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Integrated mRNA and micro RNA profiling reveals epigenetic mechanism of differential sensitivity of Jurkat T cells to AgNPs and Ag ions

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HIGHLIGHTS

- mRNA and micro RNA microarrays revealed more DEGs and DE miRNAs were induced by AgNPs than by Ag ions exposure.
- AgNPs-induced MT1F and TRIB3 and Ag ion induced-ENDOGL1 expression was regulated by miR-219-5p and miR 654-3p, respectively.
- Network of miR-219-5p–MT1F and –TRIB3 pairs by AgNPs was found to be involved in various cellular processes.
- Overall results suggest that distinct epigenetic mechanism is involved in differential toxicity of AgNPs and Ag ions in Jurkat T cells.

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ABSTRACT

In our previous *in vitro* study of the toxicity on silver nanoparticles (AgNPs), we observed a dramatically higher sensitivity of Jurkat T cells to AgNPs than to Ag ions, and DNA damage and apoptosis were found to be involved in that toxicity. In this study, to understand underlying mechanism of different sensitivity of Jurket T cells to AgNPs and Ag ions, mRNA microarray and micro RNA microarray were concomitantly conducted on AgNPs and Ag ions exposed Jurkat T cells. Surprisingly only a small number of genes were differentially expressed by exposure to each of the silver (15 altered mRNA by AgNPs exposure, whereas 4 altered mRNA by Ag ions exposure, as determined 1.5-fold change as the cut-off value). miRNA microarray revealed that the expression of 63 miRNAs was altered by AgNPs exposure, whereas that of 32 miRNAs was altered by Ag ions exposure. An integrated analysis of mRNA and miRNA expression revealed that the expression of hsa-miR-219-5p, was negatively correlated with the expression of metallothionein 1F (MT1F) and tribbles homolog 3 (TRIB3), in cells exposed to AgNPs; whereas, the expression of hsa-miR-654-3p was negatively correlated with the expression of mRNA, endonuclease G-like 1 (EDGL1) in cells exposed to Ag ions. Network analysis were further conducted on mRNA-miRNA pairs, which revealed that miR-219-5p–MT1F and –TRIB3 pairs by AgNPs are being involved in various cellular processes, such as, oxidative stress, cell cycle and apoptosis, whereas, miR-654-3p and ENDOGL1 pair by Ag ions generated a much simpler network. The putative target genes of AgNPs-induced miR-504, miR-33 and miR-302 identified by Tarbase 6.0 are also found to be involved in DNA damage and apoptosis. These results collectively suggest that distinct epigenetic regulation may be an underlying mechanism of different sensitivity of Jurkat T cells to AgNPs and Ag ion. Further identification of putative target genes of DE miRNA by AgNPs and Ag ions may provide additional clues for the mechanism of differential toxicity. Overall results suggest that epigenetic mechanism is involved in toxicity of AgNPs and Ag ions in Jurkat T cells.

1. Introduction

Silver nanoparticles (AgNPs) are widely used in biomedical applications, textiles, cosmetics, food storage containers, personal care and numerous household products, due to their potent

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antimicrobial properties (Benn and Westerhoff, 2008; Fabrega et al., 2009; Kim et al., 2007; Luoma, 2008; Pal et al., 2007; Shahverdi et al., 2007). The toxicity of AgNPs has been documented by a number of *in vitro* and *in vivo* studies (Bilberg et al., 2012; Foldbjerg et al., 2011, 2009; Kawata et al., 2009). Despite many recent studies on the mechanism of toxicity of AgNPs (Prabhu and Poulose, 2012; Völker et al., 2013; Yang et al., 2012) the mechanism of AgNPs-induced global gene expression change and its influence on toxicity still remains unclear (Gagné et al., 2012; Kaveh et al., 2013; Poynton et al., 2012).

MicroRNAs (miRNAs) have recently emerged as important regulators of gene expression and protein translation. miRNAs are endogenous single-stranded RNA genes which are 22–25 nucleotides in length, and play important regulatory roles in organisms. They exhibit a broad range of biological functions in differentiation, proliferation, apoptosis, and necrosis, as well as physiological processes (Bartel, 2004; Kloosterman and Plasterk, 2006). Although numerous studies of the roles of miRNAs have been carried out, few have reported the alteration of miRNA expression in a toxicological context (Avissar-Whiting et al., 2010; Bollati et al., 2010; Izzotti et al., 2009; Fukushima et al., 2007; Elyakim et al., 2010).

Previously, we conducted *in vitro* toxicity screening of AgNPs and Ag ion exposure, which revealed that Jurkat T cells exhibited a higher sensitivity to AgNPs than to Ag ions exposure among the 6 cell lines tested (Eom and Choi, 2010). Further investigations of Jurkat T cells revealed that AgNPs induced DNA damage, cell cycle arrest, and apoptosis, which were not observed in Ag ion exposed cells. In this study, to understand the underlying mechanism of this different sensitivity, global gene expression analysis was conducted on AgNPs and Ag ion exposed Jurkat cells using mRNA microarray. As only limited gene expression was altered by AgNPs and Ag ion exposure, and no significant difference was found at the transcriptomic level. We further conducted miRNA microarray followed by mRNA–miRNA integrative analysis and network analysis. This integrated approach can provide a comprehensive understanding of the comparative toxicity of AgNPs and Ag ions on Jurkat T cells. Moreover, the identification of potential mRNA target genes of miRNA which were altered by exposure to AgNPs and Ag ions would provide a better understanding of the role of miRNAs in the toxic mechanism of AgNPs.

