molecular pharmaceutics

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Evaluating the Gene-Expression Profiles of HeLa Cancer Cells Treated with Activated and Nonactivated Poly(amidoamine) Dendrimers, and Their DNA Complexes

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Received December 8, 2009; Revised Manuscript Received March 2, 2010; Accepted April 15, 2010

Abstract: Using dendrimers in cancer therapy as nonviral vectors for gene delivery seems promising. The biological performance of a dendrimer-based gene delivery system depends heavily on its molecular architecture. The transfection activity of dendrimers is significantly improved by processes activated in the heat degradation treatment of solvolysis. However, very little is known about the molecular mechanisms that dendrimers produce in cancer cells. We studied the changes in global gene-expression profiles in human cervical cancer HeLa cells exposed to nonactivated and activated poly(amidoamine) (PAMAM) dendrimers, alone or in complexes with plasmid DNA (dendriplexes). Real-time quantitative reverse transcriptase-polymerase chain reaction was used to confirm four regulated genes (PHF5A, ARNTL2, CHD4, and P2RX7) affected by activated dendrimers and dendriplexes. Activated and nonactivated dendrimers and dendriplexes alike induced multiple gene expression changes, some of which overlapped with their dendriplexes. Dendrimer activation improved transfection efficiency and induced additional gene expression changes in HeLa cells. Dendrimers and dendriplexes principally affect genes with the molecular functions of nucleic acid binding and transcription activity, metal-ion binding, enzyme activity, receptor activity, and protein binding. Our findings provide a deeper insight into the changes in gene expression patterns caused by the molecular structure of PAMAM dendrimers for gene-based cancer therapy.

Keywords: Poly(amidoamine) (PAMAM) dendrimers; nonviral vectors; microarrays; human cervical cancer HeLa cells; activation

Introduction

The lack of a safe and efficient vector system for gene delivery is a major limiting factor for gene therapy.[1,2] Nonviral vectors are safer and more versatile than their more efficient viral counterparts, which may induce adverse immunological responses *in vivo*.[3] Generally, nonviral vectors interact with anionic DNA to form highly condensed complexes for increased binding to the cell surface, enter the cell via a vesicular transport mechanism, protect DNA from endosomal degradation, and are subsequently transported into the nucleus for delivered gene expression.[4] Among nonviral vectors, cationic dendrimers such as poly(propylene imine) (PPI) and poly(amidoamine) (PAMAM) have unique nanocharacteristics and deliver nucleic acid *in vitro*

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10.1021/mp900303s
Published on Web 04/15/2010

and *in vivo*.[5] The biological performances of dendrimer-based gene delivery systems depend heavily on their molecular architecture. For example, processes activated in solvolytic heat-degradation treatment significantly improve dendrimer transfection activity.[6−8] Activated dendrimers are more flexible structures that are compact in a DNA complex and swell when released from the DNA complex.[7]

Cationic dendrimers induce multiple biological effects *in vitro* and *in vivo*. The intrinsic antitumor property of cationic PPI dendrimers synergistically improves the therapeutic effects of anticancer gene therapy.[9,10] Cationic dendrimers, when delivering plasmid and small interfering RNA (siRNA), cause marked global gene expression changes in human cells.[11−13] The gene expression altered by dendrimers includes defense responses, cell proliferation, and apoptosis. Cationic PPI dendrimers in DNA complexes (dendriplexes) altered gene expression profiles.[11] Also, differences in the structural architecture of the dendrimers have opposing effects on targeted gene expression.[12] The extent of dendrimer-induced gene expression changes depends on the dendrimer's architecture and on the cell type.[11] Understanding the influences and consequences of dendrimer-induced gene changes should help us design and develop gene-based delivery systems.

The primary objective of this study was to explore, using high-density microarray analysis, the alteration of whole genomic responses in human cervical HeLa cancer cells treated with nonactivated (intact) and activated PAMAM dendrimers alone or with their dendriplexes.

Materials and Methods

Materials. Starburst PAMAM dendrimer (Generation 5 (PAMAM 5.0; MW 28826 Da; 128 N-terminal amines)) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). PAMAM dendrimers were supplied in methanol, which was removed using vacuum evaporation, and stored with phosphate buffered saline (PBS) at 4 °C. The activated dendrimers were prepared at a concentration of 2 mg/mL in a 50 °C water bath for the times indicated. pDNA (pSG5*lacZ*, 8 Kb), which encodes the *lacZ* gene for β-galactosidase, was driven by a SV40 promoter to assess gene expression. pSG5*lacZ* was amplified in *Escherichia coli* and purified using column chromatography (NucleoBond PC 2000; Macherey-Nagel GmbH & Co., Düren, Germany). The purity of pSG5*lacZ* was established using ultraviolet (UV) spectroscopy (E260 nm/E280 nm ratio; range: 1.80−1.90). All other chemicals were of the highest grade available and were purchased locally.

Cell Culture. A human cervical cancer cell line (HeLa) was maintained in Dulbecco's modified Eagle medium (Gibco DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco FBS; Invitrogen) and 100 U/mL of penicillin/100 μg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 at 37 °C.

Cytotoxicity Assay. The activity of dehydrogenases (an indicator of cell viability) in the cells (treated or untreated with dendrimers, alone, or their DNA complexes) was simultaneously assessed. Negative control cells contained no PAMAM or their DNA complexes. The incubation conditions of cells were the same as the incubation conditions of the transfection assay described in the next section. To measure cell viability, 10 μL of a cell-counting kit solution, a tetrazolium salt that produces a highly water-soluble formazan dye upon biochemical reduction in the presence of an electron carrier (1-methoxy PMS) (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan), was added to a 100 μL culture medium and incubated for 4 h. The amount of yellow formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture medium. The absorbance at 450 nm was obtained using an ELISA reader with a reference wavelength of 595 nm. Results are reported as the cell viability percentage (average optical density (OD)/average negative-control OD) $\pm$ standard deviation (SD).

