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期末報告

陽離子性高分子對細胞自我吞噬作用之影響及應用(第3年)

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計 畫 主 持 人： 郭榮華
共 同 主 持 人： 詹明修
計畫參與人員： 博士班研究生-兼任助理人員：林嘉緯

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中 華 民 國 105 年 10 月 04 日

中 文 摘 要 ： 目前在文獻上應用生物晶片來評估陽離子性高分子PEI基因傳送系統對於細胞相關基因之調控仍著重於蛋白質編碼 RNA之探討，相對地陽離子性高分子如何影響miRNA表現之研究仍待補足。我們以生物晶片研究陽離子性高分子PEI如何影響MEF細胞之miRNA表現模式，並應用生物資訊工具篩選與證實出三條miRNA (mmu-miR-3090-5p; mmu-miR-346-3p; mmu-miR-494-3p) 參與細胞毒性機轉中mTOR特定訊息傳導路徑並誘導細胞自我吞噬。我們更應用聚合酶連鎖反應 PCR 確認miRNA 共同作用於目標基因生長因子Igf1，並以西方墨點法 Western Blot 確認Igf1及相關指標蛋白質之表現，使我們更完整架構陽離子性高分子引發細胞毒性之確切分子路徑網路。

中 文 關 鍵 詞 ： 聚乙烯亞胺, 自體吞噬, 微型核糖核酸, mTOR信號傳送途徑, 生物晶片, 類胰島素生長因子, p53信號傳送途徑

英 文 摘 要 ： Although the toxicology of poly(ethylenimine) (PEI) in gene expression levels has been
previously investigated, little is known about the effects of PEI on the expression of microRNAs (miRNAs) that regulate gene expression at the post-transcriptional level. In this study, we explored miRNA expression profiles related to cell death mechanisms in mouse embryonic fibroblast (MEF) cells treated with PEI by applying microarray analysis. Based on the analysis of the mTOR signaling pathway, three upregulated miRNAs (mmumiR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) were verified in MEF cells treated with PEI at 24 h using real-time quantitative reverse transcriptase-polymerase chain reaction. We further demonstrated that these three upregulated miRNAs resulted in the decrease of gene and protein expressions of the target gene growth factor Igf1 in MEF cells treated with PEI or transfected with three upregulated miRNA mimics. However, these three upregulated miRNAs are not all cell-specific. Finally, we demonstrated that the mTOR signaling pathway
is inhibited by autophagy induction and that the cell viability decreases in MEF cells treated with PEI or transfected with these three miRNA mimics. Collectively, our data suggested that PEI may affect the regulation of miRNAs in target cells.

英 文 關 鍵 詞 ： polyethylenimine (PEI), autophagy, microRNAs (miRNAs), mTOR signaling pathway, microarray, Igf1, p53 signaling pathway

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Exploring MicroRNA Expression Profiles Related to the mTOR Signaling Pathway in Mouse Embryonic Fibroblast Cells Treated with Polyethylenimine

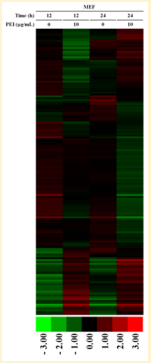
Chia-Wei Lin,[†] Ming-Shiou Jan,[*,†,‡] and Jung-Hua Steven Kuo[*,§]

[†]Institute of Microbiology and Immunology and [‡]Division of Allergy, Immunology, and Rheumatology, Medical College of Chung Shan Medical University, 110 Jianguo North Road, Section 1, Taichung 40201, Taiwan

[§]Department of Pharmacy, Chia Nan University of Pharmacy and Science, 60 Erh-Jen Road, Section 1, Jen-Te, Tainan 717, Taiwan

Supporting Information

ABSTRACT: Although the toxicology of poly(ethylenimine) (PEI) in gene expression levels has been previously investigated, little is known about the effects of PEI on the expression of microRNAs (miRNAs) that regulate gene expression at the post-transcriptional level. In this study, we explored miRNA expression profiles related to cell death mechanisms in mouse embryonic fibroblast (MEF) cells treated with PEI by applying microarray analysis. Based on the analysis of the mTOR signaling pathway, three upregulated miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) were verified in MEF cells treated with PEI at 24 h using real-time quantitative reverse transcriptase-polymerase chain reaction. We further demonstrated that these three upregulated miRNAs resulted in the decrease of gene and protein expressions of the target gene growth factor *Igf1* in MEF cells treated with PEI or transfected with three upregulated miRNA mimics. However, these three upregulated miRNAs are not all cell-specific. Finally, we demonstrated that the mTOR signaling pathway is inhibited by autophagy induction and that the cell viability decreases in MEF cells treated with PEI or transfected with these three miRNA mimics. Collectively, our data suggested that PEI may affect the regulation of miRNAs in target cells.



KEYWORDS: *polyethylenimine (PEI), autophagy, microRNAs (miRNAs), mTOR signaling pathway, microarray, Igf1, p53 signaling pathway*

INTRODUCTION

Poly(ethylenimine) (PEI) is one of the most intensively used cationic polymers in nonviral nucleic acid delivery.[1−3] PEI exhibits efficient delivery *in vitro* and *in vivo* because it provides better buffering capacity than other cationic polymers in the lysosomes due to the so-called "proton-sponge" effect.[4,5] However, high quantities of positive amines in PEI also cause intrinsic cytotoxicity that limits the clinical applicability of PEI.[6] Moreover, PEI has been shown to influence the regulation of gene expression *in vitro* and *in vivo*, and this may interfere with relevant gene expression via off-target effects.[7−10] The vast number of these regulated genes in PEI-treated cells has been found to be extensively involved in the molecular mechanisms of cell death. In a previous study, we identified the regulated genes related to autophagy in PEI-treated mouse embryonic fibroblast (MEF) cells.[11] Hence, it is important to obtain a deep understanding of the genetic information regarding cell death induced by PEI for designing safe nucleic acid delivery systems.

Previous studies on PEI-induced gene regulation have primarily been based on encoding mRNAs that are translated into proteins. However, recent studies have demonstrated that noncoding RNAs are closely related to complex cellular development systems and various human diseases.[12,13] These noncoding RNA molecules include microRNAs (miRNAs), small interfering RNAs, PIWI-interacting RNAs, and long noncoding RNAs.[12] Among these noncoding RNAs, miRNAs are approximately 22 nucleotides long and primarily play important roles in the post-transcriptional regulation of gene expression, making them potential targets for therapeutic applications.[14,15] However, the expressions of miRNA in PEI-treated cells, especially in relation to the molecular mechanisms of cell death, remain unexplored. In this study, we explored the regulated miRNAs and identified their target genes in PEI-treated MEF cells by analyzing the molecular pathways of cell death. In order to better understand the effects of regulated miRNAs on molecular mechanisms of cell death, we further investigated the functional and phenotypic outcomes of these regulated miRNAs on autophagy induction and cytotoxicity.

EXPERIMENTAL SECTION

Materials. Branched PEI 25 K (M_w = 25 000 g/mol) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and neutralized (final pH = 7.4) to desired aqueous concentrations with HCl.

Cell Culture. MEF cell lines were generously provided by Dr. T. Yoshimori (Department of Genetics, Osaka University Graduate School of Medicine, Japan). Mouse Lewis lung

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carcinoma (LLC1) and human cervical cancer (HeLa) cells were obtained from American Type Culture Collection (CRL-1642 for LLC1 cells and CCL-2 for HeLa cells; Manassas, VA, USA). The cells were maintained at 37 °C in 5% CO_2 and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen). The MEF cells were maintained in RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA), while DMEM medium (Invitrogen) was used for the LLC1 cells and HeLa cells.

