A Genome-wide Screening of Target Genes Against Silver Nanoparticles in Fission Yeast

Ah-Reum Lee,*1 Sook-Jeong Lee,†1 Minho Lee,‡ Miyoung Nam,* Sol Lee,* Jian Choi,* Hye-Jin Lee,* Dong-Uk Kim,§,2 and Kwang-Lae Hoe*,2

*Department of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, Republic of Korea; †Department of Bioactive Material Science, Chonbuk National University, Jeonju 54896, Republic of Korea; ‡Catholic Precision Medicine Research Center, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea; and §Department of Aging Research Center, KRRB, Daejeon 34141, Republic of Korea

1These authors contributed equally to this work.

2To whom correspondence should be addressed at Department of Aging Research Center, KRRB, Daejeon 34141, Republic of Korea, E-mail: Dongku@kribb.re.kr (Dong-Uk Kim) and Department of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, Republic of Korea, E-mail: kwanghoe@cnu.ac.kr (Kwang-Lae Hoe)

ABSTRACT

To identify target genes against silver nanoparticles (AgNPs), we screened a genome-wide gene deletion library of 4843 fission yeast heterozygous mutants covering 96% of all protein encoding genes. A total of 33 targets were identified by a microarray and subsequent individual confirmation. The target pattern of AgNPs was more similar to those of AgNO₃ and H₂O₂, followed by Cd and As. The toxic effect of AgNPs on fission yeast was attributed to the intracellular uptake of AgNPs, followed by the subsequent release of Ag⁺, leading to the generation of reactive oxygen species (ROS). Next, we focused on the top 10 sensitive targets for further studies. As described previously, 7 nonessential targets were associated with detoxification of ROS, because their heterozygous mutants showed elevated ROS levels. Three novel essential targets were related to folate metabolism or cellular component organization, resulting in cell cycle arrest and no induction in the transcriptional level of antioxidant enzymes such as Sod1 and Gpx1 when 1 of the 2 copies was deleted. Intriguingly, met9 played a key role in combating AgNP-induced ROS generation via NADPH production and was also conserved in a human cell line.

Key words: fission yeast; met9; NADPH; ROS; silver nanoparticles; systematic screening.
dissolving DNA, lipids, and proteins (Halliwell and Aruoma, 1991; Jakobsson-Borin et al., 1994; Stadtman, 1993). The more rapidly the area of nanoparticle-containing products grows in the market, the more studies addressing their toxicity mechanism with respect to human health and the environmental impact become urgently required.

To date, the toxic effects of AgNPs have been attributed to the intracellular release of Ag⁺ from AgNPs (Damm and Münsstedt, 2008; Olga et al., 2014). Notably, Ag⁺ is well known to exhibit bactericidal effects via a mechanism similar to that by AgNPs (Drake and Hazelwood, 2005; Yamanaka et al., 2005). Ag⁺ interacts with the cell membrane, nucleic acids, and proteins and results in membrane damage and inhibition of the thiol-containing enzymes and proteins (Jung et al., 2008).

Yeast species have long been model organisms for studying cell division and the cell cycle due to their facile genetics (Nurse, 1994). Particularly, the rod-shaped fission yeast Schizosaccharomyces pombe provides an excellent model system to study cell morphogenesis and cell division cycles. We have constructed a genome-wide gene deletion library in fission yeast after budding yeastSaccharomyces cerevisiae provides an excellent model system to study cell morphogenesis and cell division cycles. We have constructed a genome-wide gene deletion library in fission yeast after budding yeast.Saccharomyces cerevisiae to study cell morphogenesis and cell division cycles. We have constructed a genome-wide gene deletion library in fission yeast after budding yeast. For the first time, we report that several novel essential genes are critical for tolerance to AgNP-induced cytotoxicity. An aliquot of 3.5 ×10⁷ cells was harvested every 5 generations up to 20 generations. An aliquot of 3.5 ×10⁷ cells was harvested every 5 generations up to 20 generations.

MATERIALS AND METHODS

Chemicals and cell culture. AgNPs, AccuSilverSol silver nano colloid in water (Catalog no. TS-2010-1; stock solution = 1%; diameter = 17.4 nm), were purchased from Bioneer (Daejeon, Korea). AgNPs, AgNO₃, N-acetylcyesteine (NAC), and 2', 7'-dichlorodihydrofluorescin diacetate (H₂DCFDA) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Molecular Probes (Eugene, Oregon), respectively. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), unless otherwise stated. Yeast cells were cultivated in complete YES medium (0.5% yeast extract, 3% glucose, and appropriate amino acid supplements) or Edinburgh minimal medium with appropriate supplements at 30°C unless otherwise specified (Moreno et al., 1991). Human embryonic kidney (HEK) 293 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B) at 37°C in a humidified atmosphere containing 5% CO₂. All reagents and chemicals for cell culture were purchased from WELGENE (Gyeongsan, Korea) and Sigma-Aldrich, respectively, unless otherwise stated.