2. Materials and methods

2.1. Cells and silver nanoparticles

Human Jurkat T cell, Jurkat clone E6-1, were maintained in RPMI 1640 (GIBCO, Rockville, MD, USA), supplemented with 10% (v/v) fetal bovine serum and 1% penicillin streptomycin, at 37 °C in a humidified atmosphere of air and 5% CO_2. AgNPs (size <100 nm, Sigma–Aldrich Chemical, St. Louis, MO, USA) were homogenously dispersed in tetrahydrofurane (THF, Sigma–Aldrich) by sonication for 3 h (Branson-5210 sonicator, Branson Inc., Danbury, CT, USA), with stirring for 3 days to volatilize the THF, refilled with deionized water, and filtered through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Japan) to remove the AgNP aggregates.

2.2. mRNA and miRNA microarray

Cells were exposed to 0.2 mg/L of AgNPs and Ag ions for 24 h, and total RNA from exposed and control cells then extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany) for gene expression analyses and Trizol reagent (Invitrogene) for miRNA expression analyses. For the mRNA microarray, total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to yield biotinylated cRNA and labeled cRNA samples, which were hybridized to Illumina HumanRef-8 v3 Expression BeadChip (Illumina, Inc., San Diego, CA, USA) for 16–18 h at 58 °C, according to the manufacturer's instructions (Illumina). Detection of the array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK), following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner, with array data export processing and analysis performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). For the miRNA microarray, from 100 ng of total RNA, mature microRNAs were amplified with the Illumina Human MicroRNA Expression Profiling Assay V.2 (Illumina). The quality of hybridization and overall chip performance were monitored by visual inspection of both the internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina BeadStudio v3.1.3 Gene Expression Module v3.3.8). All data analyses and visualization of DEGs were conducted using ArrayAssist® (Stratagene, La Jolla, USA) and R statistical language v. 2.4.1. Normalized and raw intensity values of the microarray data have been deposited in the NCBI Gene Expression Omnibus database, under the accession numbers GSE 20692.

2.3. miRNA–mRNA integrative analysis

Integration of the mRNA–miRNA was performed for matched samples in each data set. First, statistically differentially expressed mRNAs and miRNAs were identified by |fold change| ≥ 1.5 for each data set. Computationally predicted targets mRNAs associated with the DE miRNAs were obtained from the miRBase Targets from Wellcome trust Sanger Institute (http://microrna.sanger.ac.uk/targets/v5). This approach assumed that the expression of a given miRNA is negatively correlated with the mRNA expression of its targets. We identified negative correlation between any putative pairs of miRNA and mRNA. For miRNAs have negative correlation with mRNA, hypergeometric test was performed to determine if miRNA was a statistically significant at p-value < 0.05.

2.4. mRNA qRT-PCR

For quantitative real time-polymerase chain reaction (qRT-PCR) analysis, Jurkat T cells were exposed to 0.2 mg/L of AgNPs and Ag ions for 24 h, and gene expression was analyzed using IQ™ SYBR Green Supermix (Bio-Rad). Quantitative RT-PCR was carried out on selected genes using a Chromo4 Real-Time PCR detection system (Bio-Rad). qRT-PCR conditions were optimized, and efficiency and sensitivity tests were performed for each gene prior to the main experiment. Three biological replicates were conducted for each qRT-PCR analysis.

2.5. miRNA TaqMan qRT-PCR analysis

Among the differentially expressed miRNAs, two miRNAs (hsa-miR-219-5p, hsa-miR-654-3p) were selected for confirmation analysis by miRNA TaqMan qRT-PCR assays (Applied Biosystems, Foster City, CA, USA). Small nucleolar RNA, RNU6 was used as an endogenous control in all qRT-PCR experiments. According to the Applied Biosystems TaqMan MicroRNA Assay protocol, cDNA was reverse transcribed from 10 ng of total RNA using specific looped miRNA RT primers, which allow for specific RT reactions for mature miRNAs only. The cDNA was then amplified by PCR, which uses TaqMan minor groove binder probes containing a reporter dye (FAM dye) linked to the 5′ end of the probe, a minor groove binder at the 3′ end of the probe, and a non-fluorescence quencher at the 3′ end of the probe. The design of these probes allows for more accurate measurement of reporter dye contributions than possible with conventional fluorescence quenchers.

2.6. Pathway analysis

Biological network and functions of miRNA–mRNA target pairs were generated using Pathway Studio v9.0 program (Ariadne Genomics, Inc.).

2.7. Prediction of miRNA target genes and pathway analysis

Computationally predicted mRNA targets of the significantly differentially expressed miRNAs were identified by TarBase 6.0 database (http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=tarbase/index) (Vergoulis et al., 2012). DIANA-mirPath software (Vlachos et al., 2012) analysis, which was used to analyze the KEGG pathway enrichment of the miRNA target genes, was also performed on 21 DE miRNAs by AgNPs and Ag ions. p-values were calculated with the default parameter settings (target genes predicted by Tarbase, union of target genes, p-value threshold as 0.05, and FDR correction applied).

2.8. Data analysis

Statistical differences between the control and treated cells were examined with the aid of ANOVA test using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA). An alpha level of 0.05 was used to determine the significance in all statistical analyses.