miRNA Microarray. The miRNA microarrays were carried out in Mouse & Rat miRNA OneArray v5 (Phalanx Biotech Group, Hsinchu, Taiwan), which contains 1265 mouse sequence probes and 48 control sequence probes from Sanger miRBase Release 19 with three repeats. MEF cells were grown overnight at 2×10^6 cells per 10 cm dish and then were treated with 10 μg/mL PEI for 12 and 24 h. Untreated cells after 12 or 24 h incubation were used as negative controls. After treatment, total RNA was extracted using Trizol (Invitrogen) and isolated with a miRVana miRNA Isolation Kit (Life Technologies, NY, USA). The quantity and purity of the purified RNA was assured using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The analysis of miRNA content in the total RNA samples was performed by small RNA assay with an Agilent Bioanalyzer 2100 according to the manufacturer's protocol. The intensity of the 18 and 28 S rRNA bands and the contamination of genome DNA were evaluated by 1% formaldehyde−agarose gel electrophoresis. The samples with an optical density (OD) ratio of 260 nm/280 nm > 1.8, an OD ratio of 260 nm/230 nm > 1, an RNA integrity number (RIN) > 6, 28 S/18 S > 0.7, and no contamination of genome DNA were then subjected to microarray analysis. According to the manufacturer's protocol, the fluorescent targets were labeled from 2.5 μg of total RNA using a ULS miRNA labeling kit (Kreatech Diagnostics, Amsterdam, The Netherlands) and hybridized to the microarray at 37 °C for 14−16 h after a prehybridization procedure to reduce background signals. The hybridized microarrays were scanned using an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) after washing and spin drying. The data was analyzed using GenePix 4.1 software (Molecular Devices) and normalized by 75% media-scaling normalized method. Normalized intensities were converted into miRNA expression $\log_2$ fold change between untreated controls and PEI-treated cells. Standard selection criteria to identify differentially expressed miRNAs were established at the absolute values of $\log_2$ fold change equal to or greater than 0.8 and *P*-value less than 0.05. The regulated miRNAs were demonstrated by unsupervised hierarchical clustering analysis. The potential target genes of the regulated miRNAs were predicted by DIANA tools (http://diana.imis.athena-innovation.gr/DianaTools), and the pathway analysis of target genes was performed using DIANA miRPath v.2.0, which is based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[16]

Confirming miRNA Expression Using Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-QPCR). The target miRNA expression was confirmed and quantified using RT-QPCR in a BIO-RAD Mini-Opticon real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The same samples used for the miRNA microarrays were used for confirming miRNA expression. RNA samples (400 ng per reaction) were reverse transcribed into cDNA using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the standard protocol of the supplier. The amplifications were performed under the following conditions: 95 °C for 2 min followed by 45 cycles (95 °C for 5 s, 55 °C for 20 s) using kappa probe Fast qPCR master mix kits (Kapa Biosystems, Inc., Wilmington, MA, USA). The relative expression levels of miRNAs to the internal control 18 S were reported using the Livak method ($\Delta\Delta C_q$). A melting curve analysis (60 to 99 °C) was conducted after the thermo profile by detecting only one peak produced to verify specificity in the amplification. The sequences and primers used in this study are described in Supplementary Table 1 in the Supporting Information. Standard curves with 5-fold dilutions of primer were performed to calculate reaction efficiency. The PCR reactions were all linear over a range of primer concentrations, and the calculated PCR efficiencies were all above 95%.[17]

Confirming Target Genes Related to Regulated miRNAs. RT-QPCR was used to confirm the target genes related to the regulated miRNAs. The same total RNA isolated for the miRNA microarrays was used for RT-QPCR. Three independent samples were performed, and each reaction included 20 ng of cDNA, 500 nM forward and reverse primers, and Fast SYBR Green PCR Master Mix (Applied Biosystems). A BIO-RAD CFX Connect real-time PCR machine (Bio-Rad Laboratories) was used with the following program: 95 °C for 2 min, followed by 39 cycles (95 °C for 5 s, 60 °C for 30 s). A peak produced in the melting curve analysis (60 to 99 °C) was checked to verify specificity in the amplification. The data analysis was performed by BIO-RAD CFX Manager Version 3.0 software (Bio-Rad Laboratories). Target gene RT-QPCR data was normalized to the reference gene, GAPDH. The primers used in this study are included in Supplementary Table 2 in the Supporting Information. The PCR efficiencies were all above 95% based on the primer dilution assay.[17]

Western Blot. The levels of the expression of proteins were determined by Western blot analysis as described in our previous study.[11] Rabbit anti-Igf1, rabbit anti-Beclin 1, rabbit anti-p62, and rabbit anti-LC3 antibodies were obtained from Novus Biologicals, LLC (Littleton, CO, USA). Anti-PI3K, anti-phospho-PI3K, anti-Akt, anti-phospho-Akt, anti-mTOR, and anti-phospho-mTOR antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-IRS-1 antibody was obtained from BD Biosciences (San Jose, CA, USA).

Transient Transfection of miRNA Mimics. For further confirmation of the direct effects of the regulated miRNAs on target genes, regulated miRNA mimics based on Sanger miRBase (synthesized by Guangzhou RiboBio Co., Ltd. Guangzhou, China) were transfected into MEF cells using Lipofectamine 2000 (Invitrogen) according to the supplier's protocol. Briefly, 2×10^5 cells cultured overnight were used for miRNA transfection and the medium was replaced by a serum-reduced medium (Opti-MEM (Invitrogen) prior to transfection. The cells were incubated with miRNA mimics/Lipofectamine 2000 complexes (1 (50 nM):1, v/v) in 2 mL of Opti-MEM medium for 6 h, and then the medium was replaced by a growth medium. The cells were cultured for 24 h and then subjected to further analysis. The expression of miRNAs in transfected cells was investigated by RT-QPCR as described in Confirming miRNA Expression Using Real-Time

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Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-QPCR). The target genes in the transfected cells were identified by RT-QPCR as described in Confirming Target Genes Related to Regulated miRNAs. The expression of proteins in transfected cells was determined by Western blot as described in Western Blot. The cells transfected with miRNA mimic with random sequences served as the negative controls.

Cell-Specific Effect of PEI on Regulated miRNAs. To determine whether the regulation of miRNAs in PEI-treated MEF cells is cell-specific or not, the PEI-treated LLC1 and HeLa cells were subjected to RT-QPCR as described in Confirming miRNA Expression Using Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-QPCR) for confirmation of the expression of regulated miRNAs.

Cytotoxicity Assay. Cytotoxicity was assessed by the detection of the activity of dehydrogenases in MEF cells. The experimental protocols have been described in our previous study.[18]

Statistical Analysis. The data is presented as mean ± standard deviation (n = 3). The differences between untreated control and PEI-treated samples were analyzed using Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Microarray Analysis of miRNA Expression. The cytotoxic profiles and autophagy induction of PEI in MEF cells undergoing up to 12 h of incubation have been investigated in a previous study.[11] We selected a lower 10 μg/mL dose of PEI and longer incubation times of 12 and 24 h in this study for the occurrence of autophagy at indicated conditions and so as to avoid early membrane damage in PEI-treated cells. In comparison with untreated controls, the numbers of differentially expressed miRNAs in PEI-treated MEF cells for 12 or 24 h incubation are shown in Table 1.

Table 1. Numbers of Differentially Expressed miRNAs in PEI-Treated MEF Cells

PEI dose (μg/mL)	10	10
incubation time (h)	12	24
upregulation	32	21
(overlapped)	(14)	(14)
downregulation	58	74
(overlapped)	(14)	(14)

Fourteen overlapped miRNAs were up- or downregulated at both 12 and 24 h of PEI incubation (see Supplementary Table 3 in the Supporting Information for quantitative data). The numbers of downregulated miRNAs are much higher than those of upregulated miRNAs in each of the indicated treatment conditions. Similar regulated miRNAs were clustered from the hierarchical analysis and are illustrated in Figure 1 in the form of a heat map.

Although aberrantly expressed miRNAs, including up- and downregulated miRNAs, are highly associated with the regulation systems of various cellular processes, in this study, we focused only on upregulated overlapped miRNAs after both 12 and 24 h of PEI incubation. Due to much larger numbers of downregulated miRNAs, analyzing the outcomes of down-regulated miRNAs would increase the complexity of the study. Likewise, in our previous study,[11] it was demonstrated that most of the autophagy-related regulated genes in MEF cells