Genome-wide screening of the heterozygous gene deletion library in fission yeast by microarray. For the systematic screening of sensitive target genes for AgNPs, we used the heterozygous gene deletion library constructed in a previous study (Kim et al., 2010). Briefly, the library was constructed by homologous replacement of each gene into the KanMX marker gene as a selection marker based on the parental strain of the SP286 wild-type (ade6-M210/ ade6-M216, leu1-32/leu1-32, ura4-D18/ura4-D18 h/ h~ ). The library was pooled and aliquoted into 100 μl vials for each screen, and the vials were kept frozen at ~80°C until use. Notably, each deletion mutant has a pair (up- and down-tag) of unique built-in molecular bar codes for a parallel analysis. The systematic screening of AgNP target genes was performed as previously reported (Han et al., 2013). Briefly, a vial of frozen pool was activated in 50 ml of YES media for 24–30 h up to OD₆₀₀ = 2 (approximately 4.4 ×10⁷ cells/ml). The cells were then diluted in 50 ml of YES media to OD₆₀₀ = 0.05 and cultivated up to OD₆₀₀ = 1.6 (approximately 3.5 ×10⁷ cells) with or without AgNPs (0.2 μg/ml), which was repeated 4 times every 5 generations up to 26 generations. An aliquot of 3.5 ×10⁷ cells was harvested every 5 generations and genomic DNA was prepared using the ZR-Fungal/Bacterial DNA kit (Zymo Research, Irvine, California). The microarray experiment was performed using a custom-made GeneChip (48 K KRRB, SP2, ThermoFisherScientific, Waltham, Massachusetts) and the fluorescence-labeled probe prepared by PCR of the pair of bar codes (Kim et al., 2010). Three independent microarray experiments were performed. The primary 44 target strains were selected by the criterion that the results indicated relative growth fitness (RF) < 0.9 (p < .05) twice.

Confirmation of the primary target genes by spotting assay. The primary candidates were confirmed one by one based on an individual growth analysis using a spotting assay. For the spotting assay, cells in log phase were diluted to OD₆₀₀ = 0.5 in YES media and spotted in 5-fold serial dilutions onto YES agar plates with or without 0.2 μg/ml AgNPs. Their sensitivity against AgNPs was scored by the following criterion: severe (SS) when their survival rate was decreased by more than 3 serial dilutions (>75-fold sensitivity); moderate (M) by 2 to 3 serial dilutions (25- to 75-fold sensitivity); and mild (S) by 2 serial dilutions (~25-fold sensitivity). The total of 33 target genes was confirmed and subject to gene ontology (GO) analysis using GO term finder (http://go.princeton.edu/cgi-bin/GOTermFinder).

Observation of cellular AgNPs. The cellular uptake of AgNPs by yeast cells was measured using inductively coupled plasma mass spectrometry (ICP-MS) and visualized using transmission electron microscopy (TEM). Cells in the log phase were treated with AgNPs or AgNO₃ at the indicated concentrations for 12 h. The treated cells were washed 3 times with PBS (pH 7.4), harvested, and subjected to ICP-MS and TEM analyses. In order to quantify intracellular Ag that was absorbed by the cells, the harvested cells were digested by acid treatment using the HotBlock digestion system (Environmental Express, Charleston, South California). Briefly, HNO₃ was added to the sample, followed by irradiation at 110°C for 5–6 h. After digestion, the samples were diluted with water and quantified using the ELAN DRC II ICPMS (PerkinElmer, Waltham, Massachusetts). The AgNPs accumulated inside the cells were also observed by TEM. Briefly, the harvested
cells were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4), followed by fixing in 2% osmium tetroxide in the same buffer for 1 h. The cells were dehydrated using a series of ethanol concentrations and subsequently embedded in the Epon 812 resin (Hexion, Columbus, Ohio). After sectioning the samples using an ultramicrotome (Leica Microsystems, Vienna, Austria), they were stained with uranyl acetate and lead citrate. The stained samples of AgNPs were placed onto a carbon-coated copper grid and air-dried. The images were observed using the FEI Tecnai G2 T-20S TEM (FEI Europe B.V., Eindhoven, the Netherlands), equipped with the Gatan ORIUS SC1000 CCD camera.

