained 1.5 in all conditions. Thus, cell permeabilization depends on the cell radius. The effect of cell synchronization on permeabilization efficiency was checked in this study.

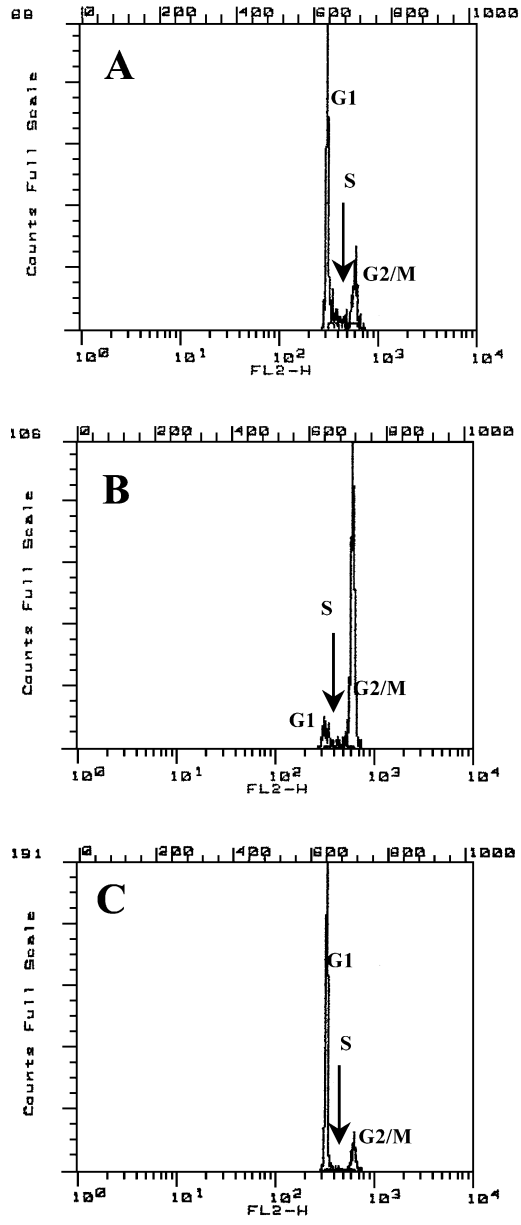

Fig. 1. Cell cycle histograms of CHO cells. (A) Control cells. (B) Aphidicolin synchronized cells in G2/M. (C) Sodium butyrate synchronized cells in G1 phase.

CHO cells in suspension are spherical with a mean diameter of 13.5 ± 1.4 μm. The mean diameter of synchronized cells in G1 phase, equal to 12.6 ± 1.35 μm, was not significantly different from control cells ($P = 0.01$). After the aphidicolin treatment, cells synchronized in G2/M phase significantly increased in size, their diameter reaching

