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Modulation of estrogen receptor gene transcription in breast cancer cells by liposome delivered decoy molecules

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Abstract

It is well known that breast carcinomas without estrogen receptor (ER) have a poor prognosis and do not respond to antiestrogenic therapy. In analyzing the question of the lack of ER gene expression, we have considered the possibility to modify the ER gene expression by transfecting ER-negative breast cancer cells with a polymerase chain reaction product mimicking a putative negative regulatory region (− 3258/ − 3157) inside the P3 ER gene promoter. Here we have demonstrated the efficacy of the selected sequence used as a decoy molecule in restoring the ER gene transcription. When this DNA was complexed and delivered by cationic liposomes (PC:DOTAP) a significant increase in the decoy effect was obtained. Breast cancer cells receiving the combination treatment responded substantially better to reactivation of quiescent ER gene than cells that had received DNA with calcium phosphate. This information may be useful for a series of in vitro transfections and also for in vivo application of the decoy strategy that is a potential therapeutic tool to control disease-related genes such as ER gene in breast cancer.

Keywords: Cationic liposomes; Estrogen receptor gene; Decoy; Breast cancer

1. Introduction

It is well known that there are different ways to study and to modulate the gene expression including antisense nucleic acids, rybozymes, transdominant mutation proteins and suicide genes. The 'decoy' strategy that allows a modulation of gene expression at transcriptional level is a more recent approach; it is based on the competition for *trans*-acting factors between the endogenous *cis*-elements present on the target gene and exogenously added double stranded oligonucleotides containing a DNA sequence identical to that of the specific *cis*-element [1–4]. This competition results in an inhibition or attenuation of the 'authentic' interaction of trans-factor(s) with the *cis*-element(s). This approach represents a method for both (i) testing the biological involvement of genomic sequences in the modulation of gene expression and in the maintenance of a specific phenotype and (ii) modulating the gene expression as a therapeutic tool. In this respect, we are interested in elucidating the regulation of the human estrogen receptor (hER) gene expression, in order to understand the role of ER protein in the induction and maintenance of the breast cancer phenotype [5–7]. In a previous study, the presence of regulatory sequences was demonstrated inside the P1 canonical promoter and the P3 distal promoter of ER gene [8]. In particular, when used in direct or calcium phosphate mediated transfection, a specific PCR-generated DNA fragment that contains the sequence − 3258/ − 3157 belonging to P3 upstream promoter (namely DNA-102), was able to act as decoy molecule, increasing ER RNA level in ER-positive breast cancer cells [8].

The present study describes the delivery, by cationic liposomes, of DNA-102 decoy molecule in MDA-MB-231 ER-negative breast cancer cells. The aim of this

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work was to investigate (i) the role of the selected sequence in the lack of ER gene transcription and (ii) the possibility of potentiating its biological activity as decoy molecule using cationic liposomes as gene delivery formulation. In this respect, it is important to underline that (a) the absence of ER gene expression is associated with a more aggressive disease course [6], (b) breast cancer cells usually represent a difficult target for DNA transfection [9] and (c) liposomes have recently been proposed as cell transfection agents for DNA molecules intended for decoy strategies [10–12].

2. Materials and methods

2.1. Materials

Egg phosphatidyl choline was purchased from Lipid Products (Surrey, England). The cationic surfactant *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methyl-sulfate, was purchased from Sigma Chemical Company.

As nucleic acids molecules, two DNA fragments generated by polymerase chain reaction (PCR) were used: a 102 bp genomic fragment (DNA-102) belonging to the 5′ region of hER gene [8] and a 150 bp plasmidic fragment (DNA-150). pBLCAT8ERCAT1 [8] and pGEX-2TK [13] recombinant plasmids were used as templates for PCR-102 and PCR-150, respectively. After amplification DNAs were purified by ultrafiltration procedure with the Microcon-30 system (Amicon, Beverly, USA) as previously described [14].

2.2. Liposome preparation

Cationic liposomes, composed of egg phosphatidyl choline (PC) and the cationic surfactant *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methyl-sulfate (DOTAP), PC:DOTAP (8:1 mol/mol), were prepared by reverse phase evaporation followed by three extrusion cycles through 200 nm pore size polycarbonate membranes. The extrusion step was performed in order to obtain unilamellar liposomes with a homogeneous size distribution as confirmed by the freeze-fracture electron microphotographs [15].

2.3. Analysis of the electrophoretic mobility of liposome–DNA complexes

For DNA binding measurements, complexes with different amounts of vesicles and ng of PCR product were made as described [15]. The complexes were incubated at room temperature for 10 min and then electrophoresed. The charge ratio was calculated by measuring the amount of lipid needed to bind all the DNA, i.e. the amount of lipid at which PCR product no longer migrated into the gel.