3. Results and discussion

3.1. mRNA microarray expression

Previously, we reported that Jurkat T cells showed a different sensitivity to AgNPs than to Ag ions. To identify involvement of the genes and pathways in such different susceptibilities, mRNA microarray was conducted on cells exposed to AgNPs and Ag ions,

as well as on unexposed cells (Fig. 1). Global gene expression profiling revealed that there were a total of 15 differentially expressed genes (DEGs) upon exposure to AgNPs, but only 4 upon exposure to Ag ions, using 1.5 fold changes as cut-off values (Fig. 1A). Besides the total number of DEGs, the intensities of expression compared to the control were greater in cells exposed to AgNPs than in cells exposed to Ag ions. DEG analysis indicated that metallothioneins (MTs) 2A, 1H, 1F, and 1A and endonucleases G like 1 (ENDOGL1) were up-regulated more than 2-fold upon exposure to AgNPs, compared to the control, whereas Ag ion induced an approximate 1.5-fold increase in the expression of two MT genes (*i.e.* MT2A and MT1H), compared to control. It was not surprising that five MT genes were induced upon exposure to AgNPs, as it is well known that dissolved Ag from AgNPs largely contributes to AgNPs toxicity, and MT is a typical biomarker protein for metal exposure. This result indicates that the expressions of MT genes may be involved in the toxicity of AgNPs toward Jurkat T cells, which has been observed in previous microarray studies on the human lung epithelial cell line A549, hepatoma cell line HepG2, and cervical cancer cell line HeLa (Foldbjerg et al., 2012; Kawata et al., 2009; Xu et al., 2012). Increased expressions of MT genes on exposure to AgNPs, as determined using real time PCR, was also reported in HeLa cells (Miura and Shinohara, 2009), and in the nematode *Caenorhabditis elegans* (Roh et al., 2009). Our microarray results of the up-regulation of MTs as major DEGs by AgNPs exposure reinforce the importance of dissolved metallic ions on AgNPs toxicity. However, only two MTs were included by Ag ion exposure, and to a lesser degree than those of AgNP exposure, and these varying mechanisms may be related to the different sensitivities of Jurkat T cells to Ag ion and AgNP exposures.

Hierarchical clustering analysis was also conducted on 18 DEGs from 9 independent microarray results upon exposure of Jurkat T cells to AgNPs and Ag ions, as well as a control, which revealed that the expression pattern of Ag ion exposure was clustered with that of the control while AgNPs showed a distinct expression pattern (Fig. 1B). This clustering result suggests that AgNPs may exert toxicity *via* a distinct pathway from that of Ag ions. A functional analysis using an mRNA microarray revealed that immunity, cell defense and the cell cycle were the biological processes most affected, and that nucleic acid binding and ligase were the molecular processes most affected (Fig. 1C). The results of functional analysis support our previous study that reported oxidative stress, the cell cycle and DNA damage as possible mechanisms of the toxicity of AgNPs (Eom and Choi, 2010).

3.2. *miRNA microarray expression*

Although an extensive amount of information has been generated from studies of gene expression profiling in response to toxicants using toxicogenomics tools, it is still difficult to find a clear signature for the exposure or effect of toxicants from microarray data. Indeed, our mRNA microarray results revealed that surprisingly small numbers of genes were differentially expressed by AgNPs, given the severe toxicity phenomenon observed at the cellular level (Eom and Choi, 2010), making it difficult to understand the mechanism behind toxicity. Recently, an important role of epigenetic changes in many cell regulation processes has been discovered through the use of miRNA profiling as a response to toxic compounds, and is now being applied to toxicological research (Hudder and Novak, 2008; Yauk et al., 2011). As more than one third of human genes have been predicted to be miRNA targets, miRNAs are a broad ranging class of regulatory molecules (Lewis et al., 2005). Therefore, miRNA microarray was additionally conducted in an attempt to identify fine regulator molecules in AgNPs induced toxicity to Jurkat T cells. miRNA expression profiling was investigated in AgNPs and Ag ions exposed Jurkat T cells using the Illumina miRNA expression chip (Fig. 1D), which indicated 63 differentially

(A)

Treatment	Description	Fold changes
AgNPs	metallothionein 2A (MT2A)	2.88
	metallothionein 1H (MT1H)	2.34
	metallothionein 1F (MT1F)	2.27
	endonuclease G-like 1 (ENDOGL1)	2.24
	metallothionein 1A (MT1A)	2.18
	argininosuccinate synthetase 1 (ASS1), transcript variant 1	1.77
	argininosuccinate synthetase 1 (ASS1), transcript variant 2	1.67
	ferritin, heavy polypeptide-like 12 (FTHL12)	1.60
	phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2)	1.57
	metallothionein 1G (MT1G)	1.52
	tribbles homolog 3 (Drosophila) (TRIB3),	1.51
	ferritin, heavy polypeptide-like 3 pseudogene (FTHL3P)	1.51
	microfibrillar-associated protein 4 (MFAP4)	1.50
	ferritin, heavy polypeptide-like 11 (FTHL11)	1.50
	actin, gamma 2, smooth muscle, enteric (ACTG2),	-1.53
Ag ions	metallothionein 2A (MT2A)	1.56
	metallothionein 1H (MT1H)	1.51
	sapiens endonuclease G-like 1 (ENDOGL1)	1.51
	glutathione peroxidase 1 (GPX1)	-1.59