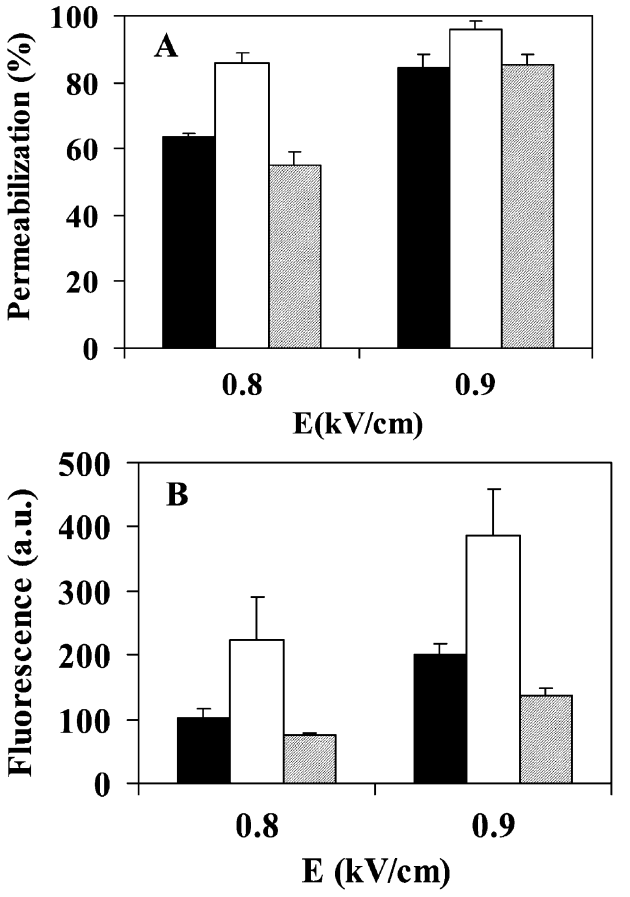

Fig. 2. Effect of cell synchronization on permeabilization. Control cells (■), aphidicolin synchronized cells (□) and sodium butyrate synchronized cells (⊠) were submitted to 10 pulses of 5 ms duration in pulsing buffer containing propidium iodide. Permeabilization was analyzed by flow cytometry. It was determined based on total number of cells. (A) Percentage of permeabilized cells. (B) Associated mean fluorescence level of the permeabilized cells. Values represent means ± S.E. ($n=3$).

16.3 ± 1.5 μm ($P<0.001$). Indeed, in aphidicolin synchronized cells, synthesis of proteins and lipids has been shown to be still present while cell cycle was blocked [34]. These syntheses led to an increase of cell volume, which is in agreement with our observations.

Cells were pulsed under optimum conditions for gene transfer. Ten pulses, lasting 5 ms, were applied. Under those conditions, 60% of control cells were permeabilized at 0.8 kV/cm and 80% at 0.9 kV/cm (Fig. 2A). The associated fluorescence intensity, related to the number of molecules incorporated into the electropermeabilized cells (Fig. 2B), increased with an increase in the electric field intensity. The amount of electroloaded molecules in permeabilized cell increased with the electric field intensity, in agreement with previous results [39]. The percentage of permeabilized cells and the permeabilization efficiency for cells synchronized in G1 phase were slightly smaller than for control cells (Fig. 2A,B). Cells synchronized in G2/M phase exhibited a 1.4-fold increase in the percentage of permeabilization at 0.8 kV/cm and a slight increase at 0.9 kV/cm (Fig. 2A). This synchronization effect was more pronounced when fluorescence intensities were compared. Electropermeabilized cells in G2/M phase exhibited a two-fold increase in fluorescence intensity at 0.8 and 0.9 kV/cm (Fig. 2B). However, this increase had to be associated with the fact that in G2/M phase, cells had 4*N* instead of 2*N* chromosomes and therefore two times more sites for PI interaction [40].