Cross-sensitivity comparison by hierarchical clustering. For cross-sensitivity comparison, in addition to the top 10 target genes showing higher sensitivity than SS, 17 additional genes which were related to the relevant GO terms were used. The genes used were as follows: signal transduction (wnt1, sty1, pap1, af1, and wsi1 in addition to wls4, mcs4, and SPPC1827.07c), sulfur compound metabolism (sua1, sir1, cys12, met10, cys11, and met14 in addition to hmt2, rdl2, gc1, and gc2), (met11, shm2, bfh1, df1, mtd1, and fdi1 in addition to met9), and cellular component organization (sph1 and pep1). Additionally, 35 genes were randomly selected and included in the above analysis. Their growth rate was measured by plating serial 5-fold dilutions onto YES plates supplemented with 0.2 μg/ml AgNPs, 0.4 μg/ml AgNO₃, 2 mM H₂O₂, 0.4 mM Cd, or 0.5 mM As. All experiments were performed at least 3 times. The results were analyzed by hierarchical clustering using Euclidean distance as the measure of similarity. R version 3.3.3 and R package “gplots” were used for analysis and visualization, respectively.

Measurement of ROS levels and relative growth. To measure intracellular ROS levels, the redox-sensitive green fluorescent H₂DCFDA probe was used along with propidium iodide (PI) dye to distinguish living cells from dead ones. Briefly, cells were exposed to AgNPs for 3 h at 30°C and washed twice with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). The cells were then incubated with 40 μM H₂DCFDA for 1 h and 3 μg/ml PI for 10 min at 30°C in the dark. To measure both NADP and NADPH, 4 mM sodium arsenite (scrambled, catalog no. K347-100; BioVision Inc; Milpitas, California). The PCR primers were synthesized by Bioneer. The primer sequences of fusion yeast genes were as follows: srd1, forward 5′-GTCACTCGCC TTCTTAGTACAAGG-3′ and reverse 5′-CCCATATGAAAACAC TCTCACAT-3′; gpx1, forward 5′-AGCGAGCAAAATGTTGGATC T-3′ and reverse 5′-AATTGAGCGATTTCTTCGTCAGA-3′; act1 (a normalization control), forward 5′-TCTTTCAACCGGAAAGATG TAC-3′ and reverse 5′-CATACCGAGGAGCAAAAGAC-3′. The primer sequences of human genes are as follows: MTHFR, forward 5′-TTGGAAGACACATTGGAGC-3′ and reverse 5′-CAAGAGAA GACCGACTGT-3′; β-actin as a normalization control, forward 5′-ATGTCACCGCAAATGCTTCTA-3′ and reverse 5′-AAGCCAG TGCCAAATCTTACATTGGT-3′.

Measurement of NADP and/or NADPH concentration. To measure NADP/NADPH concentration, the NADP/NADPH quantification colorimetric kit was used by following the manufacturer’s protocol (catalog no. K347-100; BioVision Inc; Milpitas, California). To measure both NADP and NADPH, 4 × 10⁶ cells were harvested and lysed in extraction buffer. An NADP cycling mix was added and incubated to convert NADP to NADPH. Finally, NADPH developer was added, and the OD₄₅₀ was measured after a reaction for 1–4 h. NADPH concentration was calculated using a standard curve of NADPH. To measure NADPH only, NADPH was decomposed from the cell extracts by heating samples to 60°C for 30 min. Samples were normalized using the BCA protein assay kit (ThermoFisherScientific).

siRNA assay. To mimic the heterozygous effect of yeast in humans, gene transcription was reduced by siRNA knockdown. The siRNA oligonucleotides of MTHFR and a negative control (scrambled, catalog no. 4390843) were purchased from ThermoFisherScientific. Sequences of MTHFR were as follows: sense 5′-GCACAUCCGAGUGAUUU (dTdT)-3′ and antisense 5′- AAACUCAUCCGAGUGU (dTdT)-3′. The oligonucleotides were transfected into cells using the HiPerFect kit (Qiagen) according to the manufacturer’s instruct. After incubation for 72 h, the extent of knockdown by siRNA was measured by q-PCR.

MTT assay and 8-OHdG measurement. HEK293 cells (2 × 10⁴ cells in a 48-well plate) were treated with 0.6 μg/ml AgNPs, and then their viability was measured using an MTT-based cell viability assay. To measure oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG) was analyzed using the OxiSelect Oxidative DNA damage ELISA kit (Cell Biolabs Inc, San Diego, California) by following the manufacturer’s protocol. Briefly, genomic DNA was converted to single-stranded DNA and 8-OHdG was quantified using a standard curve by quantitative ELISA assay.
Statistical analysis. All experiments were performed using triplicate samples and repeated at least 3 times. Data are presented as mean ± SD, and statistical comparisons between groups were performed using a Student’s t-test. For multiple comparisons among groups, 2-way ANOVA with the Student-Newman-Keuls method was performed using GraphPad Prism (La Jolla, California). The p-values < .05 were considered significant.