Electrophoresis was carried on in 2% agarose gel at constant voltage (25 mV) for 2 h. The relative band migration was determined after staining the gels with ethidium bromide.

2.4. DNA stability studies

The stability of the liposome/DNA complexes towards the nucleases contained in fetal calf serum (FCS) was studied following the above protocol. The liposome/DNA complexes (liposomes/DNA ratios of 10:1 w/w) were incubated at 37°C in a thermostatic bath. At different time intervals, between 0 and 7 days, samples were withdrawn and stored at −20°C until electrophoretic analysis was performed. In order to displace the DNA/liposome complexes, after the indicated length of time, the samples were treated with trypsin, phenol-extracted and loaded on agarose gel. The quantitative analysis of band intensity was performed by a computerized scanning analysis of digitalized images.

2.5. Cytotoxicity studies

The cytotoxicity of cationic liposomes was determined on in vitro cultured human cell lines. Standard conditions for cell growth were α-medium (Gibco BRL, Gaithersburg, MD), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10% FCS (Boerhinger, Manneheim) in 5% CO_2 at 80% humidity. MDA-MB-231 ER-negative breast cancer cells were maintained at 37°C in 5% CO_2, in phenol red-free MEM medium (Gibco BRL, Gaithersburg, MD) plus 10% charcoal-stripped foetal bovine serum (FBS; estrogen-depleted medium). Cell growth was determined by counting with a Model ZF Coulter Counter (Coulter Electronics, Hielah, FL). Counts of viable cells were performed after colorimetric assay with MTT (thiazolyl blue) [16].

2.6. Analysis of DNA transfection efficiency

Calcium phosphate and liposome-mediated transfection methods were compared.

Six hundred nanograms of DNA-150 were used to transfect cells at 60% confluence plated in 31-mm diameter plates. The calcium phosphate-DNA precipitates were left on the cells for 16 h, then removed with phosphate-buffered saline, the cells were fed with fresh medium for 24 h and then harvested. In the other case, DNA was mixed with cationic liposome suspension (lipid:DNA ratio 10:1 w/w) in a final volume of 200 μl. After 15 min incubation at room temperature, 200 μl of serum-free medium was added to the liposome/DNA complex and used to transfect one well. 24 h later, transfection solution was replaced with complete

medium. After transfection the cells were washed five times with PBS and incubated with DNase (1 ml of a 1 ng/ml DNase solution) for 15 min. After inactivation of the DNase with 50 mM EDTA pH 8 and harvesting, the cells were disrupted with 100 μl of Triton X-100 2% solution. An aliquot of each cellular lysate, corresponding to 10^4 cells, was used to test, by PCR, the efficiency of transfection by evaluating the presence of the 150 bp plasmidic DNA fragment.

2.7. Analysis of gene transcription

Expression of ER and β-actin mRNA was detected by reverse transcription polymerase chain reaction (RT-PCR) on total RNA (5 μg) from MDA-MB-231 transfected cells.

RT-PCR reactions were performed in one step by using the Titan™ One Tube RT-PCR Kit (Boehringer, Manneheim) and a Violet Thermal Cycler with 30 cycles for ER and 20 cycles for β-actin cDNA. The following primers and conditions were used:

ER: F = 5′-CTATATGTGTCCAGCCACCAACC-3′ (exon 3)

R = 5′-CTCTACACATTTTCCCTGGTTCCT-3′ (exon 6)

the cycles were 60 s at 94°C, 60 s at 57°C and 60 s at 72°C and

β-actin: F = 5′-TGACGGGGTCACCCACACTGT-GCCCATCTA-3′

R = 5′-CTAGAAGCATTTGCGGTGGA-CGATGGAGGG-3′

the cycles were 45 s at 94°C, 45 s at 60°C and 45 s at 72°C.

All amplifications were compared with a negative control (primers without RNA) and the levels of ER mRNA were normalized against the β-actin mRNA content using a densitometric analysis. RT-PCR products were separated on agarose gel, electrophoresed and, for ER mRNA analysis, were subsequently blotted onto nylon membrane using standard procedures [17], and hybridized with ^{32}P-labelled pOR15 probe [5].