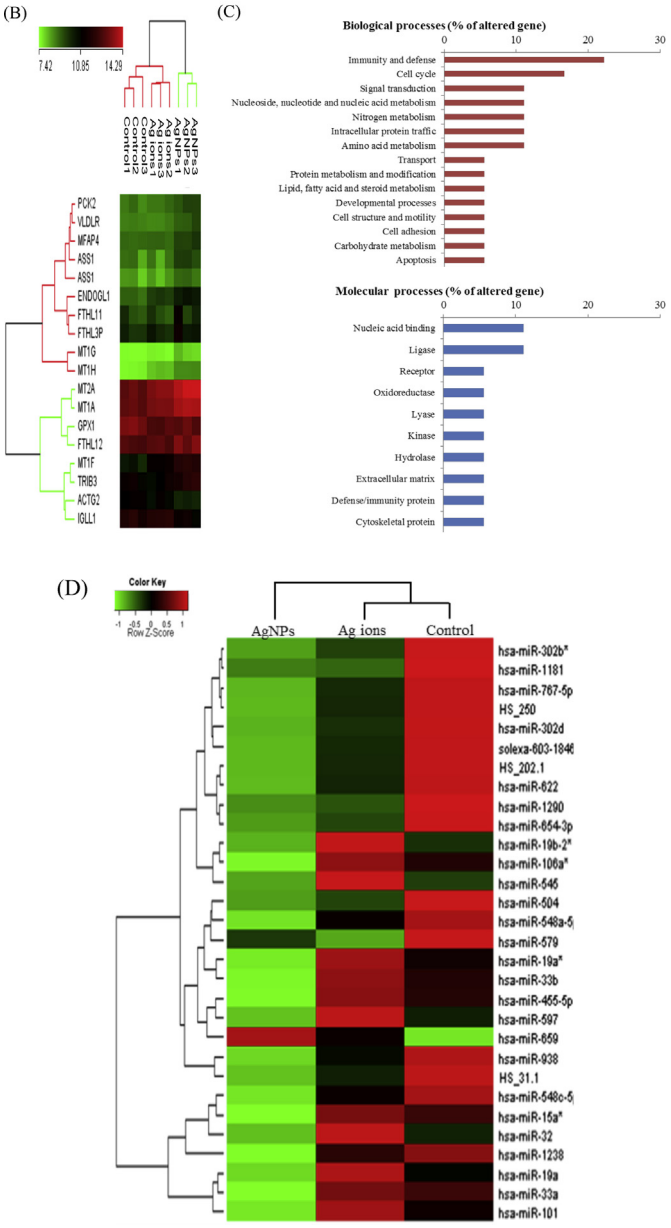

Fig. 1. Differentially expressed genes (A), hierarchical cluster (B) and functional analysis (C). mRNA microarray analysis was conducted on Jurkat T cells exposed to 0.2 mg/L AgNPs and Ag ions for 24 h, and each of which were performed in triplicate. Hierarchical cluster analysis of differentially expressed miRNAs (D). miRNA microarray analysis was conducted on Jurkat T cells which were exposed to 0.2 mg/L AgNPs and Ag ions for 24 h.

Table 1
Differentially expressed miRNAs. miRNA microarray was conducted in cells which were exposed to 0.2 mg/L of AgNPs and Ag ions for 24 h (miRNA* represents the passenger strand of miRNA).

AgNPs				Ag ions	
miRNA	Fold changes	miRNA	Fold changes	miRNA	Fold changes
hsa-miR-659	2.16	hsa-miR-32	−1.59	hsa-miR-32	2.29
hsa-miR-616	1.92	hsa-miR-1248	−1.60	hsa-miR-597	2.11
hsa-miR-483-3p	1.88	hsa-miR-153	−1.62	hsa-miR-545	1.82
hsa-miR-1826	1.81	hsa-miR-219-5p	−1.64	hsa-miR-19b-2*	1.77
hsa-miR-1224-5p	1.73	hsa-miR-1259	−1.69	hsa-miR-19b-2*	1.77
hsa-miR-1229	1.73	hsa-miR-106a*	−1.7	hsa-miR-1229	1.63
hsa-miR-1178	1.68	hsa-miR-490-5p	−1.71	hsa-miR-92a-2*	1.58
hsa-miR-29b-1*	1.63	hsa-miR-548d-5p	−1.73	hsa-miR-23b*	1.56
hsa-miR-935	1.63	hsa-miR-362-3p	−1.75	hsa-miR-659	1.54
hsa-miR-760	1.62	hsa-let-7g*	−1.75	hsa-miR-302a	1.54
hsa-miR-628-3p	1.60	hsa-miR-571	−1.76	hsa-miR-19a	1.54
hsa-miR-23b*	1.58	hsa-let-7c	−1.76	hsa-miR-1281	−1.53
hsa-miR-23a*	1.57	hsa-miR-632	−1.81	hsa-miR-571	−1.53
hsa-miR-1257	1.57	hsa-miR-15a*	−1.82	hsa-miR-580	−1.54
hsa-miR-603	1.54	hsa-miR-579	−1.84	hsa-miR-153	−1.55
hsa-miR-877*	1.53	hsa-miR-1225-3p	−1.85	hsa-miR-1259	−1.57
hsa-miR-138-1*	1.53	hsa-miR-181c	−1.99	hsa-miR-610	−1.62
hsa-miR-92a-2*	1.51	hsa-miR-455-5p	−2.00	hsa-let-7c	−1.66
hsa-miR-214	1.50	hsa-miR-1181	−2.02	hsa-miR-767-5p	−1.72
hsa-miR-18b	−1.50	hsa-miR-548c-5p	−2.09	hsa-miR-632	−1.77
hsa-miR-141	−1.50	hsa-miR-548a-5p	−2.2	hsa-miR-490-5p	−1.80
hsa-miR-101	−1.50	hsa-miR-302b*	−2.2	hsa-miR-302b*	−1.86
hsa-miR-101	−1.50	hsa-miR-767-5p	−2.2	hsa-miR-1248	−1.87
hsa-miR-610	−1.51	hsa-miR-33b	−2.37	hsa-miR-1181	−1.94
hsa-miR-566	−1.51	hsa-miR-33a	−2.53	hsa-miR-579	−2.26
hsa-miR-19a	−1.52	hsa-miR-1290	−2.86	hsa-miR-654-3p	−2.48
hsa-miR-19a	−1.52	hsa-miR-654-3p	−3.07	hsa-miR-1290	−2.49
hsa-miR-16-1*	−1.52	hsa-miR-302d	−3.89	hsa-miR-622	−2.55
hsa-miR-1281	−1.52	hsa-miR-622	−4.15	hsa-miR-302d	−2.60
hsa-miR-140-5p	−1.55	hsa-miR-504	−5.67	hsa-miR-1238	−3.04
hsa-miR-597	−1.55	hsa-miR-938	−26.94	hsa-miR-504	−3.98
hsa-miR-580	−1.55	hsa-miR-1238	−66.54		
hsa-miR-19a*	−1.55				