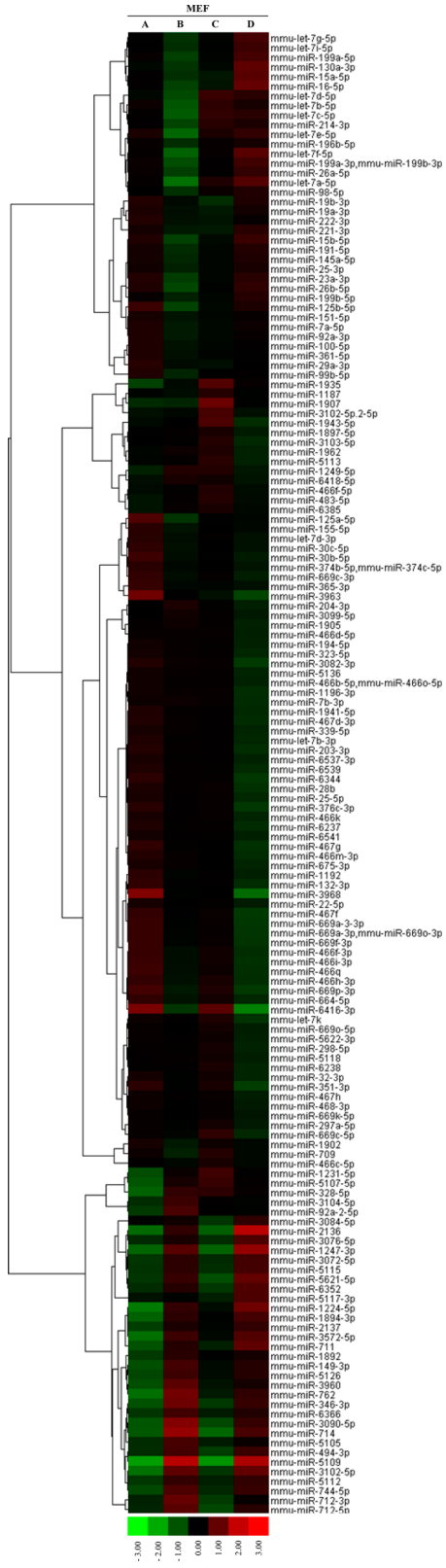

Figure 1. Heat map of differentially expressed miRNAs in PEI-treated MEF cells. The A and C columns represent untreated MEF cells for 12 and 24 h. The B and D columns show the MEF cells treated at a dose of 10 μg/mL PEI for 12 and 24 h. Green colors indicate upregulation, and red colors represent downregulation.

treated with PEI were downregulated, and this finding supports a greater focus on the study of upregulated miRNAs due to the

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Table 2. Potential Pathway Analysis of Overlapped Upregulated miRNAs in PEI-Treated MEF Cells

overlapped upregulated miRNAs	KEGG pathway	*P*-value	no. of target genes	no. of miRNAs involved
mmu-miR-1224-5p	glutamatergic synapse	1.87×10^{-9}	23	7
mmu-miR-1247-3p	long-term depression	6.23×10^{-7}	12	7
mmu-miR-2136	mucin type O-glycan biosynthesis	9.08×10^{-7}	5	3
mmu-miR-3072-5p	proximal tubule bicarbonate reclamation	4.27×10^{-5}	6	2
mmu-miR-3090-5p	long-term potentiation	4.27×10^{-5}	12	5
mmu-miR-3102-5p	endocrine and other factor-regulated calcium reabsorption	7.80×10^{-5}	10	5
mmu-miR-346-3p	nicotine addiction	0.000115	8	6
mmu-miR-494-3p	axon guidance	0.000133	20	7
mmu-miR-5109	phosphatidylinositol signaling system	0.000143	10	7
mmu-miR-5115	retrograde endocannabinoid signaling	0.000143	17	8
mmu-miR-5621-5p	Wnt signaling pathway	0.000143	22	9
mmu-miR-711	mTOR signaling pathway	0.000167	11	6
mmu-miR-712-5p	GABAergic synapse	0.000174	12	6
mmu-miR-714	Ssynaptic vesicle cycle	0.000336	11	4
	MAPK signaling pathway	0.000336	28	9
	D-glutamine and D-glutamate metabolism	0.000357	2	1
	dopaminergic synapse	0.000634	17	6
	glioma	0.000716	11	7
	transcriptional misregulation in cancer	0.00083	23	9
	focal adhesion	0.000988	22	9
	neurotrophin signaling pathway	0.003994	14	6
	cyanoamino acid metabolism	0.00407	2	2
	melanoma	0.00407	10	8
	Fc gamma R-mediated phagocytosis	0.005169	11	7
	gastric acid secretion	0.005465	10	5
	VEGF signaling pathway	0.005465	9	7
	salivary secretion	0.006286	10	6
	calcium signaling pathway	0.006659	19	8
	lysine degradation	0.007071	6	4
	vitamin B6 metabolism	0.00731	2	2
	circadian rhythm	0.008854	6	2
	vascular smooth muscle contraction	0.008981	15	7
	Notch signaling pathway	0.01008	7	6
	melanogenesis	0.015804	11	8
	taurine and hypotaurine metabolism	0.015837	3	2
	ErbB signaling pathway	0.024683	9	6
	inositol phosphate metabolism	0.024683	7	7
	GnRH signaling pathway	0.027878	10	6
	ubiquitin mediated proteolysis	0.02803	15	5
	pancreatic secretion	0.035946	11	6
	PI3K-Akt signaling pathway	0.036213	29	9
	B cell receptor signaling pathway	0.037999	9	7
	p53 signaling pathway	0.042673	8	4

fact that the aforementioned downregulated genes may result from the upregulated miRNAs. In addition, overlapped upregulated miRNAs at both 12 and 24 h incubation were further selected because they would represent common significant consequences of gene expression changes in PEI-treated cells. Therefore, these 14 upregulated overlapped miRNAs were subjected to potential pathway analysis using DIANA miRPath v.2.0 database for further prediction of the biological functions of regulated miRNAs and their target genes (Table 2).

Based on comparisons of the target genes that belong to a particular pathway with the predicted target genes of all the reference pathways, the Fisher's exact *P*-value < 0.05 was set as a threshold for the selection of pathways potentially affected by the miRNAs and target genes.[16] Among these potential pathways, five pathways (Wnt signaling pathway, mTOR signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and p53 signaling pathway) are closely related to mechanisms of cell death. Since these potential pathways of cell death are complex and highly interconnected, in this study, we focused on the mTOR signaling pathway, which is also a key regulator of autophagy.[19] There were 6 upregulated miRNAs (mmu-miR-1224-5p, mmu-miR-3072-5p, mmu-miR-3090-5p, mmu-miR-346-3p, mmu-miR-494-3p, and mmu-miR-712-5p) related to the mTOR signaling pathway (Figure 2A). Among these 6 involved upregulated miRNAs, 4 upregulated miRNAs (mmu-miR-3072-5p, mmu-miR-346-3p, mmu-miR-494-3p, and mmu-miR-712-5p) are also related to the p53 signaling pathway, and this verified the interconnection of regulated miRNAs between the mTOR and p53 signaling pathways (Figure 2B). Therefore, the involved 6 regulated miRNAs were subjected to further RT-QPCR validation.

Confirmation of miRNA Upregulation Using RT-QPCR. Based on the prediction of the bioinformatic software Beacon Designer (PREMIER Biosoft (Palo Atto, CA, USA)), mmu-miR-3072-5p and mmu-miR-712-5p were excluded from the above 6 regulated miRNAs for RT-QPCR confirmation due to the easy formation of dimers between forward primers and reverse primers. At 12 h, mmu-miR-1224-5p and mmu-miR-494-3p were downregulated while the expressions of mmu-miR-346-3p and mmu-miR-3090-5p were upregulated as compared with the untreated controls (Figure 3A). Upregulated mmu-miR-346-3p and mmu-miR-3090-5p were consistent with the results from microarray analysis. At 24 h, three upregulated miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) were quantitatively consistent with the

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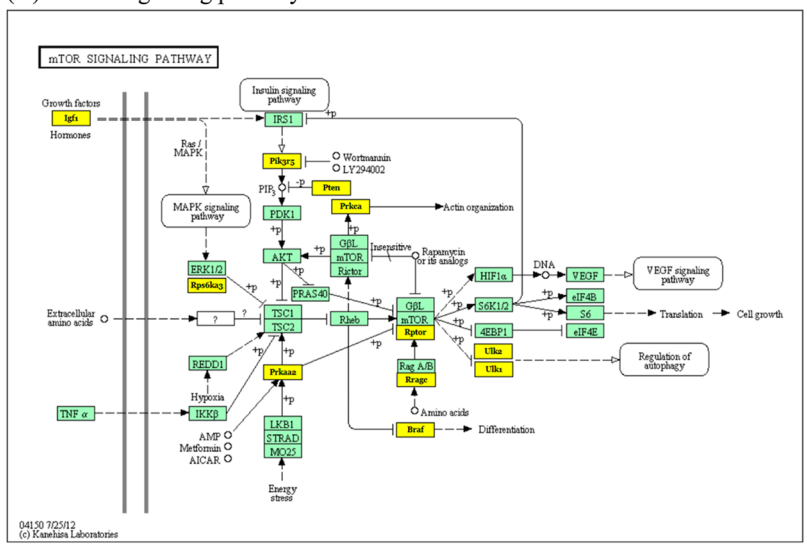
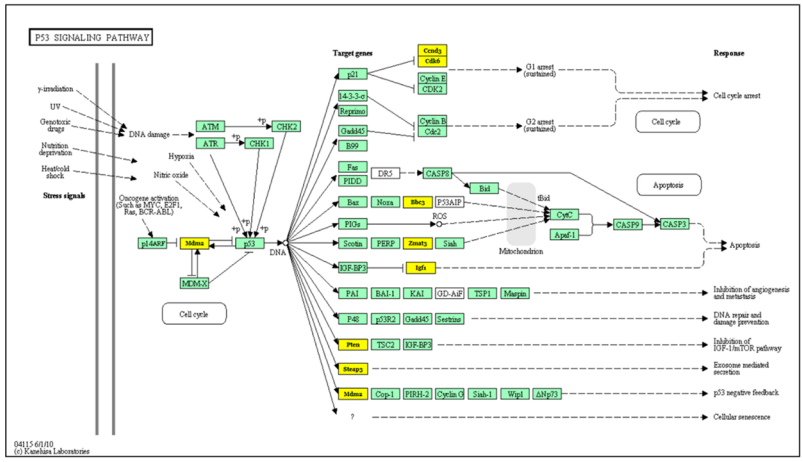
(A) mTOR signaling pathway



miRNA	mmu-miR -1224-5p	mmu-miR -3072-5p	mmu-miR -3090-5p	mmu-miR -346-3p	mmu-miR -494-3p	mmu-miR -712-5p
Target genes	*Ulk1* *Braf*	*Pik3r5*	*Rragc*	*Igf1*	*Prkaa2* *Ulk2* *Rps6ka3* *Pten*	*Prkca* *Rptor*