2.8. Analysis of estrogen receptor protein expression

Cell extracts from MCF7 and MDA-MB-231 breast cancer cells were separated by 10% SDS-PAGE essentially according to Laemmli [18], and proteins were then transferred to nitrocellulose membrane (Hybond C). After the electroblotting, proteins were visualized using PONCEAU S reagent (Sigma). The blots were blocked for 2 h at room temperature with 1X phosphate-buffered saline containing 0.1% TWEEN 20 (PBST) and 3% BSA, incubated for 2 h with purified monoclonal antibody (290 ng/ml) to the human ER (H222, dil 1:1000) in blocking solution and then washed three times with PBST for 30 min. The blots were incubated for 45 min with PBST containing Alkaline Phosphatase-conjugated goat anti-rat IgG antibody (Promega) dil 1:4000 and washed three times with PBST for 30 min. Immunoreactive proteins were visualized by using ProtoBlot Western Blot AP Systems (Promega).

The intensity of ER specific band of 66 kDa was densitometrically evaluated using the Biorad Model GS-700 Imaging Densitometer.

2.9. Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis of the data was performed using the student's paired t-test. Differences were significant with $P < 0.01$.

3. Results

Our experiments were initially designed to analyze the effect of cationic liposomes on DNA-102 stability in the presence of serum and the cytotoxicity of liposomal-DNA complexes on in vitro cultured MDA-MB-231 cells. In a second set of experiments the efficiency of DNA transfection and the biological activity of PCR-DNA delivered by cationic liposomes, in terms of modulation of gene expression, were examined.

3.1. Protective effect of liposomes on serum nuclease-mediated degradation of PCR products

The formation of the liposome/DNA-102 complex was accomplished by mixing increasing amounts of 'preformed' unilamellar cationic liposomes with a solution containing 125 ng of DNA-102, resulting in a quantitative 'association' yield when a liposome to DNA-102 ratio of 5:1 (w/w) was used (see Fig. 1, panel A).

To test the protective effect, liposome/DNA-102 was incubated at 37°C in the presence of 10% FBS, mimicking the conditions of in vitro cell growth. After different length of time spanning from 0 to 7 days, samples were subjected to a treatment able to displace the liposome/DNA-102 complex, allowing a correct quantitative analysis of the DNA (Piva et al., manuscript in preparation). Samples were treated with trypsin (0.4 units for 30 min at 37°C), phenol/chloroform extracted and then analyzed by electrophoresis on 1.5% agarose gel.

As shown in panel B of Fig. 1, naked DNA-102 was found, as expected, to be very sensitive to nuclease digestion completely since it was degraded within 1 h. On the contrary, when DNA-102 was complexed to cationic liposomes, a significant serum resistance was found. In fact, more than 95% of DNA-102 was still intact after exposure to serum nucleases for 7 days.