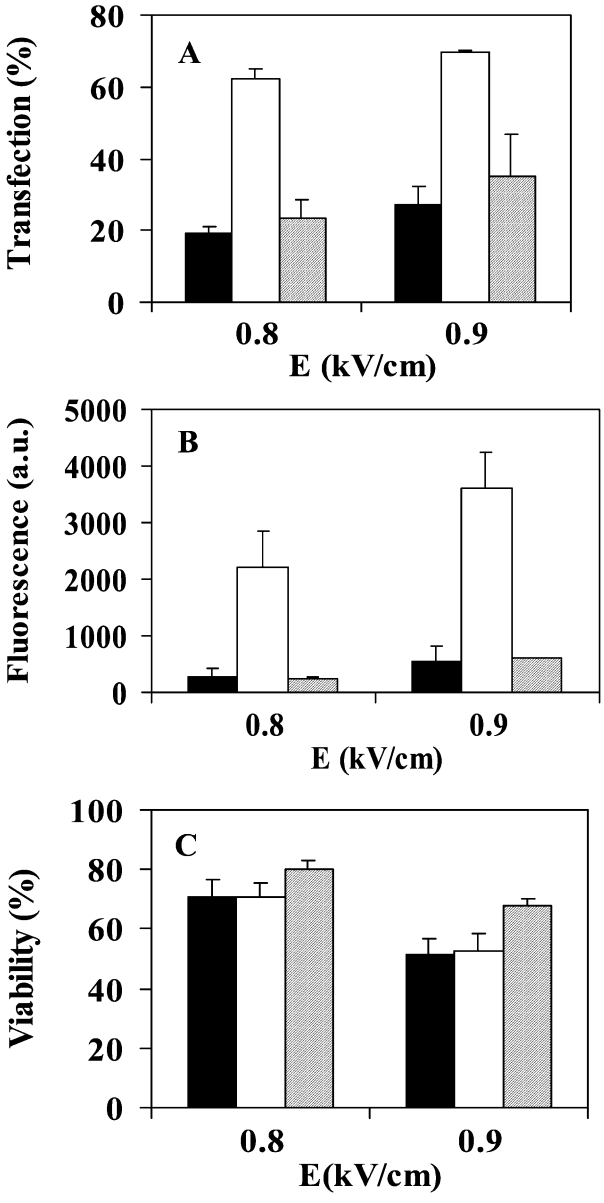

Fig. 3. Effect of cell synchronization on gene transfer and viability. Control cells (■), aphidicolin synchronized cells (□) and sodium butyrate synchronized cells (⊠) are submitted to 10 pulses of 5 ms duration in pulsing buffer containing the pEGFP-C1 plasmid. Transfection was analyzed by flow cytometry 24 h after electric treatment. It was determined based on viable cells. (A) Percentage of electrotransfected cells. (B) Associated mean fluorescence level of GFP expressing cells. (C) Cell viability. Cells were pulsed in the absence of DNA. Viability was assayed by crystal violet staining 24 h after electric treatment. Values represent means ± S.E. ($n=3$).

3.3. Cell transfection

Twenty percent of control cells were transfected at 0.8 kV/cm and 30% at 0.9 kV/cm (Fig. 3A). The associated fluorescence intensity, related to the number of GFP molecules produced into the electrotransfected cells, i.e. transfection efficiency, was assessed as well (Fig. 3B). It increased with an increase in the electric field intensity, in agreement with previous results [21,41]. Similar percentages of transfected cells and transfection efficiency were obtained when cells were synchronized in G1 phase (Fig. 3A,B). Cells (after aphidicolin treatment) submitted to electric pulses when in G2/M phase, exhibited a 3-fold increase in the percentage of transfected cells at 0.8 kV/cm and a 2.5-fold increase at 0.9 kV/cm (Fig. 3A). This increase in transfection was emphasized as far as the transfection efficiency was concerned. Cells in G2/M phase exhibited an 8-fold increase in gene expression at 0.8 kV/cm and a 6.5-fold increase at 0.9 kV/cm (Fig. 3B). These data, based on viable cells, are comparable when expressed on total number of cells. Such an increase in transfection efficiency cannot simply be due to the slight increase in permeabilization. As shown in Fig. 2A, control cells pulsed at 0.9 kV/cm and G2/M synchronized cells pulsed at 0.8 kV/cm led to the same percentage of permeabilization, i.e. 86%. However, the percentages of transfected cells and the transfection efficiencies were different (Fig. 3A,B). Indeed, electric field conditions leading to the same permeabilization efficiency clearly lead to a significant increase in transfection efficiency for cells in the G2/M phase. Under such electric field conditions, viability of the cells was kept constant at 70% at 0.8 kV/cm and 50% at 0.9 kV/cm in control and G2/M phase (Fig. 3C). Viability was slightly preserved in G1 phase probably due to the slight decrease of cell diameter in this phase.