(B) p53 signaling pathway



miRNA	mmu-miR -3072-5p	mmu-miR -346-3p	mmu-miR -494-3p	mmu-miR -712-5p
Target genes	*Steap3*	*Igf1* *Ccnd3* *Zmat3*	*Bbc3* *Pten* *Cdk6*	*Mdm2*

Figure 2. Prediction of miRNAs and their target genes involved in (A) mTOR signaling pathway and (B) p53 signaling pathway. The figures of the above pathways are based on the KEGG database.

results from the microarray while mmu-miR-1224-5p was downregulated (Figure 3B). Therefore, these three well-verified miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) at 24 h were selected to identify their target genes.

Identification of Target Genes Associated with Confirmed Upregulated miRNAs. According to DIANA tools and KEGG pathway analysis related to the mTOR and p53 signaling pathways, the expression levels of 12 target genes (*Rragc*, *Igf1*, *Prkaa2*, *Ulk2*, *Pten*, *Ccnd3-1*, *Ccnd3-2*, *Ccnd3-3*, *Zmat3*, *Rps6ka3*, *Bbc3*, and *Cdk6*) associated with the three upregulated miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) were determined using RT-QPCR in 10 μg/mL PEI-treated MEF cells after 24 h incubation (Figure 4A). Four genes (*Igf1*, *Prkaa2*, *Pten*, and *Ccnd3-3*) were downregulated, and among these four target genes, the gene exhibiting the biggest degree of downregulation was the growth factor *Igf1*. We next investigated the expressions of these four genes in 10 μg/mL PEI-treated MEF cells at 12 h using RT-QPCR (Figure 4B). Compared to the untreated controls, three genes (*Igf1*, *Ccnd3-3*m, and *Pten*) were downregulated while *Prkaa2* was slightly upregulated. This may indicate that some of the gene regulation in PEI-treated MEF cells is dynamically

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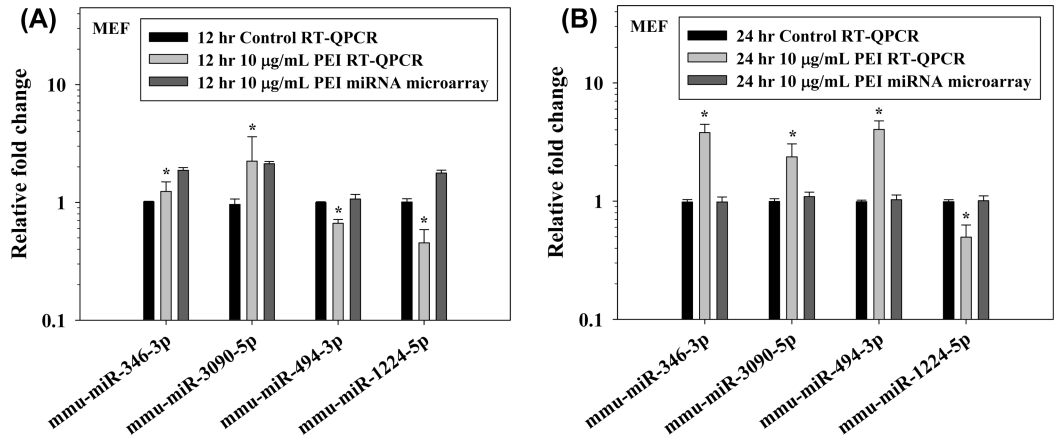

Figure 3. Confirmation of microarray results in PEI-treated MEF cells at (A) 12 h and (B) 24 h by RT-QPCR. The data from microarray are represented with mean values, and the data from RT-QPCR are shown as means ± standard deviation (n = 3). *P < 0.05 vs untreated controls.

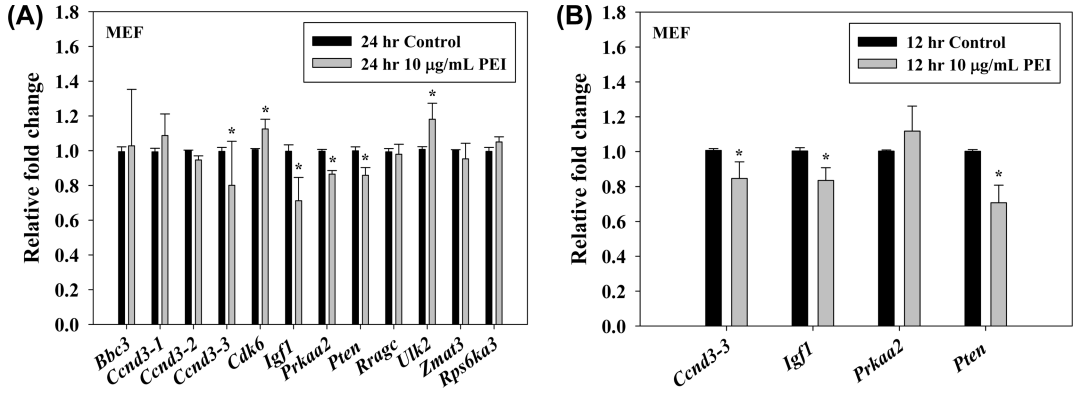

Figure 4. Quantification of target genes in PEI-treated MEF cells at (A) 24 h or (B) 12 h by RT-QPCR. Bars represent means ± standard deviation (n = 3). *P < 0.05 vs untreated controls.

controlled. However, the downregulation of *Igf1* in 10 μg/mL PEI-treated MEF cells at 12 h was not consistent with the upregulation of the gene found using microarray in a previous study.[11]

Verification of Regulated miRNA-Target Gene Interaction. To further verify the regulated miRNA−target gene interaction, MEF cells were transfected with three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) and then the expression levels of the four target genes (*Igf1*, *Prkaa2*, *Pten*, and *Ccnd3-3*) were determined using RT-QPCR (Figure 5). The increases of intracellular miRNA for each of the three miRNA were observed in transfected MEF cells, through which successful transfection by these three miRNA mimics was demonstrated (Figure 5A). The gene expression of *Ccnd3-3* in MEF cells transfected with these three miRNA mimics was not significantly different from that in the negative controls (Figure 5B). The expression levels of *Igf1* were significantly lower in MEF cells transfected with the three miRNA mimics compared to the negative controls (Figure 5C). The biggest decrease of *Igf1* gene expression came from the interaction of an mmu-miR-346-3p mimic. However, the expression levels of *Prkaa2* in MEF cells transfected with the three miRNA mimics were not statistically distinguishable from the negative controls (Figure 5D). MEF cells transfected with the three miRNA mimics induced the upregulation of *Pten* (Figure 5E). Therefore, only the decrease of *Igf1* gene expression was associated with the upregulation of mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p.

Western Blot of *Igf1*. The hormone *Igf1* (the insulin-like growth factor 1, also called somatomedin C) regulates various important cellular responses in biological systems such as cell proliferation and differentiation, cell death and survival, and migration.[20] The protein expression levels of *Igf1* in PEI-treated MEF cells are shown in Figure 6.

As compared with untreated MEF cells, the protein expression levels of *Igf1* were decreased in PEI-treated MEF cells at 12 and 24 h. Also, as observed in Figure 7, the protein expression levels of *Igf1* were decreased in MEF cells transfected with three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) as compared with negative controls. Therefore, the decreases in *Igf1* protein expression were consistent with the downregulation of gene expressions of *Igf1* in MEF cells treated with PEI or transfected with three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p).