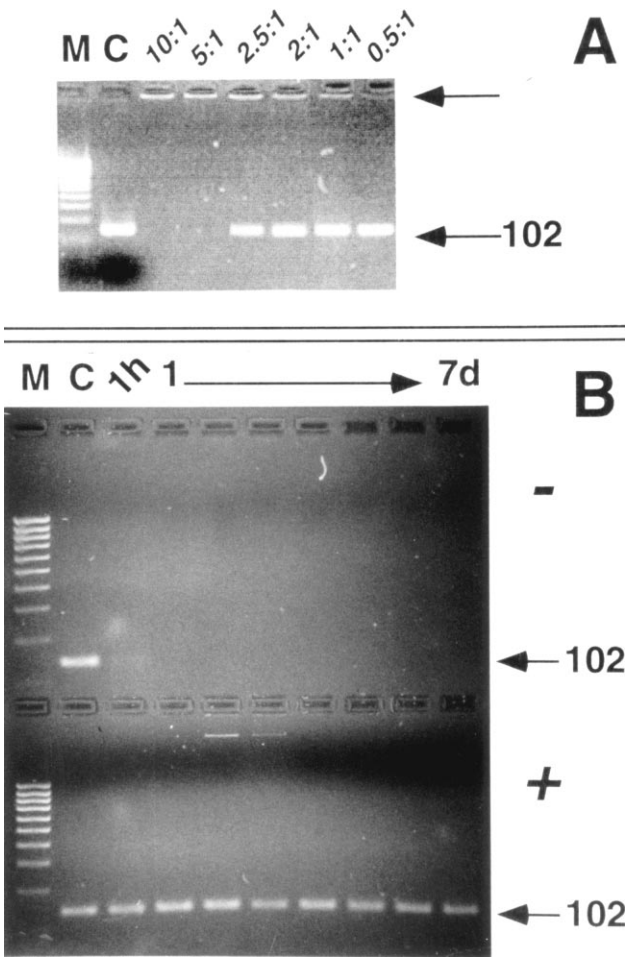


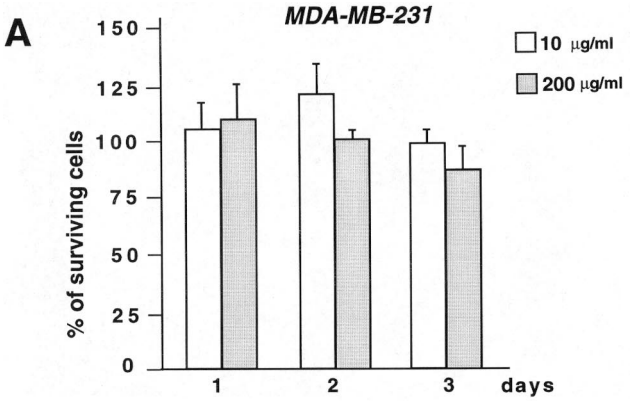
Fig. 1. Panel A. DNA–liposome association assay. Samples containing 125 ng of DNA-102 and liposomes in various liposome-to-DNA ratios (w/w) were incubated at room temperature for 30 min and loaded on agarose gel. The associated DNA is indicated by the arrow at the top of the gel; the non-associated DNA is arrowed as 102. M = molecular weight marker (100 bp DNA ladder); C = uncomplexed PCR-102. Panel B. Protective effect of liposomes on serum nuclease-mediated degradation of DNA-102. DNA-102 was incubated in the presence of 10% FBS in water at 37°C for 0 (C), 1 h and 1, 2, 3, 4, 5, 6, 7 days, in the presence (+) or in the absence (−) of liposomes. The amount of intact DNA-102 was determined by densitometric measurements of the fluorescence signals in agarose gel after ethidium bromide staining. pBLCAT8ERCAT1 [5] recombinant plasmid was used as template for DNA-102. After amplification DNAs were purified by ultrafiltration procedure with Microcon-30 system (Amicon, Beverly, USA) as previously described [14]. M = molecular weight marker (100 bp DNA ladder).

3.2. Effect of PC:DOTAP cationic liposomes on cell survival

As demonstrated, the liposomal formulation was clearly able to increase the stability of the PCR product; nevertheless, data from the literature indicate that liposomes can display a differential toxicity when analyzed on in vitro cultured cell lines [19,20]. Therefore, the effect of liposomes was investigated on MDA-MB-231 breast cancer cells that represent the target cell for the decoy experiments. Cells were treated with different amounts of cationic liposomes and viable cells were quantified by a colorimetric assay with thiazolyl blue (MTT) [16]. The percentage of surviving cells after 3 days of liposome treatment is reported in panel A of Fig. 2. Notably, treatment with 10 μg/ml of liposomes, corresponding to the lowest concentration at which the DNA/liposome complex is formed, did not decrease cell viability; on the contrary, it even slightly increased it. Also when higher concentrations, up to 200 μg/ml, were used only a faint cytotoxic activity was observed after 3 days of treatment (not statistically significant; from 1 to 3 days the P-values were 0.92, 0.73 and 0.057, respectively). The same results were obtained when other human cell lines, such as K562 erythroleukemic cells and MCF7 breast cancer cells, were subjected to liposome treatment (see panel B). In addition, the effect of PC:DOTAP on cell proliferation was assayed up to 7 days determining the cell growth by coulter counter. As reported in panel C of Fig. 2, no significant block of MDA-MB-231 proliferation was observed (day 1: $P = 0.354$, day 2: $P = 0.189$, day 3: $P = 0.35$, day 4: $P = 0.058$, day 7: $P = 0.158$).

3.3. Transfection efficiency of cationic liposomes

Cationic liposomes were then tested for their transfection efficiency. For this purpose, a non-genomic DNA in the form of a plasmidic PCR product 150 bp in size (briefly DNA-150), was used. This choice was made in order to reveal, by PCR, the amount of DNA inside the cells after transfection. Liposome-mediated transfection efficacy was compared with the calcium phosphate procedure in MDA-MB-231 cells. After transfection the percentage of cells which take up the DNA was about 90% as demonstrated using fluorescently labelled PCR products [14]. The transfected cells were then subjected to DNase treatment, disrupted and subjected to 20 cycles PCR amplification to detect the PCR product inside the cell. After 20 PCR cycles the DNA-150 was identified in cells transfected with both conditions. From the results reported in panel A of Fig. 3 cationic liposome-mediated transfection was found to be about four times more efficient, as reflected by the densitometric analysis of the DNA band visualized with ethidium bromide staining of agarose gel. Transfections



B

liposome composition	amount of liposome formulation (μg/ml)	toxicity, % of non surviving cells K562	MCF7	MDA
PC:DOTAP	10	0	0	0
PC:DOTAP	200	0	0	12

