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DNA Induces Apoptosis in Electroporated Human Promonocytic Cell Line U937

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Experimental gene transfer has permitted a wide variety of studies on gene regulation and function. However, possible effects of the introduced DNA on cellular metabolism are not well understood. Here we demonstrated that introduction of DNA into a promonocytic cell line, U937, by electroporation caused extensive cell death. The toxicity of DNA was concentration-dependent. Various DNAs including plasmid and genomic DNAs all caused cell death, indicating that the toxicity is nucleotide sequence-independent. DNA-induced cell death was associated with internucleosomal DNA fragmentation, a decrease in cell size, and a considerable proportion of cells outside cell cycle. From these results, we concluded that cells died by apoptosis. Our findings have experimental implication for an important issue concerning the interpretation of experiments using gene transfer. In addition, we propose that our observed phenomenon may be relevant to an important immune defense mechanism in monocytes/macrophages that facilitates a response to certain viral infections.

Key Words: **U937 cells; apoptosis; electroporation; DNA; gene transfer.**

Experimental gene transfer into animal cells has permitted a wide variety of studies on gene regulation and function. However, possible effects of the introduced DNA on cellular metabolism are not well understood. Several reports have shown cytotoxic effects of unintegrated viral DNA accumulating in eukaryotic cells during viral infection (1, 2). Holter *et al.* (3) have demonstrated that transfection of cloned retroviral DNA into Hela cells resulted in sequence-specific cell death, and suggested that cytopathic effects of some retroviruses might result from sequence-specific interactions between accumulated promoter sequences and recipient cell transcription factors. Recently, Stacey *et al.* (4) have also reported that introduction of DNA into mouse primary macrophages by electroporation caused extensive cell death. However, this DNA toxicity cannot be explained by competition for sequence-specific DNA-binding proteins because no nucleotide sequence specificity has been observed (4).

While cytotoxic effect of DNA has been suggested to be relevant to the pathogenic mechanisms of viral infection so far (1–3), the nature of cell death induced by DNA has not been well characterized. Eukaryotic cell death is generally caused either by necrosis or by apoptosis (5). Necrosis results from direct or indirect damage to the plasma membrane and occurs under nonphysiologic conditions (5, 6). On the other hand, apoptosis is internally regulated and is a physiological process that occurs as a part of cell differentiation, proliferation, and growth of normal and malignant cells (6). Although Stacey *et al.* (4) have proposed that the DNA-dependent cell death observed in macrophages may have a physiological role in defense against certain viral infections, whether the cell death actually occurs by apoptosis remains to be determined. Here, we document that introduction of DNA into human promonocytic cell line, U937, by electroporation causes extensive cell death and demonstrate that this is caused by apoptosis.

MATERIALS AND METHODS

Cell culture. The human cell lines employed in this study were the promonocytic U937 and THP-1 cell lines, the promyelocytic cell line HL60, the B lymphoblastoid Burkitt's cell line Daudi, the T cell leukemia Jurkat cell line, and the epithelioma cell line Hela. All the cell lines were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in RPMI1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Abbreviations used: FSC, forward light scatter; PBS, phosphate-buffered saline; PI, propidium iodide; RLU, relative light unit(s); SV40, simian virus 40.

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DNA preparation. pBluescript SK (Stratagene, La Jolla, CA) and pGL3-control (Promega, Madison, WI) were prepared by Qiagen column (Qiagen, Hilden, Germany). Endotoxin-free plasmid DNA was prepared by EndoFree Plasmid kit (Qiagen) designed to remove endotoxin. *Escherichia coli* DNA (strain B) and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). For DNase I digestion of DNA, 120 μg of pBluescript at 1 mg/ml in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, and 1 mM dithiothreitol was incubated with 84 units of RNase-free DNase I at 37°C for 7 h. As a control for the effect of DNase I itself, 84 units of DNase I was incubated in 120 μl of the same buffer in parallel with the plasmid digestion. For linearization of DNA, pBluescript was digested with *Eco*RI, followed by phenol/chloroform extraction, ether extraction, and ethanol precipitation. Complete digestion was confirmed by agarose gel electrophoresis. For denaturation of DNA, pBluescript was boiled for 5 min and then cooled on ice.