Transfection Assay. Cells were seeded into 24-well cell-culture plates at a density of 3×10^5 cells/well and grown overnight (60% to 75% confluence). Immediately before transfection, the cells were rinsed with PBS and supplemented with 1 mL of fresh DMEM per well. The pDNA (5 μg) and a range of concentrations of intact or activated PAMAM were each diluted into 500 μL of DMEM solution. PAMAM dendrimers were added to the pDNA solution at

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room temperature and allowed to sit for 15 min to allow dendrimer−DNA complexes to form. The cell medium was removed and the cells were then exposed to transfection mixtures for 4 h in the absence of serum, after which they were supplemented with 10% FBS and 1% antibiotics. After 24 h of incubation, β-galactosidase gene expression was analyzed using a β-Gal assay kit (Invitrogen) and a BCA protein assay reagent kit (Pierce, Rockford, IL). The transfection activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG)/min/mg protein.

Light Scattering and Zeta Potential Analysis. The particle size and zeta potential of dendriplexes in DNase/RNase-free ultrapure water suspensions were measured using light scattering and capillary electrophoresis (Zetasizer 3000HS; Malvern Instruments Ltd., Malvern, Worcestershire, U.K.).

Purifying RNA, and Microarray Analysis. Under the same incubation conditions as in the transfection assay, total RNA was extracted from HeLa cells treated and untreated with intact and activated PAMAM alone or their dendriplexes, using a reagent (Trizol; Invitrogen) and purified using a kit (RNeasy Mini Kit; Qiagen GmbH, Hilden, Germany). To avoid the interaction between dendrimers and total RNA, the RNA pellets were washed in 2 M NaCl solution for 12 h.[14] The purified RNA was quantified at an optical density (OD) of 260 nm using a spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE) and qualitatively analyzed using a bioanalyzer (2100; Agilent Technologies, Inc., Santa Clara, CA). Microarray experiments were done following the manufacturer's protocols. Briefly, 0.5 μg of total RNA was amplified using a kit (Fluorescent Linear Amplification Kit; Agilent) and labeled with Cy3-CTP or Cy5-CTP (CyDye; PerkinElmer, Waltham, MA) during *in vitro* transcription. RNA from cells treated with intact and activated PAMAM dendrimers or their dendriplexes was labeled using Cy5, and RNA from untreated cells was labeled using Cy3. Cy-labeled cRNA (0.825 μg) was fragmented to an average size of about 50−100 nucleotides (nt) using incubation with a fragmentation buffer (Agilent) at 60 °C for 30 min. Correspondingly fragmented labeled cRNA was then pooled and hybridized to an oligo microarray kit (Whole Human Genome Microarray [4 × 44K] kit; Agilent) at 60 °C for 17 h. After they had been washed and dried with a nitrogen gun, the microarrays were scanned (microarray scanner; Agilent) at 535 nm for Cy3 and 625 nm for Cy5. Scanned images were analyzed (Feature Extraction Software 9.5.3; Agilent) using an image analysis and normalization software to quantify signal and background intensity for each feature. The data were substantially normalized using the rank-consistency-filtering LOWESS method. Genes whose expression increased $\geq$2 times or decreased $\leq$0.5 time, and that had a p value <0.05, were considered to be induced.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qPCR). The same RNA isolated for the microarrays was used for qPCR. To prepare a cDNA pool from each RNA sample, total RNA (5 μg) was reverse-transcribed using reverse transcriptase (Moloney murine leukemia virus [M-MLV]; Promega, Madison, WI); the resulting samples were diluted 40 times by volume with nuclease-free water. Each cDNA pool was stored at −20 °C until it was subjected to additional real-time qPCR with specific oligonucleotide primer pairs from the Roche Universal Probe Library (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN). Four genes (PHF5A, ARNTL2, CHD4, and P2RX7) were investigated using the following primer sequences: PHF5A, sense GGCACACCATATCGGAGAAA and antisense TCACAGTCAGTTCCTCAAGCA; ARNTL2, sense TGGCACACTGTCCTCTTGAA and antisense CACACCAATTCTCCCCATCT; CHD4, sense TGTAAAGGAACAGCCCCAGT and antisense GCAGGAACCCACAACAGTTT; and P2RX7, sense CTGCCGTCCCAAATACAGTT and antisense GTGCCAAAAACCAGGATGTC. Human PPIH (peptidylprolyl isomerase H (cyclophilin H)) was used as the reference gene. The specificity of each primer pair was tested using rat common reference RNA (Stratagene, La Jolla, CA) as a template for real-time PCR, and then using a DNA 500 chip run on the Bioanalyzer 2100 to check the size of the PCR product. Primer pairs that yielded the predicted product at the predicted size, and no other primary or secondary products, were chosen for the real-time PCR reaction (LightCycler Instrument 1.5 using LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Cat. 03 515 885 001); Roche, Castle Hill, Australia). Briefly, 10 μL reactions contained 2 μL of 5× Master Mix, 2 μL of each 3.75 μM forward and reverse primer mixture, and 6 μL of cDNA (40:1 dilution). Each sample was run in triplicate. The real-time PCR program was 95 °C for 10 min, 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s. At the end of the program, a melt curve analysis was done. For each real-time PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. From each of these plots, the system automatically calculated the CP value (crossing point; the turning point corresponds to the first maximum of the second derivative curve), which we infer to be the beginning of exponential amplification.