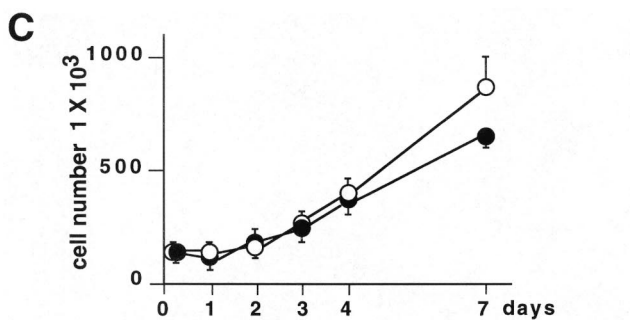


Fig. 2. Effect of PC:DOTAP cationic liposomes on cell survival of in vitro cultured MDA-MB-231 breast cancer cells. In panel A the data obtained by counting the viable cells (up to 3 days of cell culture) with a colorimetric assay based on MTT (thiazolyl blue) are reported. The viability of untreated controls has been set as 100%. Results are expressed as the percentage of surviving cells and are the mean ± SEM of at least four independent experiments. The differences were found to be not significant with $P > 0.01$ (from 1 to 3 days the P-values were 0.92, 0.73 and 0.057, respectively). The tested concentration 10 μg/ml corresponds to the lowest concentration at which the DNA/liposome complex is formed. The toxicity of PC:DOTAP cationic liposomes in K562 erythroleukemic cells, MCF7 and MDA-MB-231 breast cancer cells, after three days of treatment, is reported in panel B. Panel C: the data on cell proliferation were determined by counting, with a cell counter, the cells that were treated (○) with 200 μg/ml of PC:DOTAP or that remained untreated (●). This assay was done in duplicate and performed three times. The results (mean ± SEM) of one representative determination are shown. The proliferation rate did not change significantly ($P > 0.01$) after cells were cultured in the presence of liposomes (day 1: $P = 0.354$, day 2: $P = 0.189$, day 3: $P = 0.35$, day 4: $P = 0.058$, day 7: $P = 0.158$).

were performed in duplicate and were repeated five times. It is to be underlined that for cells such as MDA-MB-231, that are particularly difficult to transfect, the use of cationic liposome had a clear and beneficial effect resulting in a marked transfection enhancement.

3.4. The expression of estrogen receptor mRNA by delivery of a liposome encapsulated decoy DNA molecule

To investigate whether the increased transfection efficiency in MDA-MB-231 cells mediated by liposomes would correspond to an increased biological activity, the decoy effect of DNA-102, on the ER-negative breast cancer cells, was studied.

The transfection was performed in duplicate in six independent experiments; the results of a representative RT-PCR experiment are depicted in panel B of Fig. 3.

As expected, the ER mRNA level was undetectable by RT-PCR analysis on control MDA-MB-231 cells. On the contrary, after treating the cells with DNA-102 by calcium phosphate, a significative induction of ER mRNA expression was obtained; an even higher effect was found in cells treated with the same amount of DNA-102 delivered by cationic liposomes. The β-actin gene expression, used as a control in this semiquantitative RT-PCR analysis, was essentially unaffected, suggesting the specificity of DNA-102. The results of the different decoy experiments are summarized in the graph reported in panel B of Fig. 3. The values show that the reactivation of ER gene transcription was significantly higher in the cells transfected with liposome encapsulated decoy DNA-102 than in the cells transfected with calcium phosphate method (liposome vs. calcium phosphate = 9 ± 2, $P < 0.01$).

In order to evaluate any possible non-specific effects of ds-DNA molecules, transfection experiments with unrelated ds-DNA were performed. For instance, when MDA-MB-231 cells were treated with an unrelated random 150 bp PCR product, the expression of ER gene was completely unaffected, demonstrating that the effect of DNA-102 decoy can be considered specific.

We next investigated if the DNA-102 decoy molecule reactivates not only ER gene transcription, but also ER protein expression. To determine the presence of ER protein, Western blot analysis on MDA-MB-231 cells and those cells treated with the DNA-102 decoy molecule delivered with cationic liposomes was performed. Only MCF-7 ER-positive breast cancer cells, used as positive control, contained the 66 kDa protein that was recognized by the anti-ER antibody, while no signal was observed in MDA-MB-231 cells after decoy treatment, as reported in Table 1. This indicates that the reactivation of ER gene transcription is not correlated, in our experimental conditions, with the reexpression of the H-222-positive ER protein.