expressed (DE) miRNAs upon exposure to AgNPs (19 up-, and 44 down-regulated); whereas33 DE miRNAs upon exposure to Ag ions (11 up-, and 21 down-regulated). The DE miRNAs list is presented in Table 1. The hierarchical clustering on DE miRNA showed that DE miRNAs upon Ag ion exposure were clustered with those of the control, whereas DE miRNAs upon AgNP exposure showed a distinct expression pattern, which is similar as DE mRNA clustering Among. DE miRNAs, the expression of hsa-miR-1238 was the most dramatically decreased upon exposure to AgNPs, at about 67-fold compared to the control, whereas it was decreased about 3-fold upon exposure to Ag ions. The expression of hsa-miR-938 was also dramatically decreased upon exposure to AgNPs (about 27-fold compared to the control) and it was decreased to a lesser extent (about 5-fold compared to control) when exposed to Ag ions.

3.3. *miRNA–mRNA integrated analysis and network pathway*

Regulation of specific gene expressions by miRNA can be revealed by investigating the correlation between mRNA and miRNA expressions. To find the miRNA–mRNA complex involved in AgNPs toxicity, an integrated analysis of mRNA and miRNA was conducted using the web-resource MicroCosm, the miRBase Targets Version 5 database (http://microrna.sanger.ac.uk/targets/v5/). Down-regulated miRNAs were matched with the predicted up-regulated mRNA targets and *vice versa*. The miRBase Targets database uses the miRanda algorithm to identify potential binding sites for a given miRNA in a genomic sequence (Enright et al., 2003; Griffiths-Jones et al., 2006).

The result of integrated analysis indicated that the expression of miRNA, hsa-miR-219-5p, was negatively correlated with those of mRNA, MT1F and TRIB3, in cells upon exposure to AgNPs, whereas the expression of has-miR-654-3p was negatively correlated with the expression of mRNA, EDGL1 in cells upon exposure to Ag ions (Table 2). To confirm the relationship between miRNA and mRNA target pair, qRT-PCR was conducted on Jurkat T cells exposed to AgNPs and Ag ions (Fig. 2). The regulation of miR-219-5p on the expressions of target MT1F and TRIB3 was validated (Fig. 2A), and the suppression of miR-219-5p induced expression of MT1F and TRIB3 genes. MT1F gene belongs to the MT superfamily, and type 1 family is known to contain a high content of cysteine residues which bind various heavy metals. There is very limited information of miRNAs that target MT genes, however, An et al. (2013) recently identified miR-23a as a negative MT2A regulator in human gastric cancer cell lines. Our result further implicates a number of targets, such as, PLK2 and GPC3 (Huang et al., 2012; Sarachana et al., 2010), and miR-219-5p may also target MT1F gene. Considering the importance of MT in metal homeostasis and toxicity, this seems to be an important finding in relation to metal toxicity and merits further investigation. TRIB3, a mammalian homolog of *Drosophila* tribbles, is known to be an important regulatory protein involved in multiple signaling pathways, including MAPK (Kiss-Toth et al., 2004), TGF-beta (Chan et al., 2010; Hua et al., 2011), NFκB (Rzymski et al., 2008; Wu et al., 2003) and the PI3K pathway (Ding et al., 2008). The fact that miR-219-5P targets TRIB3

Table 2
Integrated analysis of miRNA-mRNA target pairs.

Treatment	miRNA–mRNA pair	mRNA log2fold
AgNPs	hsa-miR-219-5p MT1F	−1.19
	hsa-miR-219-5p TRIB3	−0.59
Ag ions	hsa-miR-654-3p ENDOGL1	−0.59