Statistical Analysis. Data were analyzed using one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

Results

Cytotoxicity Assay. To assess the cytotoxic effect of nonactivated PAMAM dendrimers and their dendriplexes, we incubated HeLa cells for 24 h with various concentrations of PAMAM dendrimers (10, 15, 30, and 50 μg/mL) and their

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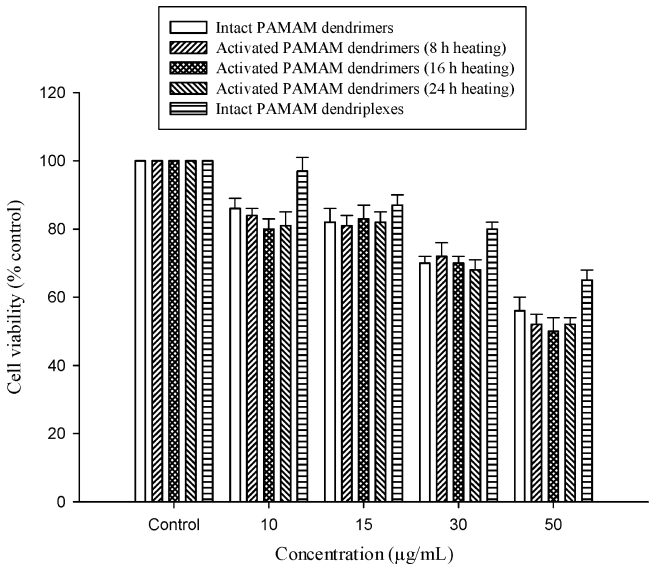

Figure 1. Cytotoxicity assays of intact PAMAM dendrimers and their dendriplexes and activated PAMAM dendrimers on HeLa cells by measuring generated dehydrogenases. Negative control cells were grown without adding either intact dendrimers and their dendriplexes or activated dendrimers. Data are cell viability percentages (average OD/average negative control OD) ± standard deviation (SD) ($n = 3$).

DNA (5 μg/mL) complexes (Figure 1). Cell viability was above 80% at the intact PAMAM dendrimer concentrations of 10 and 15 μg/mL but below 80% at 30 and 50 μg/mL. Cell viability was above 80% until 50 μg/mL for intact dendriplexes (Figure 1). The negative charge of DNA neutralized the positive charge of the PAMAM dendrimers and increased cell viability. The intact PAMAM dendrimers were then activated for 8, 16, and 24 h, after which they were analyzed using cytotoxic assays (Figure 1). The cell viability of activated PAMAM dendrimers under all incubation conditions was at best comparable and slightly more cytotoxic than that of their nonactivated counterparts.

Transfection Assay. We examined the *in vitro* gene expression of intact dendriplexes (5 μg/mL of DNA plus 10, 15, 30, or 50 μg/mL of nonactivated PAMAM) incubated for 24 h (Figure 2A). Gene expression using intact dendriplexes was more efficient than using naked DNA alone. Maximum transfection activity occurred at 15 μg/mL (Figure 2A). Taking into account transfection efficiency and cytotoxicity, 15 μg/mL of intact PAMAM dendrimers were selected and heated for different times, after which they were combined in complexes with 5 μg/mL of DNA, and then transfection activity was assessed (Figure 2B). Heating intact PAMAM dendrimers for 16 h yielded the maximum levels of gene expression (cell viability = 84 ± 3%), whereas transfection activity remained unchanged after 8 h of heating, and gene expression decreased after 24 h of heating (Figure 2B). Therefore, we chose 15 μg/mL PAMAM dendrimers (intact and activated for 16 h) and dendriplexes for microarray analysis.

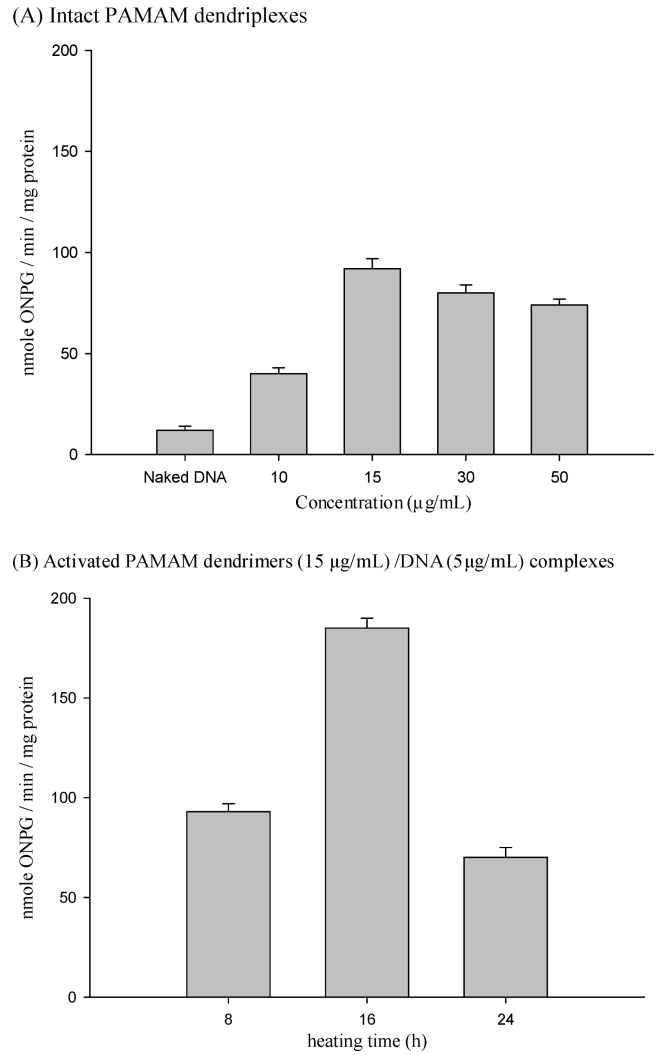

Figure 2. Transfection of HeLa cells with intact dendriplexes (A) and activated PAMAM (15 μg/mL)/DNA complexes (B). The data are means ± standard deviation (SD) ($n = 3$).