Cell-Specific Effect of PEI on Regulated miRNAs. To check whether PEI-induced upregulation of these miRNAs in cells is cell-specific or not, we examined the expression levels of four miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, mmu-miR-1224-5p, and mmu-miR-494-3p) in LLC1 cells and HeLa cells treated with and incubated in PEI for 12 or 24 h (Figure 8). For 12 and 24 h of incubation, the expression levels of these four miRNAs were downregulated in PEI-treated LLC1 cells (Figures 8A and 8B).

This indicates that the effects of PEI on regulated miRNAs in MEF cells are different from those in LLC1 cells. We next

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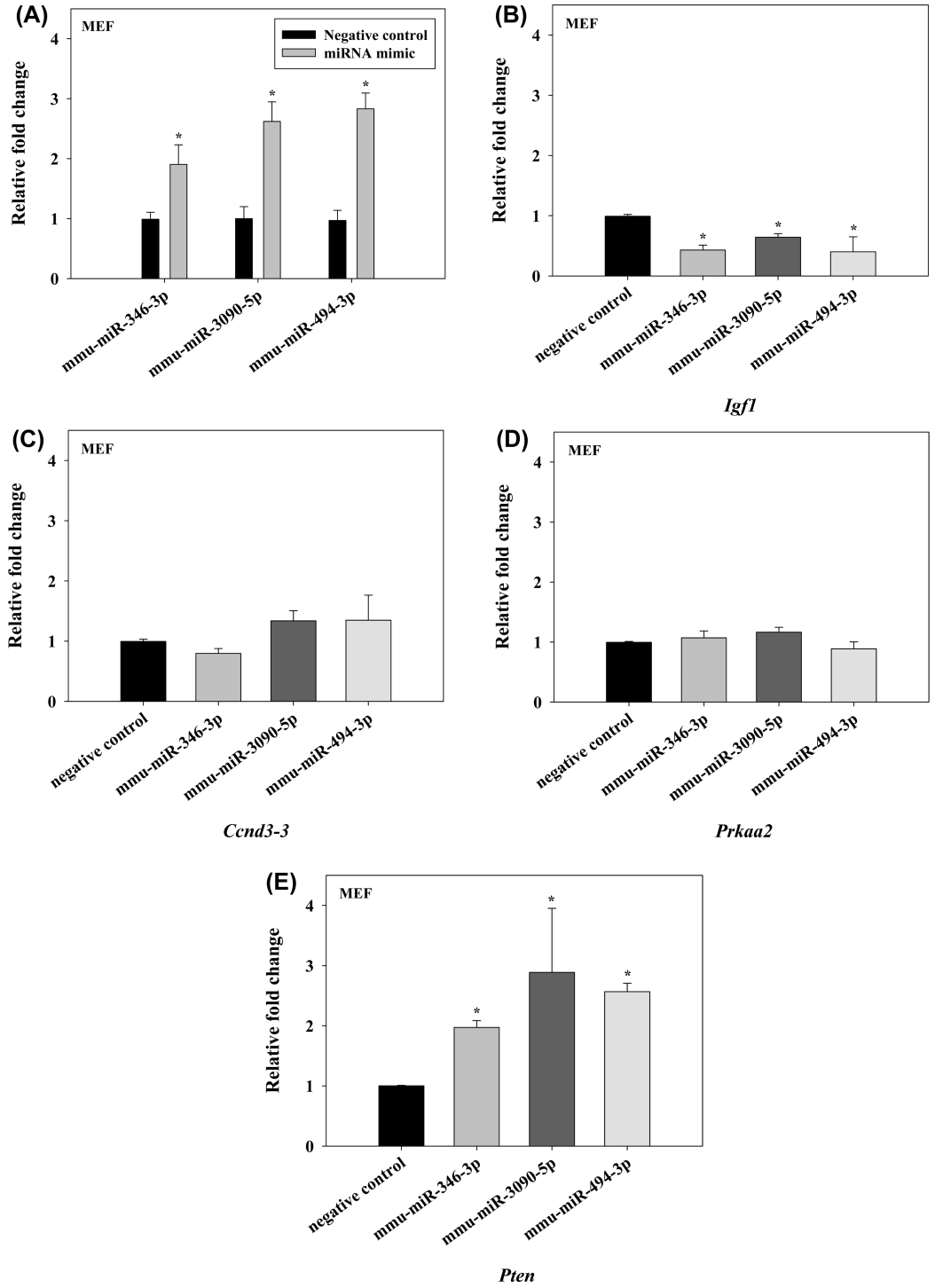

Figure 5. Quantification by RT-QPCR of miRNA (A) and target genes ((B) *Ccnd3-3*, (C) *Igf1*, (D) *Prkaa2*, and (E) *Pten*) in PEI-treated MEF cells transfected with miRNA mimics. Cells transfected with a random sequence miRNA mimic served as negative controls. Bars represent means ± standard deviation (n = 3). *P < 0.05 vs negative controls.

investigated the expression levels of these four miRNAs in PEI-treated HeLa cells, which are cells derived from a human rather than from mice. The fold changes of mmu-miR-346-3p were up to approximately 4 at 12 h and slightly above 3 at 24 h in PEI-treated HeLa cells, whereas the fold changes of the other three miRNAs were not significant (Figures 8C and 8D). Therefore, PEI also induced upregulated mmu-miR-346-3p in both HeLa and MEF cells.

The Effects of PEI and miRNA Mimics on the mTOR Signaling Pathway and Autophagy. To verify whether the mTOR signaling pathway and autophagy are involved in the effects of PEI treatment on MEF cells, Western blot analysis of key proteins involved in the mTOR signaling pathway and autophagy was conducted (Figure 9). PEI treatment resulted in downregulation of mTOR, PI3K, and AKT phosphorylation as compared with untreated controls for 24 h incubation (Figure 9A). The expression level of IRS-1 was also downregulated in

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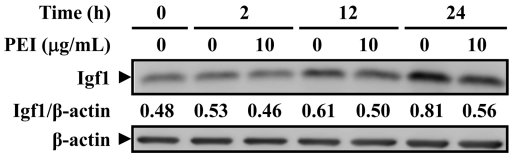

Figure 6. Western blot analysis of *Igf1* in PEI-treated MEF cells. *β*-Actin was the loading internal control. The data shown were from one representative experiment among three similar experiments. The ratios of band density were calculated with AlphaImager 2200 software.

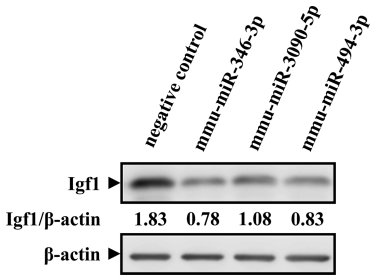

Figure 7. Western blot analysis of *Igf1* in MEF cells transfected with random sequence miRNA mimic as negative control and three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p). *β*-Actin was the loading internal control. The data shown are from one representative experiment among three similar experiments. The ratios of band density were calculated with AlphaImager 2200 software.

PEI-treated cells at 24 h (Figure 9A). Likewise, as compared with negative controls, MEF cells transfected by these three miRNA mimics also induced the decreases of IRS-1 and phosphorylation of mTOR, PI3K, and AKT (Figure 9B). This suggests that the mTOR signaling pathway is involved in both PEI-treated cells and transfected cells through these miRNA mimics.

Next, we investigated autophagic activity in cells by monitoring the expression level of a marker protein LC3. The ratio of LC3II/LC3I was significantly increased in PEI-treated MEF cells for 12 and 24 h incubation, indicating that autophagy was induced in PEI-treated cells (Figure 9C).