4. Conclusion

The results reported in this work lead to the conclusion that the G2/M phase is well suited for efficient transient transfection by electric fields. Aphidicolin and sodium butyrate synchronization methods gave different results, indicating that the observed effect was not due to the synchronization process by itself but to the fact that cells were in the G2/M phase when pulsed. This cell cycle dependence has already been observed by others on human and mouse fibroblasts [24,27], on K562 cells [32], B cells [33] and on human endothelial cells [23] using electroporation transfection method. The reason for this cell cycle dependency was suggested to be due to the loss of the nuclear membrane in the M phase [23,24,27]. However, in these experiments, the same electric field intensity was applied whatever the phase of the cell cycle. Therefore, the potential effect of cell synchronization on cell size and its consequence on cell permeabilization and transfection were not checked. This could explain why in the case of hematopoietic cells, the S-phase was associated with an increase in gene expression [26].

Another conclusion of the present work deals with the lifetime of the plasmid in the cytoplasm. In 24 h, one cell cycle has been performed; each cell has therefore reached the G2/M phase in which plasmid DNA can cross the nuclear envelope and can be expressed. Therefore, transfection levels should be the same for control and synchronized cells. However, our results showed that the transfection level of G2/M synchronized cells was greater than the transfection level of G1 synchronized cells. Thus, the plasmid could reach the nucleus after electropulsation preferentially in G2/M when the nuclear envelope was no longer present. Later, most plasmids were probably degraded or no longer efficient to transfect cells. This agrees with Lechardeur's work showing that naked plasmid DNA has a rapid turnover ($t_{1/2}$ of 90 min) in the cytoplasm [42] and with our previous work showing that a key step for gene expression after transfer by electric field is present during the first 3 h following electric pulse application [17]. Plasmid DNA crosses the nuclear envelope with a very low efficiency. Mitosis considerably enhances expression of the transgene as already observed with cationic lipid-mediated gene transfer method [25].

In summary, our results showed that DNA expression in cells was really enhanced in G2/M phase synchronized cells. A more efficient permeabilization due to the increase of the cell diameter in this phase could not explain this enhancement. Late G2 or early M phase is thought to be associated with the melting of the nuclear membrane that facilitates the access of DNA to the transcription machinery. This could contribute to the enhanced transcriptional activity of transiently transfected expressing vectors. During cell electrotransfection, the plasmid has to reach the nucleus once inside the cytoplasm. Plasma membrane is the first barrier for gene transfer. The present study reinforces the idea that the nuclear membrane is a second barrier and therefore a limiting step to cell transfection.

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References

[1] T. Tsong, Biophys. J. 60 (1991) 297–306.
[2] E. Neumann, M. Schaefer-Ridder, Y. Wang, P.H. Hofschneider, EMBO J. 1 (1982) 841–845.
[3] H. Potter, Methods in Enzymology, vol. 217, Academic Press, 1993, pp. 461–478.