Electroporation. Cells were washed with phosphate-buffered saline (PBS), and resuspended in the complete growth medium at 5×10^7 cells/ml except for Hela cells at 1×10^7 cells/ml. Unless specifically indicated, 25 μg of DNA was added to 500 μl of the cell suspension in a 0.4-cm electroporation cuvette (BioRad Laboratories, Hercules, CA). Electroporations were carried out with a BioRad Gene-Pulser electroporation apparatus (BioRad Laboratories) at 300 V, 950 μF for U937, THP-1, and HL60 cells, at 270 V, 950 μF for Daudi and Jurkat cells, and at 320 V, 950 μF for Hela cells. The cells were then transferred to a 90-mm-diameter dish containing 10 ml of the complete growth medium, and cultured for 20 h.

Luciferase assay. Luciferase assay was performed using a luminometer (Lumat LB9501, Berthold, Wildbad, Germany) and the Promega assay system. Cells were harvested in 150 μl of lysis buffer (Promega), and the luciferase activity was measured for 20 s. Results were expressed as relative light unit (RLU) per dish.

Cell viability. After one volume of trypan blue (0.2%) was added to the equal volume of cell suspension, the cells were examined by light microscopy for ingestion of the dye (×100).

DNA fragmentation. Total DNA for fragmentation analysis was isolated from cells using SepaGene (Sankyo Seiyaku, Tokyo) in accordance with the manufacturer's instructions. The DNA (1 μg) was run on 2% agarose gels in TBE (2 mM EDTA, pH 8.0, 89 mM Tris, and 89 mM boric acid), stained with ethidium bromide at 1 μg/ml, and visualized by transillumination with UV light.

Flow cytometry. The criteria for apoptosis as defined by flow cytometry were based on changes in forward light scattering properties of dead cells due to decreased cell size (7). Cells were washed with PBS and suspended at a concentration of approximately 2.5×10^6 cells/ml in PBS containing propidium iodide (PI) at a final concentration of 1 μg/ml, and then incubated on ice for 30 min. Flow cytometric analyses were carried out using a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA).

Analysis of hypoploidy. Cells were washed with PBS and resuspended in 100 μl of PBS. The cell suspension was then fixed by the addition of 5 ml of 70% ethanol, left at 4°C for more than 30 min, and centrifuged to pellet the cells. The cell pellets were resuspended in 200 μl of PBS, and treated with DNase-free RNase A (final concentration, 500 μg/ml) at 37°C for 20 min. The cells were pelleted by centrifugation, suspended at a concentration of approximately 2.5×10^6 cells/ml in PBS containing PI at a final concentration of 1 μg/ml, and then incubated on ice for 30 min. Flow cytometric analyses were carried out as described above.

RESULTS

Cytotoxic effects of the luciferase expression plasmid on electroporated U937 cells. Human U937 cells are one of the promonocytic cell lines, which has been used as recipient cells in studies for transcriptional regulation of myeloid lineage-specific genes, and optimization of transient transfection into these cells using electroporation has been reported (8). In our transient transfection experiments using U937 cells, we noticed that introduction of the plasmid containing the firefly luciferase gene driven by the simian virus 40 (SV40) promoter/enhancer (the pGL3-control vector, Promega) resulted in extensive cell death. Figure 1 shows titration of plasmid concentration. As the amount of plasmid in the electroporation was increased, cell viability was reduced while reporter gene expression driven by the SV40 early promoter/enhancer increased. Even at the lowest amount of plasmid (1.6 μg), we could detect reduction in cell viability and also low but significant luciferase activity, indicating that very low level of DNA taken up by U937 cells is sufficient for triggering cell death.