RESULTS

Primary Genome-Wide Target Screening and Secondary Individual Confirmation Revealed 33 AgNP Target Genes

Through the primary genome-wide screening against AgNPs, 44 target strains were selected with RF < 0.9 (p < .05) compared with the wild-type control in Table 1. Finally, 33 target genes were confirmed by the spotting assay as shown in Table 1. They consisted of 3 severe (SSS), 7 moderate (SS), and 23 mild (S) targets as measured by sensitivity, and 25 non-essential and 8 essential targets as measured by gene dispensability. Additionally, we observed 20 strains resistant to AgNPs (RF > 1.1, p < .05) as shown in Supplementary Table 1. Most AgNP-resistant target genes were related to the following processes: gene expression, cellular component organization, localization, and lipid metabolic process.

### Table 1. List of the 33 AgNP Targets Screened

| GO (Biological Process) | Gene Name/Systematic ID | Gene Description | Sensitivity | E/V⁺ |
|-------------------------|------------------------|------------------|-------------|------|
| Sulfur compound metabolism (p < 8.71E-04) | gcs1 | Glutamate-cysteine ligase | SSS | SS |
| | gcs2 | Glutamate-cysteine ligase regulatory subunit | SS | SS |
| | pcs2 | Phytochelatin synthetase | S | S |
| | hmt2 | Sulfide-quinone oxidoreductase | SS | S |
| | rdl2 | Mitochondrial thiosulfate sulfurtransferase | SS | S |
| | srx1 | Sulfiredoxin | S | S |
| Signal transduction (p < 1.12E-04) | mcs4 | Signal transduction response regulator | S | S |
| | uis4 | MAP kinase kinase | SS | SS |
| | SPCC1827.07c | SPX/EXS domain protein | SS | S |
| One-carbon metabolism by folate | met9 | Methylenetetrahydrofolate reductase | SSS | SSS |
| Cellular component organization | peg1 | CLASP family microtubule-associated protein | SSS | S |
| | sfr1 | RSC complex subunit | SS | S |
| | stg1 | SM22/transgelin-like actin modulating protein | S | S |
| Gene expression | cr4 | Histone H3 lysine methyltransferase | S | S |
| | sup45 | Translation release factor eRF1 | S | E |
| | tfj223 | eIF2B gamma subunit | S | NS |
| | rpl31 | 60S ribosomal protein L31 | S | S |
| | rks2 | Sulfide-quinone oxidoreductase | S | S |
| | lsg1 | Lecl1 complex gamma subunit | S | S |
| | SPAC4G8.09 | Mitochondrial leucine-tRNA ligase | S | S |
| Transport | trk1 | K⁺ transmembrane transporter | S | S |
| | msn5 | Karyopherin | S | NS |
| | ups1 | Dynamin family protein | S | S |
| | SPBC887.12 | P-type ATPase | S | E |
| Unclassified | ebs1 | EST1 family NMD pathway protein | S | S |
| | spo9 | Farnesyl pyrophosphate synthetase | S | S |
| | gid2 | GID complex ubiquitin-protein ligase E3 subunit | S | NS |
| | adn1 | Adhesion defective protein | S | S |
| | pdr1 | NLI interacting factor family phosphatase | S | NS |
| | SPC043.13 | DNAJ domain protein | S | S |
| | SPAC1805.14 | Schizosaccharomyces specific protein | S | S |
| | SPBC1604.12 | Schizosaccharomyces specific phosphoprotein | S | S |
| | SPAC9.02c | Polyamine N-acetyltransferase | S | S |