[4] S. Orlowski, L.M. Mir, Biochim. Biophys. Acta 1154 (1993) 51–63.
[5] N. Eynard, M. Rols, V. Ganeva, B. Galutzo, N. Sabri, J. Teissié, Bioelectrochem. Bioenerg. 44 (1997) 103–110.
[6] L.M. Mir, L.F. Glass, G. Sersa, J. Teissie, C. Domenge, D. Miklavcic, M.J. Jaroszeski, S. Orlowski, D.S. Reintgen, Z. Rudolf, M. Belehradek, R. Gilbert, M.P. Rols, J. Belehradek Jr., J.M. Bachaud, R. DeConti, B. Stabuc, M. Cemazar, P. Coninx, R. Heller, Br. J. Cancer 77 (1998) 2336–2342.
[7] M.R. Prausnitz, V.G. Bose, R. Langer, J.C. Weaver, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10504–10508.
[8] A. Titomirov, S. Sukarev, E. Kistanova, Biochim. Biophys. Acta 1088 (1991) 131–134.
[9] R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, C. Nicolau, FEBS Lett. 389 (1996) 225–228.
[10] M.P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros, J. Teissie, Nat. Biotechnol. 16 (1998) 168–171.
[11] H. Aihara, J.-I. Miyazaki, Nat. Biotechnol. 16 (1998) 867–870.
[12] V. Klenchin, Biol. Membr. 7 (1994) 1–16.
[13] N.I. Hristova, I. Tsoneva, E. Neumann, FEBS Lett. 415 (1997) 81–86.
[14] T. Xie, L. Sun, H. Zhao, J. Fuchs, T. Tsong, Biophys. J. 63 (1992) 1026–1031.
[15] T. Xie, T. Tsong, Biophys. J. 65 (1993) 1684–1689.
[16] P.G. de Gennes, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 7262–7264.
[17] M.P. Rols, C. Delteil, M. Golzio, J. Teissie, Eur. J. Biochem. 254 (1998) 382–388.
[18] N. Eynard, S. Sixou, N. Duran, J. Teissie, Eur. J. Biochem. 209 (1992) 431–436.
[19] V. Ganeva, B. Galutzov, J. Teissie, Biochem. Biophys. Res. Commun. 214 (1995) 825–832.
[20] M. Golzio, M.P. Mora, C. Raynaud, C. Delteil, J. Teissie, M.P. Rols, Biophys. J. 74 (1998) 3015–3022.
[21] H. Wolf, M.P. Rols, E. Boldt, E. Neumann, J. Teissie, Biophys. J. 66 (1994) 524–531.
[22] M.R. Capecchi, Cell 22 (1980) 479–488.
[23] J.L. Schwachtgen, V. Ferreira, D. Meyer, D. Kerbiriou-Nabias, Biotechniques 17 (1994) 882–887.
[24] S. Goldstein, C.M. Fordis, B.H. Howard, Nucleic Acids Res. 17 (1989) 3959–3971.
[25] V. Escriou, M. Carriere, F. Bussone, P. Wils, D. Scherman, J. Gene Med. 3 (2001) 179–187.
[26] M. Takahashi, T. Furukawa, K. Nikkuni, A. Aoki, N. Nomoto, T. Koike, Y. Moriyama, S. Shinada, A. Shibata, Exp. Hematol. 19 (1991) 343–346.
[27] T. Yorifuji, S. Tsuruta, H. Mikawa, FEBS Lett. 245 (1989) 201–203.
[28] M.P. Rols, P. Femenia, J. Teissie, Biochem. Biophys. Res. Commun. 208 (1995) 26–35.
[29] J. Kruh, N. Defer, L. Tichonicky, C. R. Soc. Biol. 186 (1992) 12–25.
[30] J. Wright, Exp. Cell Res. 78 (1973) 456–460.
[31] M.P. Rols, C. Delteil, G. Serin, J. Teissie, Nucleic Acids Res. 22 (1994) 540.
[32] G. Croaker, E. Wass, H. Iland, Leukemia 4 (1990) 502–507.
[33] K. Teshigawara, Y. Katsura, Nucleic Acids Res. 20 (1992) 2607.
[34] V. Sukhorukov, C. Djuzenova, W. Arnold, U. Zimmermann, J. Membr. Biol. 142 (1994) 77–92.
[35] S. Sixou, J. Teissie, Biochim. Biophys. Acta 1028 (1990) 154–160.
[36] J. Teissié, T.Y. Tsong, Biochemistry 20 (1981) 1548–1554.
[37] J. Teissié, M.P. Rols, Biophys. J. 65 (1993) 409–413.
[38] H.G. Sachs, P.J. Stambrook, J.D. Ebert, Exp. Cell Res. 83 (1974) 362–366.
[39] M.P. Rols, J. Teissie, Biophys. J. 58 (1990) 1089–1098.
[40] H.A. Crissman, J.A. Steinkamp, Eur. J. Histochem. 37 (1993) 129–138.
[41] G. Chu, H. Hayakawa, P. Berg, Nucleic Acids Res. 15 (1987) 1311–1325.
[42] D. Lechardeur, K.J. Sohn, M. Haardt, P.B. Joshi, M. Monck, R.W. Graham, B. Beatty, J. Squire, H. O'Brodovich, G.L. Lukacs, Gene Ther. 6 (1999) 482–497.