Light Scattering and Zeta Potential Analysis. The mean diameter of 15 μg/mL intact PAMAM/5 μg/mL DNA dendriplexes was 177 ± 24 nm with a narrow distribution (polydispersity index (PI) = 0.16; $n = 3$). We found a smaller mean particle size (108 ± 16 nm; $n = 3$) and slightly broader distribution (PI = 0.21) for 15 μg/mL activated PAMAM/5 μg/mL DNA dendriplexes. The zeta potentials were comparable between 15 μg/mL intact PAMAM/5 μg/mL DNA dendriplexes (12 ± 2 mV; $n = 3$) and 15 μg/mL activated PAMAM (16 h heating)/5 μg/mL DNA dendriplexes (11 ± 2 mV; $n = 3$).

Global Gene Expression Profiles of HeLa Cells Treated with Intact and Activated Dendrimers Alone or with Their Dendriplexes. Activated PAMAM dendrimers alone and their dendriplexes produced more upregulated and downregulated genes than did intact dendrimers alone and their dendriplexes (Figure 3). Intact dendrimers and their dendriplexes produced similar numbers of upregulated genes, but intact dendrimers produced more downregulated genes

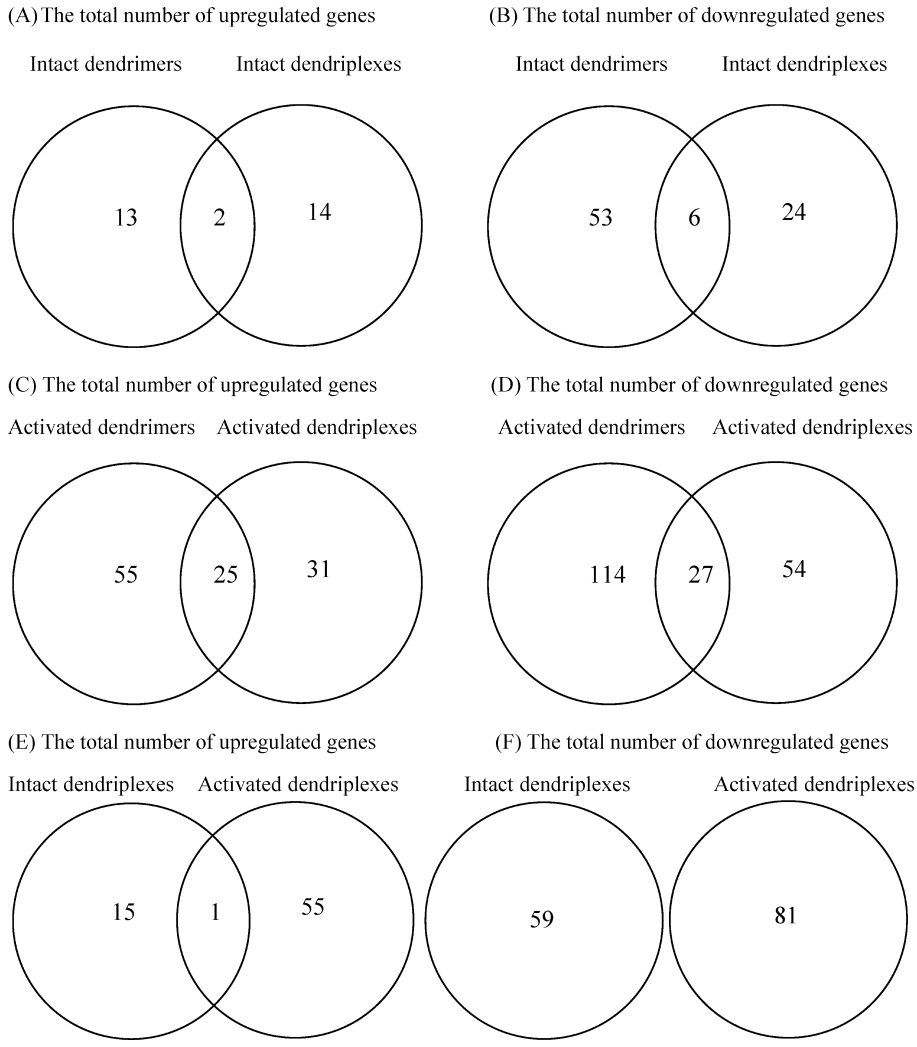

Figure 3. A Venn diagram showing the number of regulated genes in HeLa cells treated with intact dendrimers and their dendriplexes (A, B), activated dendrimers and their dendriplexes (C, D), and activated and intact dendriplexes (E, F).