aGO analysis in terms of the biological process has been analyzed using GO term finder (http://go.princeton.edu/cgi-bin/GOTermFinder).
bGene description is same as indicated in fission yeast PomBase (http://www.pombase.org) and UniProt (http://www.uniprot.org).
cSensitivity are classified as follows: severe (SSS), moderate (SS), mild (S), and not sensitive (NS).
dDispensability data are from the Schizosaccharomyces pombe PomBase (http://www.pombase.org) and confirmed by tetrad analysis in this study. ‘V’ and ‘E’ represent nonessential and essential genes, respectively.
p < 1.12E-4), one-carbon metabolism by folate (met9), cellular component organization (peg1, sfh1, and stg1), gene expression (clr4, sup45, tji223, rpl31, sks2, lsg1, and SPAC4G8.09), transport (trk1, msn5, vps1, and SPBC887.12), and unclassified process (abs1, spo9, gid2, ade3, pdr1, SPCC63.13, SPAC1805.14, SPBC1604.12, and SPAC9.02c). Among the GO terms described, the “sulfur compound metabolism” and “signal transduction” were significantly enriched, suggesting that the pathways related to reducing power production and stress signaling were important for AgNP tolerance. In accordance, other previous reports have described that both stress-activated mitogen-activated protein kinase (MAPK) cascade and redox homeostasis, including biosynthesis of glutathione and other reducing power agents are critical for the detoxification of AgNPs (Rodriguez-Gabriel and Russell, 2005). In particular, the sulfur compound metabolism and one-carbon metabolism by folate were previously reported to be involved in the production of reducing powers such as GSH (Guo et al., 2016) and NADPH (Fan et al., 2014).

For further studies, out of the 33 targets, we narrowed down the number of AgNP targets to 10 under the criterion of sensitivity higher than moderate (ie, SS or SSS), consisting of 7 nonessential and 3 essential genes. According to GO analysis of the 10 targets for the biological process, the 7 nonessential genes fall under sulfur compound metabolism (gcs1, gcs2, hmt2, and rdl2) or stress-activated MAPK kinase cascade/signaling (mcs4, wis4, and SPCC1827.07c), and the 3 essential genes fall under one-carbon metabolism by folate (met9) or (sfh1 and peg1) (Table 2). In accordance with the results, all 7 nonessential genes have been previously reported to be related to metal resistance and/or response to oxidative stress (see references in Table 2). Especially, our previous study also indicated that hmt2 plays a key role for Cd tolerance by the elimination of ROS via CoQ10 in the plasma membrane (Kennedy et al., 2008). However, the 3 essential genes have not been previously described as AgNP targets. Additionally, we analyzed whether the 33 AgNP targets were also sensitive to Ag⁺ (Table 1). The targets for AgNPs and Ag⁺ were observed to be the same except 4 target genes. These results suggest that the cytotoxic effect of AgNPs may be potentially attributed to Ag⁺ released from AgNPs.

**Table 2. List of the Top 10 AgNP Targets**

| GO* (Biological Process) | Gene name/Systematic ID | Sensitivity** (E/V) | Cross-sensitivity | Stress*** Organism | Ref* |
|--------------------------|-------------------------|---------------------|-------------------|--------------------|------|
| **Sulfur compound metabolism** | | | | | |
| Glutathione biosynthetic process | gcs1 (GCLC) | SSS (V) | Cd | Fission yeast | Kennedy et al. |
| | gcs2 (GCLM) | SS (V) | Cd | Fission yeast | Pluskal et al. |
| Cellular sulfide ion homeostasis | hmt2 (SQRDL) | SS (V) | Cd | Fission yeast | Kennedy et al. |
| | rdl2 (TSTD1) | SS (V) | Na₂Se | Budding yeast | Peyroche et al. |
| **Signal transduction** | | | | | |
| Stress-activated MAPK cascade | mcs4 | SS (V) | Cd | Fission yeast | Kennedy et al. |
| | wis4 (MAP3K4) | SS (V) | Cd | Fission yeast | Kennedy et al. |
| **Signaling** | | | | | |
| SPCC1827.07c (XPR1) | SS (V) | Metals | Budding yeast | Yu et al. |
| | | Mn | Budding yeast | Chesi et al. |
| **One-carbon metabolism by folate** | | | | | |
| Tetrahydrofolate interconversion | met9 (MTHFR) | SSS (E) | Vn | Human | Visalli et al. |
| | | | AgNPs | Fission yeast | This study |
| **Cellular component organization** | | | | | |
| Chromatin organization | sfh1 | SS (E) | AgNPs | Fission yeast | This study |
| Cytoskeleton organization | peg1 | SS (E) | AgNPs | Fission yeast | This study |

*GO analysis in terms of the biological process has been analyzed using GO term finder (http://go.princeton.edu/cgi-bin/GOTermFinder).

*Data of human orthologs are from the HomoloGene or Ensembl database (https://www.ncbi.nlm.nih.gov/homologene or http://www.ensembl.org).

*Sensitivity are classified as follows: severe (SSS), moderate (SS), and mild (S).

*Abbreviation of stresses used in the study are as follows: AgNPs (silver nanoparticles), As (Arsenite), Cd (Cadmium), and Vn (Vanadium).