As compared with negative controls, the increases of LC3II/LC3I and the decreases of p62 and beclin 1 were observed in MEF cells transfected with these three miRNA mimics (Figure 9D). The p62 protein (SQSTM1/sequestosome 1) belongs to the adaptor molecules involved in inducing autophagy. Polyubiquitin-binding p62 protein binds to LC3 and is degraded by autophagy.[21] Also, beclin 1 (the mammalian orthologue of yeast *Atg6*) is closely related with interactions between apoptosis and autophagy.[22] Our results suggested that these three miRNAs are involved in molecular mechanisms of autophagy. In our previous study, the decreases of p62 and beclin 1 have been demonstrated in PEI-treated cells.[11]

The Effects of PEI and miRNA Mimic Treatment on Cytotoxicity. To clarify cell viability, the intracellular dehydrogenase activity in mitochondria was assessed. As

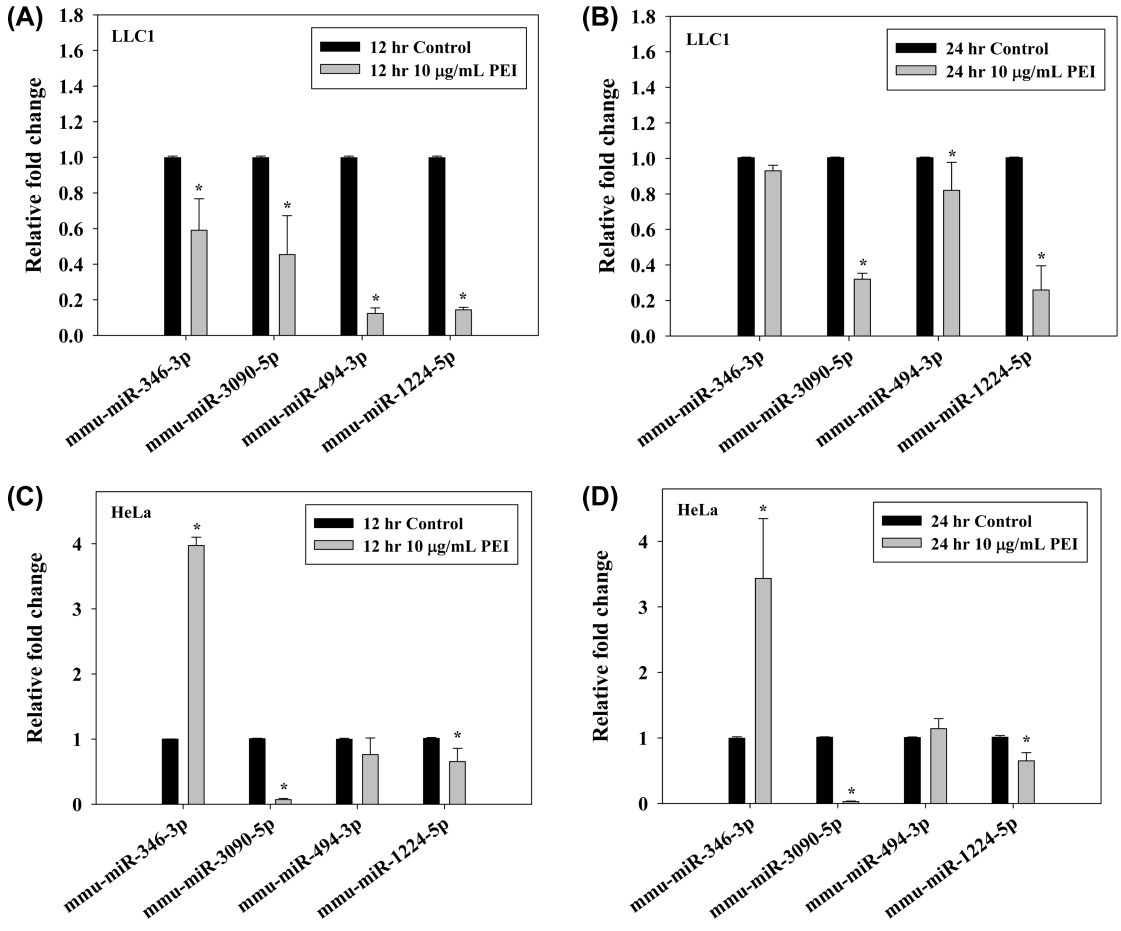

Figure 8. Quantification of miRNA expression in PEI-treated (A and B) LLC1 or (C and D) HeLa cells at 12 and 24 h by RT-QPCR. The data are shown as means ± standard deviation (n = 3). *P < 0.05 vs untreated controls.

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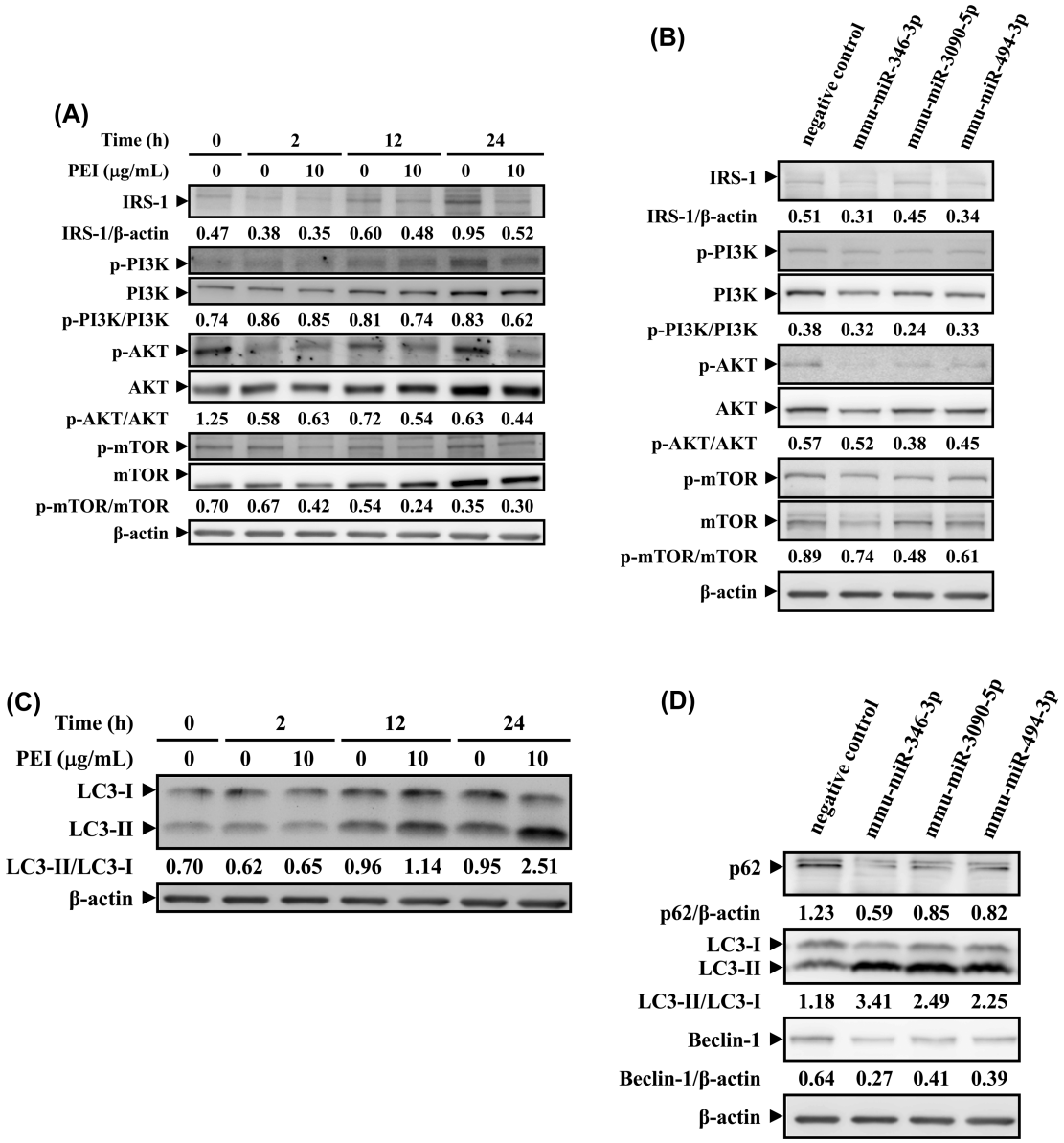

Figure 9. Western blot analysis of protein expression (IRS-1, phosphorylated PI3K (p-PI3K), PI3K, phosphorylated mTOR (p-mTOR), mTOR, phosphorylated Akt (p-AKT), and AKT) related to the mTOR signaling pathway in cells (A) treated with PEI or (B) transfected with three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) and negative controls. LC3 expression in cells treated with PEI (C). LC3, p62, and beclin 1 expressions in cells transfected with three miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) and the negative control (D). *β*-Actin was the loading internal control. The data shown are from one representative experiment among three similar experiments. The ratios of band density were calculated with AlphaImager 2200 software.

compared with untreated cells, cell viability time-dependently decreased in PEI-treated cells at a dose of 10 μg/mL (Figure 10A). For cells transfected with these three miRNA mimics, cell survival moderately decreased as compared with the negative controls (Figure 10B).