than did their dendriplexes (Figure 3A,B). The number of upregulated and downregulated genes was higher for activated dendrimers than for activated dendriplexes (Figure 3C,D); in addition, there were more downregulated genes than upregulated genes. Moreover, some expressed genes were affected by being exposed to dendrimers as well as to dendriplexes (Figure 3A−D). Tables 1−4 provide a list of 10 most upregulated and downregulated genes in HeLa cells exposed to intact and activated dendrimers and their dendriplexes. The GIPR and OR2W3 genes, which regulate membrane receptors, were both upregulated by intact PAMAM dendrimers alone and their dendriplexes. In addition to the GIPR and OR2W3 genes, the intact PAMAM dendrimers alone upregulated 13 other genes, and the intact dendriplexes upregulated 14 genes. Both intact dendrimers and their dendriplexes downregulated 6 genes. More upregulated and downregulated genes were identified for activated dendrimers alone and their dendriplexes than for intact dendrimers and their dendriplexes. This indicated that activated dendrimers and their dendriplexes produced more profound changes than did intact dendrimers and their dendriplexes in transfection efficiency and in gene upregulation and downregulation. Also, the Venn diagram of activated vs nonactivated dendriplexes demonstrated that the GIPR gene was the only upregulated gene affected by both activated and nonactivated dendriplexes, and that no downregulated genes were overlapped by both activated and nonactivated dendriplexes (Figure 3E,F). This reflected that the differences in the structural architecture of the dendrimers induced almost totally different gene-expression profiles in cancer cells. Figure 4 shows the percentage of principal molecular functions of these upregulated and downregulated annotated genes compared with the total number of annotated genes (analyzed using the Gene Ontology (GO) database). The 5 most principal molecular functions of regulated genes for intact and activated dendrimers and their dendriplexes were nucleic acid binding and transcription activity, metal-ion binding, enzyme activity, receptor activity, and protein binding. Activated dendrimers and their dendriplexes induced more molecular functions than did intact dendrimers and their dendriplexes.

Table 1. The 10 Most Upregulated and Downregulated Genes in Nonactivated Dendrimers[a]

gene name	GenBank no.	description	fold change
		Upregulated	
OR2W3[b]	NM_001001957	olfactory receptor, family 2, Subfamily W, member 3	5.350
TRIM49	NM_020358	tripartite motif-containing 49	4.834
FOLH1	NM_004476	folate hydrolase (prostate-specific membrane antigen) 1, transcript variant 1	4.747
ENST00000370419	ENST00000370419	regulating synaptic membrane exocytosis protein 1 (Rab3-interacting molecule 1) (RIM 1)	3.969
CHERP	NM_006387	calcium homeostasis endoplasmic reticulum protein	3.060
GIPR[b]	NM_000164	gastric inhibitory polypeptide receptor	3.031
ABCB1	NM_000927	ATP-binding cassette, subfamily B (MDR/TAP), member 1	2.781
RSU1	NM_012425	Ras suppressor protein 1, transcript variant	2.776
PTPRR	NM_002849	protein tyrosine phosphatase, receptor type, R, transcript variant 1	2.659
C5orf23	NM_024563	chromosome 5 open reading frame 23	2.536
		Downregulated	
KCNJ10	NM_002241	potassium inwardly rectifying channel, subfamily J, member 10	0.010
PTGFR	NM_001039585	prostaglandin F receptor (FP), transcript variant 2	0.028
SLC8A1	X91815	Na^+/Ca^{2+} exchanger isoform	0.046
USP29	NM_020903	Ubiquitin specific peptidase 29	0.068
PDE11A	NM_016953	phosphodiesterase 11A, transcript variant 4	0.070
UGT8[b]	NM_003360	UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)	0.076
ART4	NM_021071	ADP-ribosyltransferase 4 (Dombrock blood group)	0.077
RGS9BP	NM_207391	regulator of G protein signaling 9 binding protein	0.085
MATN1	ENST00000373765	cartilage matrix protein precursor (Matrilin-1)	0.128
CXYorf3	BC028151	*Homo sapiens* chromosome X and Y open reading frame 3	0.137

[a] A full list is available in the *Supporting Information*. [b] Genes affected by both nonactivated dendrimers and dendriplexes.

Table 2. The 10 Most Upregulated and Downregulated Genes in Nonactivated Dendriplexes[a]

gene name	GenBank no.	description	fold change
		Upregulated	
OR2W3[b]	NM_001001957	olfactory receptor, family 2, subfamily W, member 3	5.832
ADAMTS15	NM_139055	ADAM metallopeptidase with thrombospondin type 1 motif, 15	4.919
HIST1H2AK	BC034487	histone 1, H2ak	3.832
EFCBP2	NM_019065	EF-hand calcium binding protein 2	3.810
DPCR1	NM_080870	diffuse panbronchiolitis critical region 1	3.780
C12orf42	NM_198521	chromosome 12 open reading frame 42	3.643
ALDH1A3	BC009245	aldehyde dehydrogenase 1 family, member A3	3.350
GIPR[b]	NM_000164	gastric inhibitory polypeptide receptor	2.962
CYP2E1	NM_000773	cytochrome P450, family 2, subfamily E, polypeptide 1	2.890
AATK	AK131529	apoptosis-associated tyrosine kinase (AATK)	2.648
		Downregulated	
C1orf100	NM_001012970	chromosome 1 open reading frame 100	0.017
MSR1	NM_138715	macrophage scavenger receptor 1, transcript variant SR-AI	0.031
MUC2	NM_002457	mucin 2, oligomeric mucus/gel-forming	0.036
DDX43	NM_018665	DEAD (Asp-Glu-Ala-Asp) box polypeptide 43	0.044
PDZD3	NM_024791	PDZ domain containing 3	0.126
ERBB4	NM_005235	v-erb-a erythroblastic leukemia viral oncogene homologue 4 (avian), transcript variant JM-a/CVT-1	0.146
MAGEE2	NM_138703	melanoma antigen family E, 2	0.180
ANGPTL4	NM_139314	angiopoietin-like 4, transcript variant 1	0.190
OBSCN	NM_052843	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	0.216
PCDHGA9	NM_032089	protocadherin gamma subfamily A, transcript variant 2	0.223

[a] A full list is available in the *Supporting Information*. [b] Genes affected by both nonactivated dendrimers and dendriplexes.