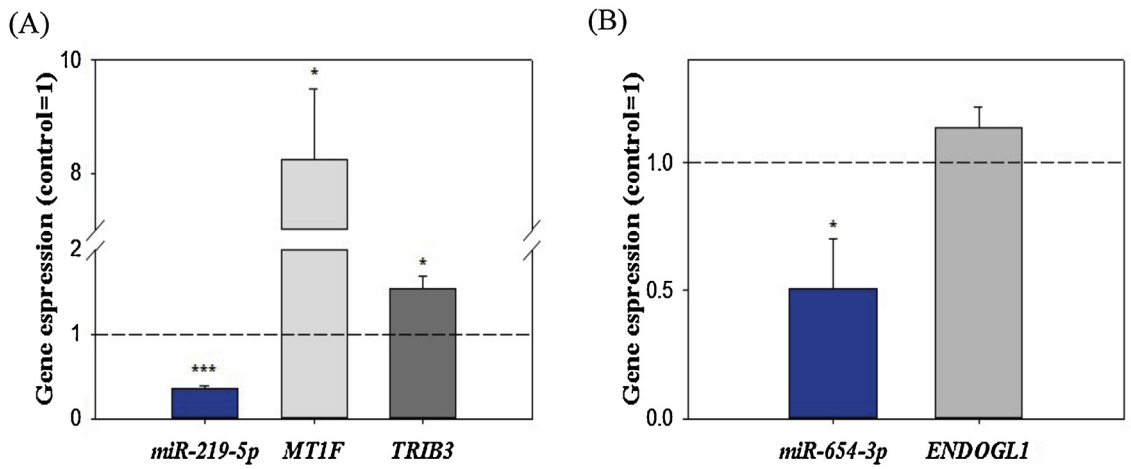

Fig. 2. The expressions of miRNA and mRNA target pairs. Quantitative RT-PCR was conducted as validation of the expression by exposure to AgNPs (A) and Ag ions (B). Data represented the expression of miR-219-5p and target genes, MT1F and TRIB3, and miR-654-3p and target gene ENDOGL1. The results are expressed as the mean values compared to the control (control = 1, $n = 3$; mean ± standard error of the mean; $^{*}p < 0.05$, $^{***}p < 0.001$).

might be related to AgNPs-induced apoptosis in Jurkat T cells, as observed in our previous study (Eom and Choi, 2010). We also determined direct binding as negative correlation between miR-654-3p and ENDOGL1 exposed to Ag ions (Fig. 2B). ENDOGL1 is a member of the DNA/RNA endonuclease family which includes human endonuclease G (ENDOG), a mitochondria-specific nuclease that is translocated to the nucleus during apoptosis. Our integrative analysis suggests that ENDOGL1 may be a potential target for miR-654-3p, for which the targets have never been identified.

To further investigate the biological networks and functions in miRNA-mRNA target pairs (*i.e.* miR-219-5p -MT1F and -TRIB3 and miR-654-3p-ENDOGL1), the involved pathways were generated using Pathway Studio (http://www.ariadnegenomics.com/products/pathway-studio/) (Fig. 3). The network analysis of the miRNA–mRNA correlated pair did not reveal direct targeting, but did reveal indirect interactions with different degrees of complexity upon exposure to AgNPs. Network analysis revealed that miR-219-5p and TRIB3 are connected through a one-step interaction, whereas miR219-5p and MT1F showed multiple interactions (Fig. 3A). miR-219-5p negatively regulates the peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PPARGC1A), which induces TRIB3 as a transcriptional co-activator (Peeters et al., 2011). Network analysis of miR-219-5p–MT1F and –TRIB3 pairs showed that various cellular processes, such as oxidative stress, cell cycle, cell survival/death and apoptosis, were being involved in these pairs. This finding supported the results from our previous study, which determined that AgNPs exposure induced oxidative stress, cell cycle arrest and apoptosis (Eom and Choi, 2010). A much simpler network was generated from the miR-654-3p and ENDOGL1 pair by exposure to Ag ions than from miR-219-5p pairs by exposure to AgNPs (Fig. 3B). Integrated miRNA–mRNA analysis and network analysis suggest that exposures to AgNPs and Ag ions induced different mechanisms of toxicity, and distinct miRNAs regulate their specific targets. This may contribute to the understanding of the differential toxicity between AgNPs and Ag ions, as previously observed (Eom and Choi, 2010).