DISCUSSION

Various molecular mechanisms of cell death induced by PEI or PEI/DNA complexes *in vitro* or *in vivo* have been observed, but these interpretations have not proven consistently conclusive. After treatment of PEI or PEI/DNA complexes, the genes or proteins related to apoptosis have been demonstrated *in vitro* and *in vivo*.[7,9,23−25] Recently, PEI and PEI/RNA (DNA) complexes have been shown to induce autophagic cell death in various cells.[11,18,26−28] In a previous study applying autophagy-related gene expression analysis, we found that autophagy and apoptosis are closely related in the PEI-induced mechanism of cell death.[11,18] However, no study of the effect of PEI on the regulation of miRNAs, which can target these regulated genes of cell death, has been conducted thus far.

In this study, we used more toxic free PEI instead of less toxic PEI/nucleic acid complexes to investigate miRNA expression in cells because we wanted to test the worst-case scenario for the cytotoxicity of PEI. In addition to causing greater cytotoxicity, free PEI also prevented particle aggregation of polyplexes and enhanced transfection efficiency *in vitro*.[29,30] Among these three upregulated miRNAs, miR-494-3p has been reported to suppress the progression and metastasis of prostate cancer.[31] Overexpressed miR-494-3p has been shown to inhibit the growth and paracrine function of mesenchymal stem cells

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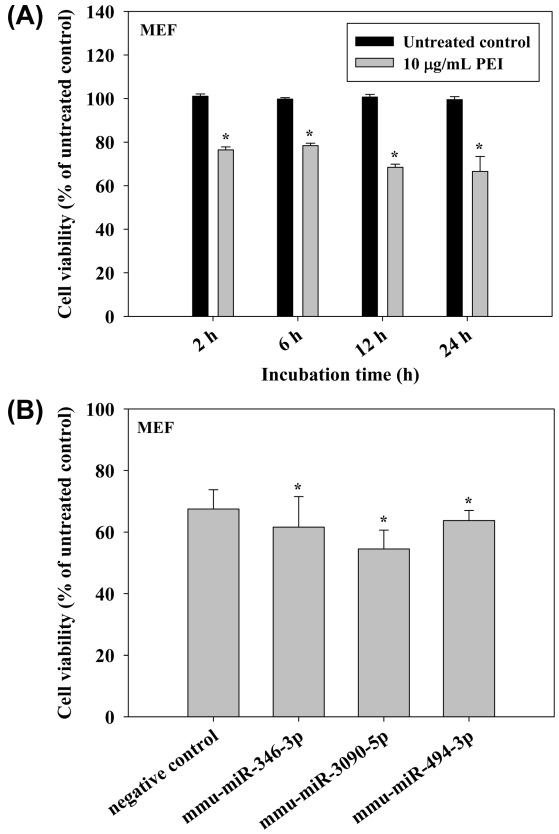

Figure 10. Cytotoxicity assays of (A) PEI and (B) three transfected miRNA mimics (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) in MEF cells by measuring generated dehydrogenases. Data are expressed as percentage of control ((average OD/average control OD) ± SD (n = 3)). Untreated cells served as controls (100% ± SD). *P < 0.05 vs untreated controls in PEI-treated cells and vs cells transfected with random sequence miRNA mimic.

by targeting of *Cdk6*, *Ccnd1*, and *VEGF*.[32] Furthermore, a mouse model has revealed that miR-494-3p is related to glioma proliferation and invasion.[33] In myeloid-derived suppressor cells, enhanced expression of miR-494-3p has been found to target the *Pten* gene and activate the Akt pathway.[34] In this study, however, upregulated *Pten* gene was identified by the transfection of miR-494-3p mimic in MEF cells (Figure 5E).

Autophagy induction has been shown to inhibit the mTOR signaling pathway.[19] Cationic Starburst polyamidoamine dendrimers have also been demonstrated to induce autophagic cell death via inhibition of the mTOR signaling pathway, a finding which is consistent with the current study.[35] PEI/DNA complexes have been demonstrated to enter various cells in endosomes and to activate the formation of tubulovesicular autophagosomes from small autophagosomes, and gene expression has been shown to be higher in $atg5^{-/-}$ cells treated with PEI/DNA complexes, indicating that tubulovesicular autophagosomes can be considered as a new barrier for gene delivery.[28] Furthermore, the transfection efficiency of PEI/DNA complexes has been shown to be affected by autophagy modulation in mouse fibroblasts.[36] The transfection efficiency was reduced by applying autophagy inhibitor 3-MA, whereas the transgene expression was enhanced by adding autophagy inducer rapamycin.[36] This indicates that the regulation of autophagy plays an important role in polyplex-mediated gene delivery. Also, the protective role of autophagy in PEI-induced cell death has previously been reported, while the fact that autophagy accelerates cell death in both hepatic and nephritic cell lines after treatment with PEI has also been demonstrated.[11,18,27] Despite autophagy induction and the decreases of cell viability that have been observed in MEF cells transfected by these three miRNA mimics, the exact role in the molecular mechanisms of cell death induced by these three miRNAs needs to be further clarified.

CONCLUSION

Presently, little is known about the effects of cationic polymers on the regulation of miRNAs related to cell death mechanisms. According to the analysis of the mTOR pathway, three miRNAs (mmu-miR-3090-5p, mmu-miR-346-3p, and mmu-miR-494-3p) were upregulated in MEF cells treated with PEI and incubated for 24 h. We further demonstrated that these three upregulated miRNAs resulted in the decrease of the target gene growth factor *Igf1*. The protein expression levels of decreased *Igf1* were consistent with its gene expressions in MEF cells treated with PEI or transfected with three upregulated miRNA mimics. However, these three upregulated miRNAs are not all cell-specific. We also demonstrated that the mTOR signaling pathway is inhibited by autophagy induction and that cell viability decreases in cells treated with PEI or transfected with these three miRNA mimics. Our data suggested that PEI may affect the regulation of miRNAs in cells.

ASSOCIATED CONTENT

Supporting Information

The sequences and primers used in confirming miRNA expression using RT-QPCR; the sequences and primers used in confirming target gene expression; and upregulated and downregulated miRNAs in PEI-treated MEF cells. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00329.

AUTHOR INFORMATION

Corresponding Authors

*(M.-S.J.) E-mail: msjan@csmu.edu.tw.

*(J.-H.S.K.) Tel: 886-6-266-4911 ext 2104. Fax: 886-6-266-6411. E-mail: kuojunghua@yahoo.com.tw.

Notes

The authors declare no competing financial interest.

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行政院科技部補助出席國際學術會議報告

105 年 7 月 29 日

報告人姓名	郭榮華 嘉南藥理大學藥學系		
時間 會議地點	07/17/2016-07/20/2014 Washington State Convention Center, Seattle, Washington, USA		
會議名稱	（中文）43 屆控制釋放協會年會暨展覽會 （英文）43rd Annual Meeting & Exposition of the Controlled Release Society		
發表論文題目	（中文）探討聚乙烯亞胺處理後老鼠胚胎纖維母細胞之 MicroRNA 相關細胞死亡路徑表現模式 (英文) Exploring MicroRNA Expression Profiles Related to Cell Death Pathways in Mouse Embryonic Fibroblast Cells Treated with Polyethylenimine		

報告內容應包括下列各項：

一、參加會議經過

本屆 2016 年的控制釋放協會年會暨展覽會（43rd Annual Meeting & Exposition of the Controlled Release Society）為期四天（07/17/2016-07/20/2016），在 Washington State 會議中心（Seattle, USA）舉行。此次會議為藥學及控制釋放領域中最為重要的會議之一，今年有超過 400 篇壁報論文、超過 60 位邀請演講、超過 30 廠商設攤展示的相關研究領域學業界參與這場盛會。來自全世界各大製藥公司的研發人員都聚集於此，一方面發表最新的研究成果，另一方面可以了解未來控制釋放與創新應用方向。本次 plenary session 所邀請的是來自 The Bill and Melinda Gates Foundation (USA) 之 Dr. Susan Hershenson 討論 Application of Controlled Release and Drug Delivery Technology to Address Global Health Needs 、CreActive Health Holdings (Korea) 之 Jun Keun Chang 討論 Turning Science to Entrepreneurship: A Journey of Passion、 University of British Columbia (Canada) 之 Prof. Helen Burt 討論 Hyperbranched Polyglycerol-Based Nanoparticles for Treatment of Superficial Bladder Cancer: Preclinical Research and Development，因此對於學界在研究成果如何轉換成兼具實用與價值的產品，都可以在這個會議中獲益無窮。而本次會議涵蓋的主題非常廣泛及實用，如 Delivery Technologies in Cosmetics, Personal care, and Household products 、Integration of Imaging and Drug Delivery 、 Oral Delivery、Manufacture, Characterization, Stability, and Regulatory Aspects、Parenteral Systemic Delivery of Biopharmaceuticals: Overcoming Product Development and Regulatory Challenges、Developing Therapeutic Options for Combating Cancer: A “One Health” Challenge for Humans and Dogs Mini Symposium、” Thinking Outside the Box” Delivery Technologies: Nanocarriers from Nature、Tissue Engineering、Transdermal Delivery、 Delivery Technologies in Nutraceuticals, Food, and Oral Products、Ocular drug Delivery、Physical Oncology: Modulating Tumor Microenvironment for Drug Delivery、Local Drug Delivery、New Processes, New Materials, New Products 、mRNA Delivery and Clinical Translation for Vaccines and Therapeutics Mini Symposium、Comparative Pharmacokinetics in Preclinical Sciences、Encapsulation and Controlled Release for Industrial Applications、Overcoming Biological Barriers in Drug Delivery、Oligonucleotide Delivery: New Applications and Opportunities、Peptides, Proteins, and Vaccines、Preclinical Science Challenges to Drug Delivery、Research Highlight Talk 等，令人目不暇給，實屬挖寶之旅。