Confirming Microarray Results Using qPCR. To compare the gene expression results obtained from the microarray analysis with the results from qPCR, we tested four genes—PHF5A, ARNTL2, CHD4, and P2RX7, responsible for nucleus gene regulation of transcription, signal transduction, and DNA binding, and membrane gene regulation of ion transport, respectively—from activated dendrimers and their dendriplexes (Figure 3). The qPCR were qualitatively consistent with those from the microarray (Figure 5).

Table 3. The 10 Most Upregulated and Downregulated Genes in Activated Dendrimers[a]

gene name	GenBank no.	description	fold change
		Upregulated	
ENST00000379879	ENST00000379879	immunoglobulin heavy chain variable region (Fragment)	8.937
ZSCAN4	NM_152677	zinc finger and SCAN domain containing 4	8.871
NPTX1	NM_002522	neuronal pentraxin I	7.116
MYH8	NM_002472	myosin, heavy chain 8, skeletal muscle, perinatal	5.371
HMGCS1	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	4.988
IGF1	NM_000618	insulin-like growth factor 1 (somatomedin C)	4.712
ENST00000374458	ENST00000374458	gametogenetin-binding protein 1	4.681
TMEM16C	NM_031418	transmembrane protein 16C	4.618
ANKRD19	NM_001010925	ankyrin repeat domain 19	4.522
SLC4A3	NM_005070	solute carrier family 4, anion exchanger, member 3, transcript variant 1	4.432
		Downregulated	
ENST00000319098	ENST00000319098	prosaposin-like protein 1	0.147
TEP1	NM_007110	telomerase-associated protein 1	0.154
ARNTL2	AF256215	cycle-like factor CLIF	0.171
C1QTNF5	NM_015645	C1q and tumor necrosis factor related protein 5	0.186
ZNF652	NM_014897	zinc finger protein 652	0.186
RNF125	NM_017831	ring finger protein 125	0.188
TEAD4	NM_003213	TEA domain family member 4, transcript variant 1	0.195
LRRC4	NM_022143	leucine rich repeat containing 4	0.205
DIRAS2	NM_017594	DIRAS family, GTP-binding RAS-like 2	0.205
ARHGAP26	NM_015071	Rho GTPase activating protein 26	0.206

[a] A full list is available in the *Supporting Information*.

Table 4. The 10 Most Upregulated and Downregulated Genes in Activated Dendriplexes[a]

gene name	GenBank no.	description	fold change
		Upregulated	
HS6ST3	NM_153456	heparan sulfate 6-O-sulfotransferase 3	11.019
GATS	NM_178831	opposite strand transcription unit to STAG3	8.069
KLF17	NM_173484	Kruppel-like factor 17	7.890
VNN3	NM_078625	vanin 3 (VNN3), transcript variant 2	5.334
HMGCS1[b]	NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	5.118
B4GALT5	NM_004776	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 5	3.774
EGR1	NM_001964	early growth response 1	3.764
PLA2G2E	NM_014589	phospholipase A2, group IIE	3.556
UBQLN1[b]	NM_013438	ubiquilin 1, transcript variant 1	3.208
SYNPO	ENST00000307662	synaptopodin	3.019
		Downregulated	
IL31	NM_001014336	interleukin 31	0.064
MED18	NM_017638	mediator of RNA polymerase II transcription, subunit 18 homologue (S. cerevisiae)	0.075
TEP1	NM_007110	telomerase-associated protein 1	0.125
DNAJC12	NM_021800	DnaJ (Hsp40) homologue, subfamily C, member 12, transcript variant 1	0.150
ARNTL2	AF256215	cycle-like factor CLIF	0.184
RNF125	NM_017831	ring finger protein 125	0.196
ZNF652	NM_014897	zinc finger protein 652	0.204
HKDC1	NM_025130	hexokinase domain containing 1	0.213
C1QTNF5	NM_015645	C1q and tumor necrosis factor related protein 5	0.228
CD74	NM_001025158	CD74 molecule, major histocompatibility complex, class II invariant chain, transcript variant 3	0.243

[a] A full list is available in the *Supporting Information*. [b] Genes affected by both activated dendrimers and dendriplexes.

Discussion

Using dendrimers in cancer therapy as nonviral vectors for gene delivery has been successful *in vivo*;[9,10] they not only delivered genes to tumors but also had a synergistic therapeutic effect.[9,15] However, little is known about the molecular mechanism that dendrimers and their dendriplexes