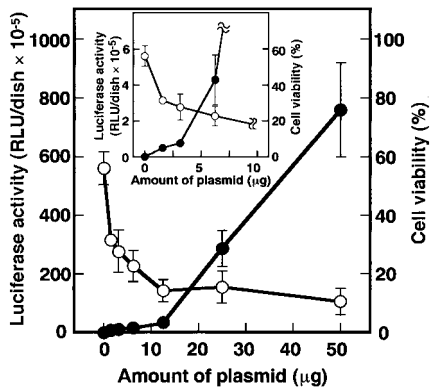

FIG. 1. Effect of introduced plasmid on transient gene expression and cell viability. U937 cells (2.5×10^7) were electroporated with various amounts of pGL3-control. Luciferase activity (closed circle) and survival rate of the cells (open circle) were evaluated 20 h after transfection. Luciferase activity was expressed as RLU. Thin bars represent the SD from three independent experiments performed in duplicates. The inset in Fig. 1 shows the same data plotted in an expanded scale to illustrate the luciferase activity and the viability of cells electroporated with smaller amounts of pGL3-control. The background was <5000 RLU/dish.

Cell death is induced by DNA itself. It has been shown that transfection of cloned retroviral DNA results in sequence-specific cell death (3). However, the pBluescript plasmid DNA, which contains neither viral sequences nor reporter gene, and pGL3-control gave equivalent degrees of cell viability (Fig. 2A, pBS and pGL3-C, respectively), indicating that neither the presence of viral promoter sequences nor the products from the reporter gene were responsible for cell death. Plasmid prepared by a method designed to remove endotoxin gave the equivalent level of cell death (Fig. 2A, pBS EndoFree). Furthermore, digestion of the plasmid DNA with DNase I prevented extensive cell death (Fig. 2A, pBS + DNase I), while no effect of DNase I itself on viability of cells electroporated without DNA was ob-

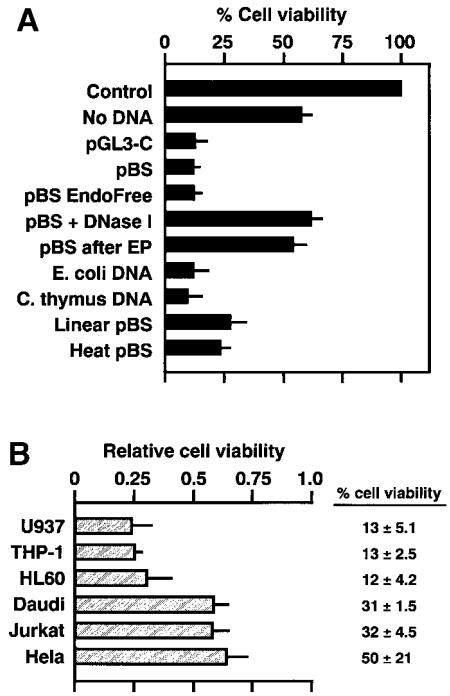

FIG. 2. (A) Effect of electroporation with various plasmids on cell viability of U937. U937 cells (2.5×10^7) were electroporated with 25 μg of various plasmids. Cell treatments were: Control, unelectroporated cells; No DNA, electroporated without DNA; pGL3-C, electroporated with pGL3-control; pBS, electroporated with pBluescript; pBS + DNase I, electroporated with DNase I-treated pBluescript; pBS EndoFree, electroporated with pBluescript prepared with Qiagen EndoFree Plasmid kit; pBS after EP, pBluescript added approximately 5 min after electroporation; *E. coli* DNA, *E. coli* genomic DNA; C. thymus DNA, calf thymus genomic DNA; Heat pBS, heat-denatured pBluescript; Linear pBS, pBluescript linearized with *Eco*RI. Cell viability was determined by trypan blue exclusion 20 h after electroporation. Cell viability from each experiment was normalized to unelectroporated cells set at 100%; thin bars represent the SD from three to seven experiments performed in duplicates. (B) Cell type specificity of DNA-induced cell death. U937, THP-1, HL60, Daudi, Jurkat, and Hela cells were electroporated with 25 μg of pGL3-control. Cell viability was determined by trypan blue dye exclusion 20 h after electroporation. Cell viability of each cell line was expressed relative to cells electroporated without DNA; thin bars represent the SD from three to five independent experiments performed in duplicates. Percent cell viability compared to unelectroporated cells was shown on the right side.

served (data not shown). These results indicate that the DNA itself but not a contaminant was responsible for cell death. In addition, DNA added without electroporation and added 5 min after electroporation caused no DNA-dependent cell death (data not shown and pBS after EP in Fig. 2A, respectively), strongly suggesting that cell death requires entry of DNA into the cells, and also that electroporation acts on the introduction of DNA into the cells rather than acts synergistically with exogenous DNA.