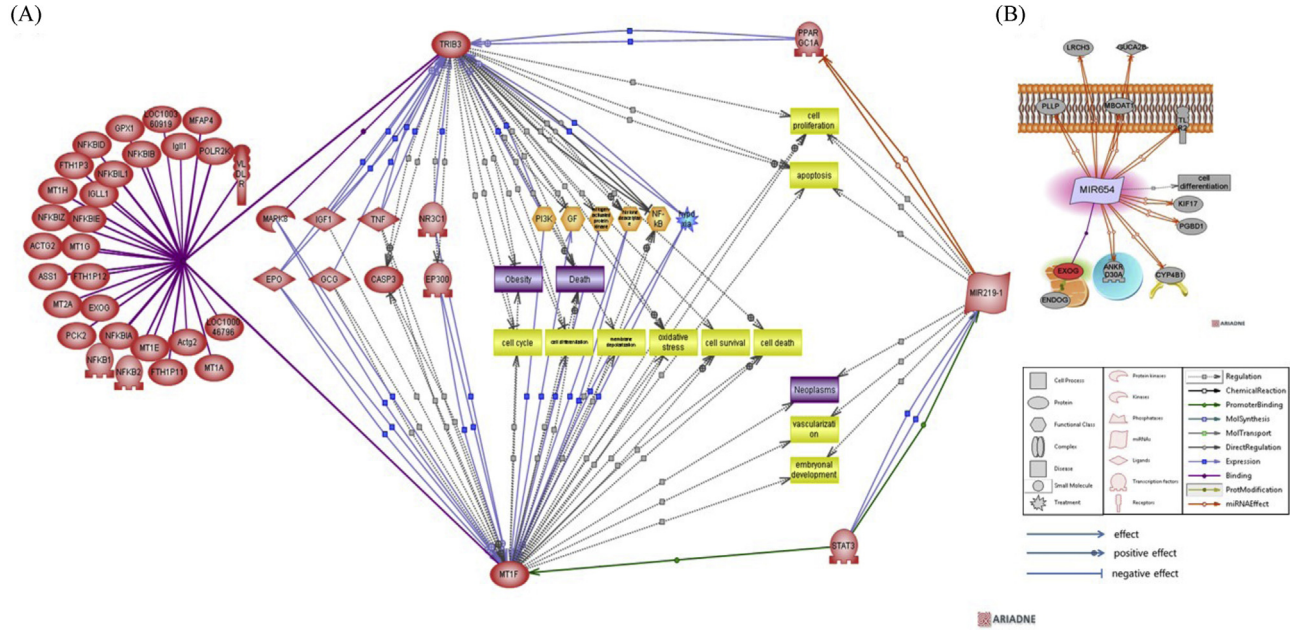

Fig. 3. Interaction network of miRNA–mRNA targets generated by Pathway Studio. The network analysis showed the connection of miR-219-5p–MT1F and TRIB3 (A) and miR-654-3p–ENDOGL1 (B). The different symbols indicated different types of proteins or small molecules, and arrows displayed the regulation, expression and miRNA effect.

Table 3
The number of miRNA targets predicted by the Tarbase 6.0 data base (miRNA* represents the passenger strand of miRNA).

Treatment	miRNA	Fold changes	Number of target genes	Ref.
AgNPs	hsa-miR-659	2.16	2	Rademakers et al. (2008)
	hsa-miR-219-5p	−1.64	2	Kocerha et al. (2009), Xiong et al. (2010)
	hsa-miR-302b*	−2.20	4	Lee et al. (2008), Lin et al. (2010), Scheel et al. (2009)
	hsa-miR-33b	−2.37	3	Najafi-Shoushtari et al. (2010), Ji et al. (2008)
	hsa-miR-33a	−2.53	71	Hafner et al. (2010), Thomas et al. (2012), Rayner et al. (2010), Najafi-Shoushtari et al. (2010)
	hsa-miR-654-3p	−3.07	1	Wu et al. (2010)
	hsa-miR-302d	−3.89	16	Tsai et al. (2011), Li et al. (2009), Barroso-delJesus et al. (2011), Lin et al. (2010), Tsai et al. (2010), Ye et al. (2008), Li et al. (2009)
	hsa-miR-622	−4.15	1	Guo et al. (2011)
	hsa-miR-504	−5.67	11	Hu et al. (2010), Huang and Li (2009), Ye et al. (2008)
Ag ions	hsa-miR-32	2.29	143	Hafner et al. (2010), Ambs et al. (2008), Pichiorri et al. (2008), Wang et al. (2008)
	hsa-miR-579	−2.26	1	Xiong et al. (2010)
	hsa-miR-654-3p	−2.48	1	Wu et al. (2010)
	hsa-miR-622	−2.55	1	Guo et al. (2011)
	hsa-miR-302d	−2.60	16	Tsai et al. (2011), Li et al. (2009), Barroso-delJesus et al. (2011), Lin et al. (2010), Tsai et al. (2010), Ye et al. (2008), Li et al. (2009)
	hsa-miR-504	−3.98	11	Hu et al. (2010), Huang and Li (2009), Ye et al. (2008)

3.4. Putative miRNA target genes and pathway analysis

Though high numbers of DE miRNAs were identified by miRNA microarray, we only found three miRNA–miRNA pairs using integrative analysis (Table 2). Therefore, we further analyzed DE miRNA using computational miRNA target prediction software Tarbase 6.0 and DIANA-mirPath. Among the altered miRNAs, we focused on the 21 most significantly altered miRNAs by AgNPs and Ag ions, and identified their putative target genes predicted by Tarbase 6.0 (Table 3). miR-504, miR-33a, miR-33b, miR-302b, miR-302d and miR-32 are well known to regulate apoptosis and cell cycle arrest. Hu et al. (2010) demonstrated that miR-504 directly inhibits the transcriptional activity of p53, and reduces p53-mediated apoptosis and cell cycle arrest in response to stress. Previously, we observed DNA damage upon AgNPs exposure, and not Ag ions exposure, evidenced by an increase of H2AX protein level (Eom and Choi, 2010). We recently found increased p53 protein expression by AgNPs exposure (Chattergee and Choi, 2014,

Table 4
KEGG pathway analysis of miRNA target genes.