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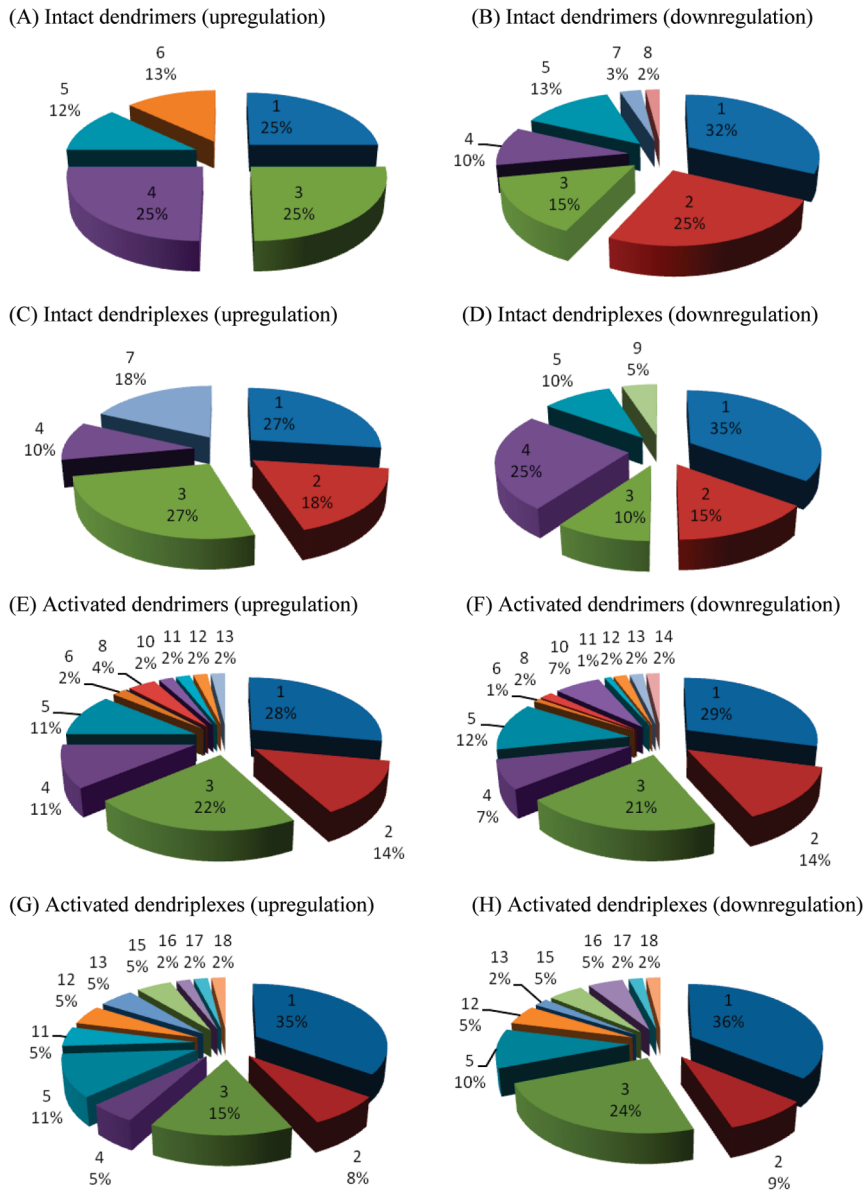

1. Nucleic acid binding and transcription activity
2. Metal ion binding
3. Enzyme activity
4. Receptor activity
5. Protein binding
6. Structural molecule activity
7. Catalytic activity
8. Chemokine activity
9. Microtubule motor activity
10. Sugar binding
11. Lipid binding
12. Signal transducer activity
13. GTP binding
14. Cytokine activity
15. Metabolism
16. Proteolysis and peptidolysis
17. Cell proliferation
18. Immune response

Figure 4. Graphical categories by principal molecular functions of regulated genes in HeLa cells treated with intact dendrimers (A, B), intact dendriplexes (C, D), activated dendrimers (E, F), and activated dendriplexes (G, H). The percentage of each category compared to the total number of annotated genes is shown.

use in cancer cells.[9] One microarray analysis[11] showed that, in human A431 cells, cationic PPI dendrimers induced changes in the expression of a variety of gene ontologies, including those involved in defense responses, cell proliferation, and apoptosis, which might have been related to their antitumor effects. Furthermore, the patterns of global gene expression were dramatically changed when PPI dendrimers were bound in complexes with plasmid DNA. It is, therefore,

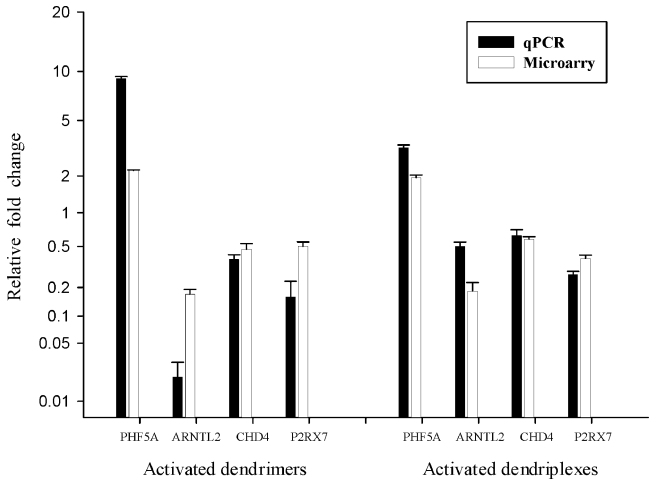

Figure 5. Confirmation of microarray results with real-time quantitative RT-PCR (qPCR). Relative expression levels of 4 genes (PHF5A, ARNTL2, CHD4, and P2RX7) in HeLa cells incubated with activated dendrimers and activated dendriplexes are shown. Data are means ± standard deviation (SD) ($n = 3$).

important to understand the influence of the molecular architecture of dendrimers on gene expression profiling.

In this microarray study, we investigated 44,000 high-density genes instead of 161 low-density genes.[11] We found that the extent of gene changes was dependent on the molecular architecture of intact and activated dendrimers,[11] and that the patterns of gene expression were different between dendrimers and their dendriplexes. However, the type of genes significantly upregulated and downregulated is different from those in the only other study on this topic.[11] These differences may depend upon on the type of dendrimer (PPI vs PAMAM), cell (A431 vs HeLa), or gene chips (low-density vs high-density) used. Fewer upregulated and downregulated genes were involved in apoptosis and immune responses than in the previous study.[11]

The activated PAMAM dendrimers and their dendriplexes used in our study showed not only higher transfection efficiencies but also more significant upregulated and downregulated genes than intact dendrimers and their dendriplexes. Our transfection results were consistent with those of other studies[7,8] reporting that a highly flexible activated structure was responsible for this effect.[7,8] We showed that dendrimer activation induced more gene expression changes in cancer cells. Genes that regulate transcription and proliferation have also been identified in macrophages treated with carbosilane dendrimers.[13]