DNA specificity for DNA-dependent cell death. The plasmid preparations used in this paper were predominantly supercoiled as determined by agarose gel electrophoresis (data not shown), but the linearized plasmid and heat-denatured plasmid significantly reduced toxicity (Fig. 2A, Linear pBS and Heat pBS, respectively). These results indicate that DNA needs to be double-stranded and supercoiled to induce cell death.

It has recently been reported that bacterial DNA but not mammalian DNA modulates lymphocyte function (9, 10), as well as stimulates macrophages (11). Bacterial DNA is known to have several types of structural determinants absent in mammalian DNA (12). However, genomic DNAs derived from *E. coli* and calf thymus both gave equivalent degrees of cell death (*E. coli* DNA and Calf thymus DNA in Fig. 2A), clearly indicating that DNA-induced cell death observed in this study is independent of the specific response to bacterial DNA. Taken together, plasmid DNA with or without eukaryotic promoter sequences, *E. coli* DNA, and calf thymus DNA were all capable of causing death of U937 cells (Fig. 2A), indicating that the toxicity of DNA is sequence-independent.

Cell type specificity of DNA-dependent cell death. Figure 2B compares the sensitivity of various cell types to DNA toxicity. The electroporation voltage was optimized for each cell line based on luciferase activity of transfected pGL3-control. As shown in Fig. 2B, whereas toxicity of DNA to cell lines belong to B and T lymphoid lineages (Daudi and Jurkat cells, respectively), and nonhematopoietic tumor line (Hela cells) was limited, the large scale of cell death seen in U937 cells was observed exclusively in promonocytic cell line THP-1 and promyelocytic HL60 cells, suggesting that extensive cell death induced by DNA is specific for myeloid cells, especially cells belonging to monocyte/macrophage lineage.

DNA-induced cell death is caused by apoptosis. To determine whether U937 cells were dying as a result of apoptosis, we examined for internucleosomal DNA fragmentation. As shown in Fig. 3, although no or very little of fragmented DNA was seen in cells without electroporation (lane 1) or cells electroporated without DNA (lane 2), respectively, considerable fragmented DNA was detected in cells electroporated with the pGL3-control and pBluescript plasmids (lanes 3 and 4, respectively). In contrast, DNA added without electroporation gave no DNA fragmentation (lane 5). Also, DNA added 5 min after electroporation did not induce DNA-dependent DNA fragmentation (lane 6). DNA fragmentation was also detected in cells electroporated with *E. coli* and calf thymus genomic DNAs (lanes 7 and 8, respectively), although the degrees were somewhat less than those seen with plasmid DNAs.

To verify that DNA induces apoptosis, the electroporated cells were further examined for a decrease in cell size and for cell cycle. As shown in Fig. 4C, when

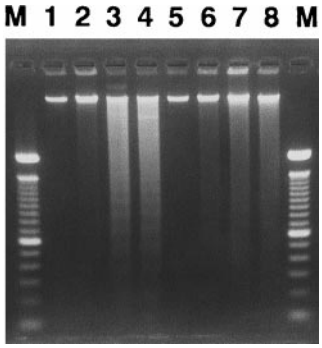

FIG. 3. Effect of electroporation with DNA on DNA fragmentation of U937 cells. U937 cells (2.5×10^7) were electroporated with 25 μg of various nucleic acids. Cell treatments were: lane 1, unelectroporated cells; lane 2, electroporated without DNA; lane 3, electroporated with pGL3-control; lane 4, electroporated with pBluescript; lane 5, pBluescript added without electroporation; lane 6, pBluescript added approximately 5 min after electroporation; lane 7, electroporated with *E. coli* genomic DNA; lane 8, electroporated with calf thymus genomic DNA. DNA fragmentation was determined 20 h after electroporation. M, molecular weight markers (the 100 Base-Pair Ladder from Pharmacia).

electroporated with plasmid, nonviable cells as determined by permeability for PI increased and showed a low forward light scatter (FSC), indicating a decrease in cell size. In contrast, very little of such population was observed when electroporated without DNA (Fig. 4B). Furthermore, when electroporated cells were fixed and stained with PI, hypoploidic cells were observed to increase in a DNA-dependent manner (Fig. 5). DNA fragmentation, a decrease in cell size, and a considerable proportion of cells outside cell cycle, all are cardinal features of apoptosis (6, 13), and these were clearly observed in the DNA-introduced cells.