*References represent the previous reports saying that the genes are associated with metal resistance and/or response to oxidative stress in yeasts and/or human.

AgNPs Penetrate Yeast Cells and Release Ag⁺

The above results prompted us to analyze whether AgNPs could penetrate cells and subsequently cause cytotoxicity. TEM images showed AgNPs inside the cells, suggesting that AgNPs could penetrate the yeast cells (Figure 1A). AgNPs were also observed in cellular organelles, such as nucleus and vesicle-like structures, but not at the cell wall. Next, the amount of intracellular Ag and its associated cytotoxicity were compared between the AgNP- and AgNO₃-treated cells (Figure 1B). The amount of Ag in the AgNP-treated cells was approximately 2-fold higher than that in the AgNO₃-treated cells, and it accumulated in a concentration-dependent manner. Based on these results, we concluded that the cytotoxicity of AgNPs was higher than that of AgNO₃ at the indicated concentrations (Figure 1C). These
results also suggest that the cytotoxic effect of AgNPs is attributed to Ag\(^{+}\) released from these NPs.

**Target Pattern of AgNPs Is More Similar to Those of AgNO\(_3\) and H\(_2\)O\(_2\)**

To elucidate the mechanism of action of the AgNP targets, we determined which stress is most similar to AgNPs (Figure 2). To increase the resolution power of cross-sensitivity, the 17 potential target genes associated with relevant GO terms and 35 randomly selected genes, in addition to the 10 target genes, were included. As shown by the hierarchical clustering analysis, the target pattern of AgNPs was more similar to those of AgNO\(_3\) and H\(_2\)O\(_2\) than to those of the metals (Cd or As). However, evidence suggests that the metal stimulants also elicit oxidative stress for inducing cellular toxicity (Valko et al., 2005) (Table 2). The GO terms of target genes were related to biological processes such as stress-activated signaling and stress defense metabolism. Intriguingly, only 3 targets were observed to be sensitive to AgNPs among the 35 randomly selected targets, suggesting that the primary target screening using microarray was useful, despite not accounting for the missing targets. Taken together, AgNP-induced cytotoxicity is potentially attributed to ROS, as reported previously.

**AgNP-Induced Growth Inhibition Is Attributed to an Increased Cellular Level of ROS**

The above results prompted us to evaluate the relationship between AgNP-induced cytotoxicity and their cellular ROS level. Upon measuring the relative cellular level of ROS induced by AgNPs, all the top 10 target strains produced higher levels of ROS (p < .001) compared with the wild-type control (Figure 3A). At the same time, treatment with AgNPs also significantly inhibited the relative growth of these strains (Figure 3B). Furthermore, pretreatment with the antioxidant NAC completely abrogated the AgNP-induced growth inhibition as described previously (Bell and Kramer, 1999; Navarro et al., 2008; Zhang et al., 2011). Taken together, the AgNP-induced cytotoxicity is attributed to ROS generation.

**Three Essential AgNP Target Genes Are Related to Cell Cycle Progression via ROS**

In this study, for the first time the 3 essential target genes, **met9**, **sfh1**, and **peg1**, have been reported to be related to AgNP-induced cytotoxicity. Therefore, the effects of AgNP treatment on cell morphology and cell cycle were observed by microscopy (Figure 4A) and FACS analysis (Figure 4B). Microscopic analysis showed that cell shape was elongated with irregular thick septa, which is a morphological hallmark of G2/M cell cycle arrest. When observed by FACS after HU release, the cell cycle showed a delayed time lag in the transition from 4°C to 8°C (the arrows in Figure 4B) owing to a G2/M cell cycle arrest (Kang et al., 2012). As shown in the lower panels of Figures 4A and 4B, the effect of AgNPs on cell morphology and cell cycle was abrogated by NAC pretreatment. The results suggest that ROS induced by AgNPs caused the observed effects on cell morphology and cell cycle.
The met9 Gene Is Related to NADPH Production

There is an accumulating body of evidence that oxidative stress affects the transcriptional level of many antioxidant defense enzymes in fission yeast (Chung et al., 2004; Lee et al., 2002). In this regard, the intracellular level of reducing power molecules such as GSH regulates the transcriptional level of antioxidant enzymes (Farrugia and Balzan, 2012; Grant, 2001). Therefore, we examined the effects of AgNPs in the transcriptional induction of the first-line antioxidant enzymes such as sod1 and gpx1. As shown in Figure 5A, all 3 heterozygous deletion strains (met9, sfh1, and peg1) showed a similar transcriptional pattern of sod1 and gpx1. Compared with the wild-type control, the 3 common essential targets showed no discrete transcriptional induction of sod1 and gpx1 at 4 h after AgNP treatment. This may be because all the 3 heterozygotes with a single copy of the respective essential gene showed ROS induction resulting in chromatin/cytoskeleton stress (Perrone et al., 2008; Uffenbeck and Krebs, 2006) or reducing power shortage (Fan et al., 2014) resulting in the lack of transcriptional induction of the antioxidant enzymes against in response to AgNP treatment.