二、與會心得

這次我報告的壁報論文，被大會安排在壁報論文場次中張貼並與其他有興趣的研究學者一同討論，在壁報張貼期間許多相關領域的專家都前來參觀壁報，也提出許多有用的意見及討論，使我能知道自己研究的盲點何在及本實驗在生技製藥界的價值及重要性，因此受益匪淺。此外在會議進行期間也有許多出版社、廠商設攤展示，進行一連串示範及演講活動，對於往後在研究中所使用相關材料及儀器亦十分有所幫助。此次獲益良多，更對未來的研究主題有正面的幫助。由於此會議所涵蓋之研究領域很廣且選擇性也多，有更多的交流，也是重要的收穫。

三、攜回資料名稱及內容

大會相關論文手冊、廠商 DM

William Klein <wklein@pcm411.com>
收件者
kuojunghua@yahoo.com.tw
3 月 29 日於 11:01 PM

Dear Jung-hua Kuo,

Your Abstract ID 1030 - "Exploring MicroRNA Expression Profiles Related to Cell Death Pathways in Mouse Embryonic Fibroblast Cells Treated with Polyethylenimine" has been accepted for **poster presentation** at the 43rd Annual Meeting & Exposition of the Controlled Release Society, July 17 – 20, 2016, at the Washington State Convention Center in Seattle, Washington, U.S.A.

Information regarding your poster number and presenter schedule will be sent to you in the coming week.

IMPORTANT INFORMATION

- **Online registration will open the week of March 28.**
- **Any changes in presenting authors** are to be submitted to CRS prior to May 10, 2016.
- **All designated presenting authors must be registered by May 10, 2016.** *Any designated presenting authors not registered by the deadline will have their abstract withdrawn from the program.*
- **CRS Student and Post-Doc members** may apply online for the Allan Hoffman Student Travel Grant Program. Deadline to submit is May 10.
- **Poster size** must not exceed the poster dimensions of 47.5 inches (120.65 cm) high by 46 inches (116.84 cm) wide.
- **Please include a photo of yourself** on your poster in the bottom right-hand corner.
- **NEW! Poster Pub** is on Monday, July 18 from 5:15 – 6:30 p.m. All presenting authors should be present.
- **Poster Set-Up and Take Down:** All posters must be set up by 2:00 p.m. on Sunday, July 17 and removed by 4:30 p.m. on Tuesday, July 19.

Contact CRS Meeting Coordinator, Bill Klein at wklein@pcm411.com with any abstract questions or presenting author changes.

Thank you for your participation in the Controlled Release Society's 43rd Annual Meeting & Exposition. We look forward to meeting you and listening to your presentation.

See you in Seattle!

Regards,

2016 CRS Annual Meeting Program Committee
Kinam Park, Chair
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Exploring MicroRNA Expression Profiles Related to Cell Death Pathways in Mouse Embryonic Fibroblast Cells Treated with Polyethylenimine

Jung-hua Kuo[1], Ming-shiou Jan[2], and Chia-wei Lin[2]

[1] Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan 717, Taiwan;

[2] Institute of Microbiology and Immunology, Chung Shan Medical University, Taichung 40201, Taiwan

kuojunghua@yahoo.com.tw

Purpose: In order to better understand the toxicology of poly(ethylenimine) (PEI) in microRNA (miRNA) expression levels, we explored miRNA expression profiles related to cell death mechanisms in mouse embryonic fibroblast (MEF) cells treated with PEI by applying microarray analysis.

Methods: The miRNA microarrays were carried out in Mouse & Rat miRNAOneArray® v5 (Phalanx Biotech Group, Hsinchu, Taiwan). MEF cells were grown overnight at 2×10^6 cells per 10-cm dish and then were treated with 10 μg/mL PEI for 12 and 24 h. Untreated cells after 12 or 24 h incubation were used as negative controls. The pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Results: In this study, we focused only on up-regulated overlapped miRNAs after both 12 h and 24 h of PEI incubation. 14 up-regulated overlapped miRNAs were subjected to potential pathway analysis for further prediction of the biological functions of regulated miRNAs (Table 1). Five pathways (Wnt signaling pathway, mTOR signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and p53 signaling pathway) are closely related to mechanisms of cell death.

Overlapped up-regulated miRNAs		KEGG pathway	P-value	Number of miRNAs involved
mmu-miR-1224-5p	mmu-miR-5109	Wnt signaling pathway	0.000143	9
mmu-miR-1247-3p	mmu-miR-5115	mTOR signaling pathway	0.000167	6
mmu-miR-2136	mmu-miR-5621-5p	MAPK signaling pathway	0.000336	9
mmu-miR-3072-5p	mmu-miR-711	PI3K-Akt signaling pathway	0.036213	9
mmu-miR-3090-5p	mmu-miR-712-5p	p53 signaling pathway	0.042673	4
mmu-miR-3102-5p	mmu-miR-714			
mmu-miR-346-3p	mmu-miR-494-3p			

Table 1 Potential pathway analysis of overlapped up-regulated miRNAs in PEI-treated MEF cells

Conclusions: Our data suggested that PEI may affect the regulation of miRNAs in target cells and various pathways of cell death involved were identified.

Acknowledgements: This work was supported by grant NSC 102-2221-E-041-013-MY3 from Ministry of Science and Technology, R.O.C.

References:

1. Akhtar S., Benter I. Adv. Drug Deliv. Rev. 2007(59) 164-182.
2. Bartel D. P. Cell. 2009 (136) 215-233.

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/04

科技部補助計畫	計畫名稱: 陽離子性高分子對細胞自我吞噬作用之影響及應用
	計畫主持人: 郭榮華
	計畫編號: 102-2221-E-041-013-MY3　　學門領域: 綠色與生醫高分子

無研發成果推廣資料

102年度專題研究計畫成果彙整表

計畫主持人：郭榮華	**計畫編號：**102-2221-E-041-013-MY3
計畫名稱：陽離子性高分子對細胞自我吞噬作用之影響及應用	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			1		科技部104年高分子學門成果發表會
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			1	篇	通訊作者: Exploring microRNA expression profiles related to the mTOR signaling pathway in mouse embryonic fibroblast cells treated with polyethylenimine. Molecular Pharmaceutics 2015; 12:2858-68. (SCI)。
		研討會論文			1		43nd Annual Meeting & Exposition of the Controlled Release Society
		專書			0	本	
		專書論文			1	章	“Autophagy, Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging”, Volume 8; Chapter 6 (Elsevier Inc., 2015) 專書中之一章”Molecular mechanisms underlying cell death caused by cationic polymers”。
		技術報告			0	篇	

		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
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參與計畫人力	本國籍	大專生			0	人次	
		碩士生			0		
		博士生			1		
		博士後研究員			0		
		專任助理			0		
	非本國籍	大專生			0		
		碩士生			0		
		博士生			0		
		博士後研究員			0		
		專任助理			0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）							

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　■申請中　□無
   技轉：□已技轉　■洽談中　□無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   在應用層面上本計畫主要在研究一種非病毒載體基因製劑，以達成調控細胞自我吞噬之運作功能，這種非病毒載體沒有在體內重組成有致病力的病毒(recombinant viruswild type virus)的可能，故在動物實驗及臨床實驗上應用的機會相當大；另外製作病毒性載體(viral vector)需要有特殊的操作空間和嚴格的感染控制，所耗的經費也相對昂貴，即使如此，在大量製作(up-scaling)病毒性載體時也因危險性的考量而有相當之困難。本計劃所設計的載體歸屬於非病毒性載體(non-viral vector)，在一般實驗室中就可以大量置備，不僅使成本下降而且減少因缺乏再現性(reproducibility)造成分析結果的困難之可能,是一種具有發展潛力達到基因治療的方式。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）