4. Discussion

The data reported in the present paper demonstrate the

increase in the reactivation of ER gene transcription by delivery of a liposome-encapsulated-decoy-DNA sequence belonging to P3 distal promoter of ER gene. The selected sequence, when exogenously transfected in MDA-MB-231 ER-negative breast cancer cells, is able to interfere with the transcriptional regulation of ER gene, most likely by competing with the cellular *cis*-element for binding putative negative factor(s). This sequence has been characterized in more detail, by footprinting and Southwestern analysis, confirming the critical role of the region in ER gene transcriptional regulation [21].

Interestingly, we obtained an increase in transfection efficiency and in the decoy activity by using PC:DOTAP liposomes that probably act by stimulating endocytosis and DNA trafficking from the cytoplasm

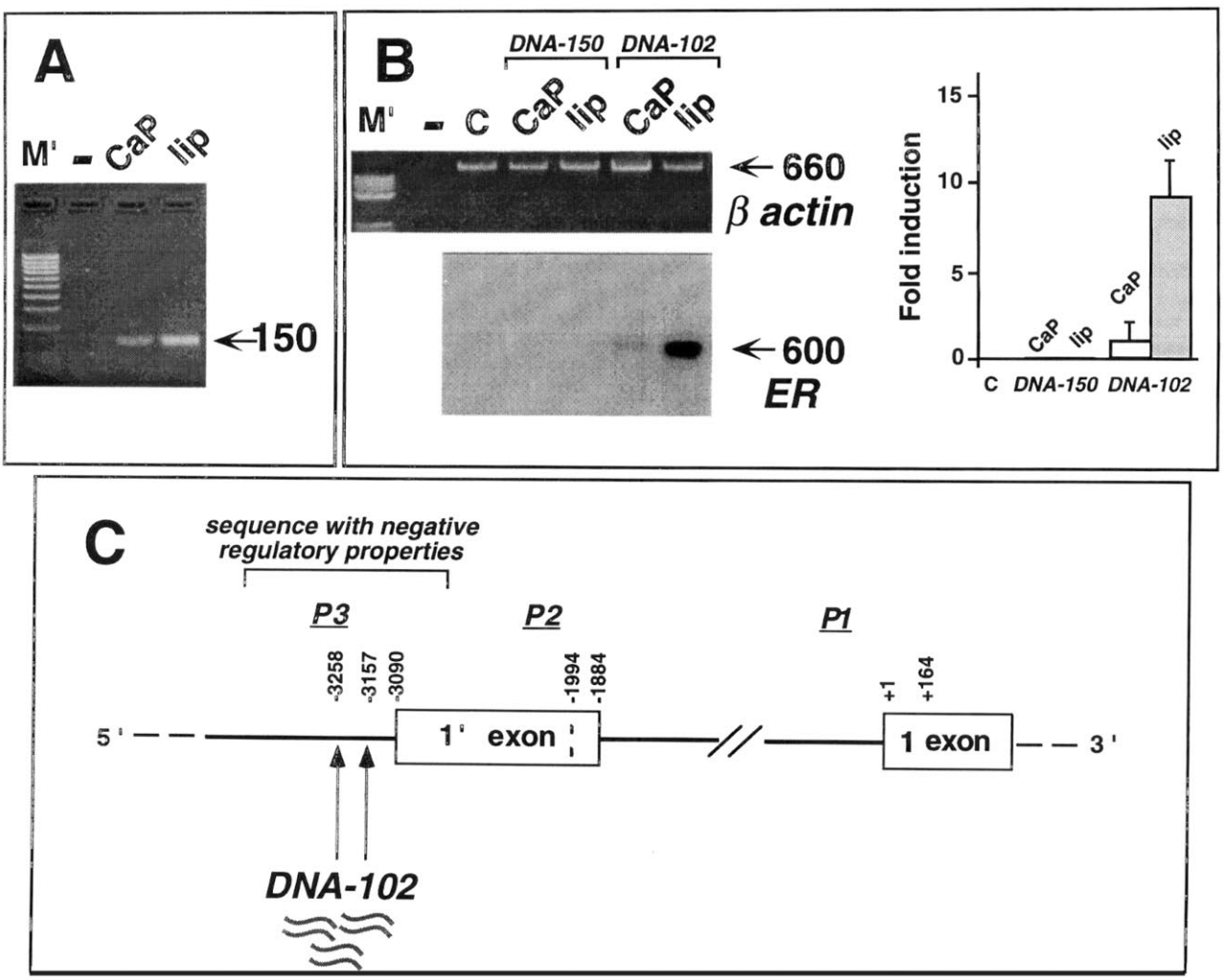


Fig. 3. Panel A. Effect of cationic liposomes on DNA transfection efficiency. MDA-MB-231 breast cancer cells were transfected with non-genomic DNA in the form of a 150 bp PCR product (PCR-150) for which pGEX-2TK [13] was used as template. Calcium phosphate and liposome-mediated transfection methods were compared. An aliquot of each lysate from transfected cells and corresponding to 10^4 cells, was used to test, by PCR, the efficiency of transfection by evaluating the presence of the 150 bp DNA fragment. M′ = 100 bp DNA ladder. Panel B. Effect of cationic liposomes on the ability of DNA-102 to modulate ER gene transcription. Expression of ER and β-actin mRNA was evaluated by RT-PCR on the same amount of total RNA from MDA-MB-231 cells after transfection with calcium phosphate or liposomes. All amplifications were compared with a negative control (primers without RNA) and the levels of ER mRNA were normalized against the β-actin mRNA content using a densitometric analysis. RT-PCR products were separated on agarose gel, electrophoresed and, for ER mRNA analysis, were subsequently blotted onto nylon membrane using standard procedures [17]. Hybridisation was performed with ^{32}P-labelled pOR15 ER specific probe [5]. The specific RT-PCR products are arrowed. M = molecular weight marker (*Hae*III restricted pBR322 DNA); (−) = negative control; C = untreated cells. Results on ER gene expression of six decoy experiments were reported in the graph. The ER mRNA levels in the cells treated with DNA-150 or DNA-102 decoys was normalized against β-actin mRNA content. The expression levels detected in cells treated with DNA-102 decoy calcium phosphate-mediated were arbitrarily ascribed a value of 1. Results are expressed as mean fold induction ± SEM of six independent transfections $P < 0.01$. In panel C a schematic representation of 5′ upstream region of the hER gene with the position of the sequence DNA-102 used in the decoy experiments is reported. The positions of 1 exon and the upstream 1′ exon with P1 canonical promoter, P2 and P3 distal promoters are also shown.