In our study, the criteria for selecting genes measured using qPCR were based both on significant expression changes and on their pharmacological functions that are closely related to antitumor actions. Of the four genes selected, three (PHF5A, ARNTL2, and CHD4) are involved in survival signaling and the proliferation of cancer cells, and one (P2RX7) is related to signaling between immune cells and target cells. PHF5A is a highly conserved chromatin-associated protein of various species and required for cell viability.[16] Our results indicate that PHF5A upregulation is the survival signal in HeLa cells treated with activated dendrimers and their dendriplexes. ARNTL2 downregulation increased cell proliferation in hepatocellular carcinoma.[17] In contrast, downregulation of CHD4, involved in the regulation of the early growth response, inhibits cell proliferation in various cells.[18,19] P2RX7 is a membrane receptor of ligand-gated cation channels and associated with diverse physiological roles including cancer, pain, inflammation, and unusual responses to infection.[20,21] In the presence of ATP and when divalent cation levels are low, P2RX7 converts to an intrinsic pore that initiates binding to ATP, which allows ions to flow.[20] P2X7 receptor is involved in signaling between macrophages or other cells involved in the immune response and target cells, and may cause cell swelling and cell death after prolonged activation.[20] The consequences of downregulating P2X7 in HeLa cells have not been reported before, and the detailed molecular mechanism remains to be clarified. In addition, some of the gene expression changes from qPCR may have been due to the presence of the transgene rather than just the delivery process. Previous studies[9,10] reported that the dendrimers not only had delivered the encoded gene for tumor necrosis factor-alpha but also had an intrinsic antitumor effect in tumor-bearing mice, and that these data demonstrated the possibilities for enhanced cancer gene therapy using dendrimers as the delivery vectors. However, the exact mechanism of antitumor activity from dendrimers remains largely unknown. Our study of these four regulated genes explained that the engagement of cell survival and cell death mechanisms, as well as immune stimulation by activated PAMAM dendrimers and dendriplexes, may be important for cancer gene therapy because of their antitumor effects. Taken together, our results suggest that activated dendrimers and dendriplexes have diverse roles in controlling gene expression changes in HeLa cells.

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Using dendrimers *in vivo* has significant potential in gene-delivery cancer therapy. Two early *in vivo* studies[22,23] reported that activated PAMAM dendrimer-mediated angiostatin and metalloproteinase (TMP)-2 inhibited tumor growth and angiogenesis, and that gene delivery of Fas ligand by activated PAMAM dendrimers induced significant growth suppression and apoptotic tumor cell death in prostate cancer. Interestingly, like polypropylenimine dendrimers, activated PAMAM dendrimers alone also delayed tumor growth, but only polypropylenimine dendriplexes lead to a significant regression of tumors.[9] Our results from exploring the gene-expression profiles of dendrimers and dendriplexes might allow a more detailed insight for understanding their molecular mechanisms in cancer cells. Our results showed that activated dendrimers and dendriplexes induced marked global gene-expression changes in cancer cells. We found that genes involved in cell proliferation and immune responses were altered by activated dendrimers and dendriplexes, a finding consistent with their direct antitumor effect. Besides that, genes involved in various cellular processes, such as nucleic acid binding and transcription activity, metal-ion binding, enzyme activity, receptor activity, and protein binding, were also altered. Furthermore, the extent of gene changes was different between activated dendrimers and activated dendriplexes, which explains their variable antitumor effects in cancer cells. Our findings suggested that a successful vector for cancer gene therapy might be designed to have desirable molecular functions, such as an antitumor activity, optimal transfection efficiency, and minimal cytotoxicity.

Finally, the transfection efficiency results of nonviral gene delivery systems depend upon overcoming extra- and intracellular barriers involved in gene delivery. Once the gene delivery systems are at the targeted tumor cells, there are still several intracellular barriers: internalization, endosomal escape, cytoplasm trafficking, nuclear translocation, and transcription. We found that activated and nonactivated dendriplexes principally affected genes with the molecular functions of nucleic acid binding and transcription activity, metal-ion binding, enzyme activity, receptor activity, and protein binding. We hypothesize that the genes involved in nucleic acid binding and transcription activity are a major factor that directly leads to the transfection efficiency of dendriplexes. Because we used in our study the same plasmid that was used in previous studies,[9,10,22,23] we conclude that dendrimer architecture is important for regulating transcription processes. Nonviral vectors compact DNA so efficiently that transcription processes are hindered.[24] It has been reported[25] that PAMAM dendrimers inhibited the initiation of transcription *in vitro* but did not affect the elongation of the RNA transcript. However, activated dendrimers are reported[7] to undergo DNA decondensation after swelling, to be more flexible structures than intact dendrimers, and, therefore, to increase transfection efficiency. We found that activated dendriplexes have more regulated genes involved in transcription processes than intact ones, and that this may assist the activities of transcription factors and reach the ultimate goal of transgene expression.

Conclusion

In summary, our results illustrate the importance of the molecular architecture of the dendrimers on the effect of global gene expression profiles in HeLa cells. Dendrimers and dendriplexes induced gene expression changes; some of the dendrimer-induced expression changes were the same as dendriplex-induced expression changes. Activating dendrimers led to higher transfection efficiencies and induced more gene expression changes in cancer cells. Dendrimers and dendriplexes principally affect genes via the molecular functions of nucleic acid binding and transcription activity, metal-ion binding, enzyme activity, receptor activity, and protein binding. We hypothesize that a successful vector for cancer gene therapy may be designed to have desirable molecular functions, such as antitumor activity, optimal transfection efficiency, and minimal cytotoxicity.

Acknowledgment. This work was supported by Grant NSC 98-2221-E-041-002 from the National Science Council, Taiwan.

Supporting Information Available: Tables of intact and activated dendrimers and dendriplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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