As all enzymatic or nonenzymatic antioxidants basically require NADPH as a reducing power (Birben et al., 2012), the above results prompted us to check the cellular level of NADPH in the 3 heterozygous strains in response to AgNP treatment (Figure 5B). Normally, AgNP treatment of the control strain increased the NADP+/NADPH ratio by 2-fold as the oxidative condition consumed NADPH for the supply of antioxidant enzymes. Similarly, after AgNP treatment, the heterozygote with a single copy of the essential gene sfh1 or peg1 displayed a 2- to 4-fold increase in the NADP+/NADPH ratio. Intriguingly, AgNP treatment...
of a heterozygote with a single copy of the essential met9 gene resulted in a shortage of NADPH reducing power, as judged by a 28-fold increase in the NADP\(^+\)/NADPH ratio which is 7 times greater induction than was observed for a heterozygote with a single copy of the essential sfh1 or peg1 gene. The results suggest that met9 plays a key role for NADPH production in the defense against oxidative stress by AgNPs. In accordance with the results, a recent paper has reported that almost half of NADPH production is related to a folate pathway containing the MTHFR (methylenetetrahydrofolate reductase, human ortholog of met9) gene in human cell lines (Fan et al., 2014).

Role of met9 Is Conserved in a Human Cell Line

We next determined whether the role of the 3 essential genes as AgNP targets was conserved in humans. According to the NCBI HomoloGene database, MTHFR gene, which sits at a gateway for both methionine and folate cycles, was revealed as the human ortholog of the fission yeast met9 gene, which is closely associated with both NADPH production and amino acid/DNA synthesis (Fodinger et al., 2000). Unfortunately, we could not find the human ortholog of sfh1 or peg1. Therefore, we focused on the functional conservation of fission yeast met9 in humans.

To mimic the heterozygous effect of fission yeast in humans, we employed siRNA knockdown in HEK293 cells. As shown in Figure 6A, the transcriptional level of the MTHFR knockdown cells showed 34% expression of the control (scrambled), as judged by a quantitative real-time PCR (qRT-PCR) using \(\beta\)-actin as a normalization control. At first, we checked whether knockdown of MTHFR induced ROS in response to AgNP treatment (Figure 6B). As expected, the AgNP treatment of the MTHFR knockdown cells showed a significant increase in the ROS induction by 30% compared with the scrambled cells. Furthermore, the phenomenon was completely abrogated by NAC pretreatment. Next, we further evaluated the effects of AgNP-induced ROS on DNA damage (Figure 6C), cell survival rate (Figure 6D), and cell cycle (Figure 6E). Upon measuring the level of 8-OHdG adducts (Figure 6C) as an established marker of oxidative stress-induced DNA lesions (Cadet et al., 2003), the AgNP treatment in the MTHFR knockdown cells resulted in a significant increase in the level of 8-OHdG adducts by 30% compared with the levels observed in scrambled cells. In accordance with the increased DNA damage, the AgNP treatment of the MTHFR knockdown cells showed a significant 40% decrease in cell viability compared with the viability of scrambled cells.
which was abrogated by NAC pretreatment (Figure 6D). Finally, the AgNP treatment of the MTHFR knockdown cells caused an extra cell cycle delay in the progression through sub-G1 phase (apoptotic cell populations) in addition to the S-phase delay in the mock control cells (Figure 6E). This effect was also abrogated by NAC pretreatment. Taken together, the AgNP treatment of the MTHFR knockdown cells caused oxidative DNA damage and subsequent cell cycle arrest in the sub-G1/S phases, leading to growth inhibition. Taken together, MTHFR plays a key role in cellular defense against ROS induced by AgNPs via NADPH production, implying that the functional role of MTHFR is well conserved from fission yeast to humans.
DISCUSSION