Table 1
Expression of ER protein in breast carcinoma cell lines valuated by densitometric analysis of Western blots[a]

Cell type	Expression of ER number	Mean (±SE)
MCF-7	4	3.42 (0.132)
MDA-MB-231	4	0
MDA-MB-231 + DNA-102	4	0

[a] Nuclear extracts were separated by 10% SDS-PAGE and blotted to nitrocellulose membrane. Four different blots were probed with monoclonal antibody to the human ER (H222), which was revealed by AP system. Films were scanned with the Biorad Model GS-700 Imaging Densitometer. Data are expressed as arbitrary units. SE is the standard error.

to the nucleus. The increase of specific functional properties of the transfected DNA molecules, results in an increase in ER gene transcription. This suggests that the adopted strategies may be considered a good approach not only to improve the transfection efficiency in breast cancer cells, that are generally a critical target for DNA transfection, but also to obtain a preliminary step towards a change in the breast cancer differentiation programme, that is a modification of the ER-negative phenotype.

We also investigated, by Western blot and immunocytochemical experiments, whether, after decoy, the ER protein is reexpressed, but we only found a faint cytoplasmic positiveness. Therefore, in our experimental conditions, we have no evidence of the expression of ER protein in the MDA-MB-231 cells. One possibility is that the decoy of DNA-102 alone, or at the doses that we have used, is not sufficient to induce a complete reactivation of ER gene expression. It could be of interest to investigate the effect of higher doses of DNA-102 in transfection and a longer exposure of the cells to this decoy molecule.

Alternatively, flanking regions or other sequences belonging to the different ER upstream promoters, may play a more critical role in the regulation of gene expression and may be used as more efficient decoy molecules. To try to address the complex question of lack of ER gene expression in breast cancer we are now focusing our analysis on the characterization of protein regulatory factor(s) that are able to bind the DNA-102 decoy sequence, and on the study of relationships between the decoy effect and other mechanisms such as (i) the interactions with the putative transcription factor that binds the decoy sequence and other proteins, (ii) the level of methylation of specific ER DNA sequences and (iii) the transcriptional regulation of the other ER gene promoters, that altogether may contribute to the lack of ER gene expression.

We can, however, conclude that the selected sequence used as decoy molecule, when delivered by cationic liposomes, is capable of markedly increasing the transcription of the ER gene, and should therefore be considered a good model system in 'in vitro' and 'in vivo' decoy strategies.