DISCUSSION

With regard to effects of DNA accumulation in cells, it has been reported that cytopathic effects of the spleen necrosis virus and the avian leukosis virus correlate with the amount of unintegrated forms of retroviral DNAs accumulating in the host cells (1, 2). Other studies have demonstrated that transfected DNA is toxic to recipient cells (3, 4). Although these reports have suggested some physiological significance of the cellular response to exogenous DNA, the nature of cell death induced by DNA has been poorly characterized. This is the first demonstration that DNA-induced cell death is caused by apoptosis, strongly suggesting that this phenomenon is mediated through a physiological process.

At present, mechanisms underlying DNA-induced apoptosis are unknown. It is possible that soluble factor(s) produced by the cells taking up foreign DNA might induce apoptosis in bystander cells. However, we detected no effects of the supernatants of transfected cells on cell growth of U937 (data not shown), suggesting the absence of such a soluble factor. In addition, treatment of U937 cells with several inhibitors of second-messenger signaling pathways including H8 (10 μM; an inhibitor of protein kinase A), H7 (10 μM; an inhibitor of protein kinase C), pyrrolidine dithiocarbamate (10 μM; an antioxidant), and N^G-methyl-L-arginine (3 mM; an inhibitor of nitric oxide synthase) failed to inhibit DNA-induced cell death (data not shown). While this suggests that the cell response to introduced DNA might not be mediated through these pathways, still we can not rule out a possibility that these agents did not function effectively at the intracellular level. Further studies are required to reach definite conclusions.

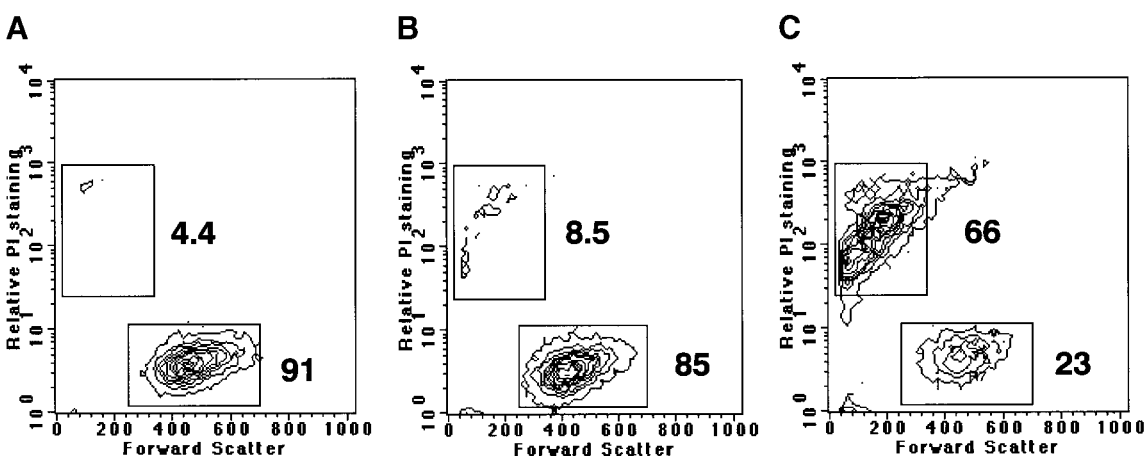

FIG. 4. Cytometric analysis of PI uptake by U937 cells electroporated with or without DNA. U937 cells (2.5×10^7) were electroporated without (B) or with 25 μg of pBluescript (C), or not electroporated (A), and cultured for 20 h. Data are presented as counter plots of cell number and FSC, a measure of relative cell size, vs fluorescence due to PI uptake. The proportion of the cells to all cells analyzed are indicated to the right of each box as percent.