KEGG pathway	AgNPs			Ag ions		
	p-value	# genes	# miRNAs	*p*-value	# genes	# miRNAs
p53 signaling pathway	4.56.E−08	6	4	2.68.E−09	7	4
Hepatitis B	1.35.E−06	6	5	3.73.E−05	7	4
Viral carcinogenesis	7.05.E−06	7	5	6.12.E−08	9	4
Pathways in cancer	1.99.E−05	9	5	2.17.E−08	13	4
ErbB signaling pathway	1.71.E−04	4	4	1.04.E−02	3	3
Bladder cancer	1.38.E−03	3	3	4.95.E−05	4	4
Cell cycle	1.86.E−03	5	5	5.03.E−07	8	4
PI3K-Akt signaling pathway	8.67.E−03	7	6	1.10.E−06	13	4
HIF-1 signaling pathway	3.73.E−06	6	6			
Tuberculosis	7.05.E−06	7	4			
Prostate cancer	2.06.E−03	4	4			
Transcriptional misregulation in cancer	2.06.E−03	5	5			
Amyotrophic lateral sclerosis (ALS)	4.30.E−03	3	3			
Apoptosis	5.07.E−03	3	2			
Epstein-Barr virus infection	5.07.E−03	6	5			
Endocytosis	5.71.E−03	5	3			
Colorectal cancer	1.03.E−02	3	3			
Neurotrophin signaling pathway	1.04.E−02	4	4			
Amphetamine addiction	3.25.E−02	2	2			
Renal cell carcinoma	3.25.E−02	2	3			
Glioma	3.50.E−02	3	3			
Valine, leucine and isoleucine biosynthesis				4.98.E−12	2	1
Prostate cancer				1.09.E−05	6	4
Chronic myeloid leukemia				1.07.E−04	4	3
Pantothenate and CoA biosynthesis				1.52.E−04	3	1
Small cell lung cancer				1.52.E−04	5	2
Oocyte meiosis				6.61.E−04	5	2
Valine, leucine and isoleucine degradation				1.85.E−03	2	1
Melanoma				1.89.E−03	4	3
Lysine degradation				1.04.E−02	2	1
Focal adhesion				1.04.E−02	6	3
HTLV-I infection				4.96.E−02	6	4

manuscript in preparation). Decreased expression of DNA damage regulator miRNA miR-504 by AgNPs might be related to DNA damage response to our previous studies (Chattergee and Choi, 2014 manuscript in preparation; Eom and Choi, 2010).

A recent article reports that the miR-33 family are regulators of cell cycle progression, targeting both CDK6 and CCND1 directly (Cirera-Salinas et al., 2012) and indirectly targeting c-Myc to reduce cyclin E expression (Gocek et al., 2011). To date, the miR-302 family (miR-302a, miR-302b, miR-302c, and miR-302d) have been reported to have roles in the regulation of embryonic cell differentiation, and to be potential tumor biomarkers (Lin et al., 2010; Murray et al., 2011). Decreased expressions of miR-33a, miR-33b, miR-302b and miR-302d by AgNPs (Table 3) may activate their target pro-apoptosis genes, though we did not measure them, and these genes trigger AgNPs-induced apoptosis, as we have previously determined (Eom and Choi, 2010).

Overexpression of miR-32 led to increased cell proliferation, migration, invasion, and reduced apoptosis by suppressed PTEN protein in CRC cells (Wu et al., 2013) and down-regulated Bim expression in human acute myeloid leukemia (AML) cells (Gocek et al., 2011). Though we did not measure miR-32 target genes, the increase of miR-32 by Ag ions, but not by AgNPs (Table 3) may explain why apoptosis was observed by AgNPs, and not by Ag ion exposure.

The published report addressed the functions of miR-622 as a tumor suppressor by targeting ING1 and K-Ras induced cell growth arrest and apoptosis (Guo et al., 2011; Han et al., 2012). miR-622 was down-regulated in about 4-folds by AgNPs exposure, whereas, only in about 2-folds by Ag ion exposure, which again further supports why apoptosis was the prevalent by AgNP exposure, but not by Ag ion exposure. Unfortunately, target genes of miR-1238 and miR-938, which were most dramatically down-regulated miRNAs by AgNPs (in about 67 and 27 folds), have not been identified with using Tarbase 6.0. Moreover, no target genes were available for 7 DE miRNAs (*i.e.* miR-455-5p, miR-1181, miR-548c-5p, miR-548a-5p, miR-767-5p, miR-597 and miR-1290) through Tarbase 6.0.

The DE miRNAs-induced pathway was therefore further analyzed using DIANA-mirPath (Table 4). This software, starting from a set of miRNAs, performs an enrichment analysis of predicted targets, giving indications of possible functions of the regulated miRNAs (Papadopoulos et al., 2009). The predicted KEGG pathway from the DIANA-mirPath revealed that p53 signaling, as well as cell cycle and PI3K-Akt signaling pathways were commonly involved upon exposure to AgNPs and Ag ions. However, HIF-1 signaling pathway, apoptosis and endocytosis pathway were only observed in AgNPs exposed cells. HIF-1 pathway was found to be involved in AgNP toxicity in our recent study of the nematode, *C. elegans* (Eom et al., 2013). Collectively, the predicted miRNA target genes and DIANA-mirPath analysis also suggests a differential toxic mode of action between AgNPs and Ag ions.

3.5. Conclusions

In the present study, mRNA and miRNA microarray analyses were conducted to identify underlying mechanisms of the different susceptibilities of Jurkat T cells to AgNPs and Ag ions, revealing that more DEGs and DE miRNAs were induced by AgNPs than by Ag ions. We further identified negatively correlated miRNA–mRNA target pairs, and analyzed their interaction and pathways *via* an integrated analysis of miRNA and mRNA expression profiles. The results of integrated analysis revealed that AgNP-induced MT and TRIB3 expressions were regulated by miR-219-5p, whereas, Ag ion-induced ENDOGL1 expression was regulated by miR 654-3p. The results also suggest the potential roles of miR-504, miR-33 and miR-302 in AgNP-induced DNA damage and apoptosis, and the expression of miR-32 and miR-622 may also provide a clue toward the differential toxicity between AgNPs and Ag ions in Jurkat T cells. Overall results suggest that distinct epigenetic mechanism is involved in differential toxicity of AgNPs and Ag ions in Jurkat T cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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