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References

[1] R. Morishita, J. Higaki, N. Tomita, T. Ogihara, Application of transcriptional factor decoy strategy as means of gene therapy and study of gene expression in cardiovascular disease, Circ. Res. 82 (1998) 1023–1028.
[2] I. Kawamura, R. Morishita, N. Tomita, E. Lacey, M. Aketa, S. Tsujimoto, T. Manda, M. Tomoi, I. Kida, J. Higaki, Y. Kaneda, K. Shimomura, T. Ogihara, Intratumoral injection of oligonucleotides to the NF kappa B binding site inhibits cachexia in a mouse tumor model, Gene Ther. 6 (1999) 91–97.
[3] C. Fraisier, A. Irvine, C. Wrighton, R. Craig, E. Dzierzak, High level inhibition of HIV replication with recombinant RNA decoys expressed from an HIV-Tat inducible vector, Gene Ther. 5 (1998) 1665–1676.
[4] Y.G. Park, M. Nesterova, S. Agrawal, Y.S. Cho-Chung, Dual blockade of Cyclic AMP Response Element-(CRE) and AP-1-directed transcription by CRE-transcription factor decoy oligonucleotide, J. Biol. Chem. 274 (1999) 1573–1580.
[5] S. Green, P. Walter, V. Kumar, A. Krust, J.M. Bornert, P. Argos, P. Chambon, Human estrogen receptor cDNA: sequence, expression and homology to v-erb-A, Nature 320 (1986) 134–139.
[6] W.A. Knight, R.B. Livingston, E.J. Gregory, W.L. McGuire, Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer, Cancer Res. 37 (1977) 4669–4671.
[7] E.C. DeConinck, L.A. McPherson, R.J. Weigel, Transcriptional regulation of estrogen receptor in breast carcinomas, Mol. Cell Biol. 15 (1995) 2191–2196.
[8] L. Penolazzi, E. Lambertini, G. Aguiari, L. del Senno, R. Piva, Modulation of estrogen receptor gene expression in human MCF7 breast cancer cells: a decoy strategy with specific PCR-generated DNA fragments, Breast Cancer Res. Treat. 49 (1998) 227–235.
[9] R.J. Weigel, E.C. deConinck, Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma, Cancer Res. 53 (1993) 3472–3474.
[10] R. Morishita, G.H. Gibbons, M. Horiuchi, K.E. Ellison, M. Nakajima, L. Zhang, Y. Kaneda, T. Ogihara, V.J. Dzau, A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo, Proc. Natl. Acad. Sci. USA 92 (1995) 5855–5859.
[11] N. Tomita, R. Morishita, S. Tomita, K. Yamamoto, M. Aoki, H. Matsushita, S. Hayashi, S. Higaki, T. Ogihara, Transcription factor decoy for nuclear factor-kappa B inhibits tumor necrosis factor-alpha-induced expression of interleukin-6 and intracellular adhesion molecule-1 in endothelial cells, J. Hypertens. 16 (1998) 933–1000.

[12] Y. Maeshima, N. Kashihara, T. Yasuda, H. Sugiyama, T. Sekikawa, K. Okamoto, K. Kanao, Y. Watanabe, Y.S. Kanwar, H. Makino, Inhibition of mesangial cell proliferation by E2F decoy oligodeoxynucleotide in vitro and in vivo, J. Clin. Invest. 101 (1998) 2589–2597.
[13] B. Nilsson, L. Abrhmsen, M. Uhlen, Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors, EMBO J. 4 (1985) 1075–1080.
[14] L. Penolazzi, M.C. Facciolo, G. Aguiari, L. del Senno, R. Piva, Direct transfection of PCR-generated DNA fragments into mammalian cells employing ethidium bromide indicator and ultrafiltration, Anal. Biochem. 248 (1997) 190–193.
[15] R. Cortesi, E. Esposito, E. Menegatti, R. Gambari, C. Nastruzzi, Effect of cationic liposome composition on their in vitro cytotoxicity and protective effect on carried DNA, Int. J. Pharm. 139 (1996) 69–78.
[16] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Meth. 22 (1986) 271–277.
[17] R. Piva, N. Bianchi, G. Aguiari, R. Gambari, L. del Senno, Sequencing of an RNA transcript of the human estrogen receptor gene: evidence for a new transcriptional event, J. Steroid Biochem. 46 (1993) 531–538.
[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriofage T4, Nature 227 (1970) 680–685.
[19] H. Schreier, L. Gagné, T. Bock, G.W. Erdos, P. Druzgala, J.T. Conary, B.W. Müller, Physicochemical properties and in vitro toxicity of cationic liposome cDNA complexes, Pharm. Acta Helv. 72 (1997) 215–223.
[20] M.C. Filion, N.C. Phillips, Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, Biochim. Biophys. Acta 1329 (1997) 345–356.
[21] L. Penolazzi, E. Lambertini, G. Aguiari, L. del Senno, R. Piva, Cis element 'decoy' against the upstream promoter of the human estrogen receptor gene, Biochim. Biophys. Acta 1492 (2000) 560–567.