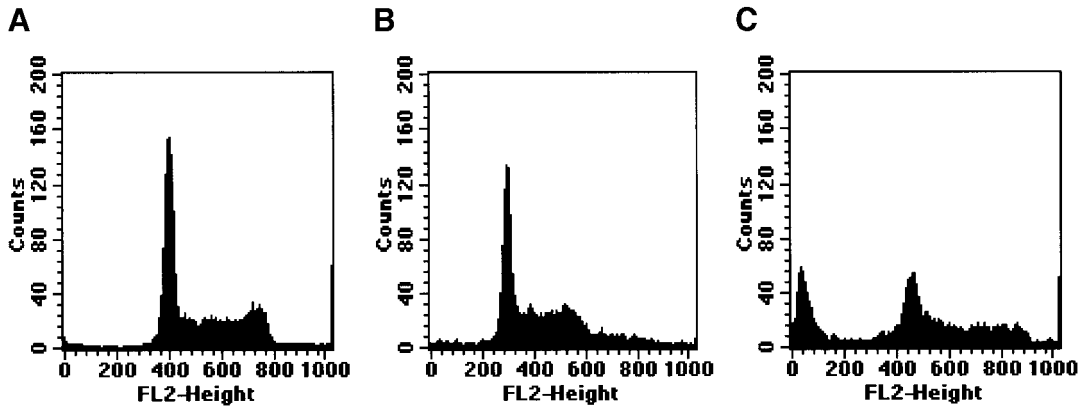

FIG. 5. Induction of hypoplody by electroporation with DNA. U937 cells (2.5×10^7) were electroporated without (B) or with 25 μg of pBluescript (C), or not electroporated (A), and cultured for 20 h.

Interestingly, we recently found that DNA-dependent cell death did not occur when U937 cells were transfected using liposomes (Lipofectamine, Gibco/BRL) or a chemical reagent (Superfect reagent, Qiagen), although these methods gave higher luciferase activity than that obtained by electroporation with 1.6 μg of pGL3-control (Fig. 1 and data not shown). Since DNA transfected by liposomes or chemical reagents is thought to enter into endocytic-lysosomal compartment by active endocytosis while electroporation-mediated DNA uptake does not necessarily involve endocytic mechanism and DNA directly enters into cytosolic compartment (14), it is likely that DNA-induced apoptosis observed here may require the existence of DNA in cytoplasmic compartment rather than vesicular compartment.

Our study using various cell lines strongly suggests that the large scale of cell death induced by DNA is specific for myeloid cells, especially cells belonging to monocyte/macrophage lineage (Fig. 2B). Stacey *et al.* (4) have also observed DNA-dependent cell death in electroporated mouse primary macrophages and proposed its physiological role in host defense against certain viral infections, although whether the cell death occurs by apoptosis remains to be determined. It has recently been accepted that many animal viruses induce an apoptotic response in infected cells (13, 15). Among them, some viruses including adenovirus (16, 17), Epstein-Barr virus (18), baculovirus (19), etc. have been reported to possess some mechanisms to inhibit apoptosis presumably to allow host cell survival for their replication. Against these viruses, apoptosis appears to work as a host defense mechanism to limit their replication. Rapid shut-down of viral or bacterial replication by death of host cells could be effective for preventing the propagation of the microorganisms. In this context, monocytes/macrophages are capable of acting as a first line of host defense against invading pathogens including viruses, and recent reports have emphasized that apoptosis may represent a defense mechanism of macrophages to infection with bacteria (20, 21) and parasites (22). These are strong evidences that monocytes/macrophages have a physiological system underlying induction of apoptosis in response to infection. Double-stranded RNA, which is thought to be normally generated during the course of viral infection, leads to antiviral state including the induction of apoptosis (23). DNA-induced apoptosis observed with the promonocytic cell line U937 may, if not all, be relevant to such physiological systems in the course of viral infection.

Finally, so far cell death has generally been viewed as a technical problem to be overcome in studies of transcription rather than as a physiologically relevant phenomenon. However, our findings suggest a possibility that the results of transfection experiment may reflect complex effects resulting from some activation of the recipient cells by transfected DNA. To resolve and exclude the unfavorable effects, further analyses for molecular mechanisms of our observed phenomena are required.

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