To elucidate the mechanism of AgNP-induced cytotoxicity in a systematic way, so far, studies have been performed using many model organisms. Yeasts (Guo et al., 2016; Okada et al., 2014) also have been employed to screen target genes against a variety of stresses such as ROS, chemicals, or toxic metals. Recently, the gene deletion library of fission yeast was constructed by our group after budding yeast (Kim et al., 2010), which opens a new era for the genome-wide screening of target genes against chemicals by the principle of drug-induced haploinsufficiency (Lum et al., 2004). These studies normally use a haploid gene deletion library only consisting of non-essential genes for the systematic target screening against a stress inducer. In this study, to find AgNP targets on a genome-wide scale, we have applied, for the first time, a fission yeast heterozygous deletion library covering nearly all essential genes as well as nonessential genes. Thus, our trial using a heterozygous deletion library has the advantage of finding novel essential target genes. Although the genome-wide microarray screening system is convenient regarding time, this

Figure 5. AgNP-induced transcriptional changes in antioxidant enzymes and depletion of NADPH in the 3 essential AgNP targets. A, Transcriptional pattern of antioxidant enzymes in the presence of AgNPs in the 3 noble heterozygous AgNP targets (Δmet9/+/met9−, Δsfh1/sfh1−, and Δpeg1/peg1−). Cells were treated with 0.2 μg/ml AgNPs for the indicated time, and their mRNA levels of sod1 and gpx1 were analyzed by q-PCR compared with the wild-type control (n = 3). B, AgNP-induced changes in NADP+/NADPH contents in the 3 noble heterozygous AgNP targets (Δmet9/+, Δsfh1/+, and Δpeg1/pegl−). Cells were treated with 0.2 μg/ml AgNPs for 4 h, and the cellular level of NADP+/NADPH was analyzed by the NADP+/NADPH quantification colorimetric kit compared with the controls (n = 3, *p < .05 and **p < .01, treated vs untreated control cells; #p < .05, ##p < .01, and ###p < .001, treated wild-type vs treated heterozygous deletion cells).
Figure 6. Functional conservation of fission yeast met9 in human cell line HEK293. A, Knockdown of MTHFR (met9 ortholog in human) in HEK293 by siRNA. Cells were transfected with scrambled or MTHFR si-RNA and their transcriptional levels were analyzed by quantitative PCR 72 h after the transfection. Relative expression levels were normalized to the mock transfection (n = 3, *p < .05). B, AgNP-induced reactive oxygen species (ROS) in si-MTHFR HEK293. After transfection with scrambled or MTHFR si-RNA, the cells were treated with or without 0.6 μg/ml AgNPs and their ROS levels were analyzed by FACS using H2DCFDA as a fluorescent dye. Next, the cells were pretreated with 2 mM N-acetylcysteine (NAC) for 3 h prior to the AgNP treatment and their ROS levels were compared with ROS levels in unpretreated cells (n = 3, *p < .05 and **p < .01, AgNP-treated vs untreated cells; ***p < .001, NAC-treated vs untreated cells). C, AgNP-induced DNA damage via ROS in si-MTHFR HEK293. After transfection with scrambled or MTHFR si-RNA, the cells were treated with or without 0.6 μg/ml AgNPs and their 8-OHdG levels were measured as a DNA damage marker using OxiSelect Oxidative DNA damage ELISA kit. Next, the cells were pretreated with 2 mM NAC for 3 h prior to the AgNP treatment and their DNA damage was compared with DNA damage in unpretreated cells (n = 3, *p < .05 and **p < .01, AgNP-treated vs untreated cells; ***p < .001, NAC-treated vs untreated cells). D, AgNP-induced growth inhibition via ROS in si-MTHFR HEK293. After transfection with scrambled or MTHFR si-RNA, the cells were treated with or without 0.6 μg/ml AgNPs and their survival rates were measured by an MTT assay. Next, the cells were pretreated with 2 mM NAC for 3 h prior to the AgNP treatment and their growth rate was compared with the growth rate of unpretreated cells (n = 3, **p < .01 and ***p < .001, AgNP-treated vs untreated cells; #p < .05 and ##p < .01, NAC-treated vs untreated cells). (E) AgNP-induced cell cycle arrest via ROS in si-MTHFR HEK293. After transfection with scrambled or MTHFR si-RNA, the cells were treated with or without 0.6 μg/ml AgNPs and their fraction at each cell cycle phase (sub-G1, G1, S, or G2/M) was measured by FACS. Next, the cells were pretreated with 2 mM NAC for 3 h prior to the AgNP treatment and their fraction at each cell cycle phase was compared with cell cycle patterns in unpretreated cells (n = 3, *p < .05 and **p < .01, AgNP-treated vs untreated cells; #p < .05, NAC-treated vs untreated cells).
system needs to be validated by a one-by-one assay for confirmation. For example, the primary genome-wide screening has shown 11 false-positive targets, as judged by the spotting assay confirming the 33 targets (Table 1) out of the 44 primary genes screened. Furthermore, primary screening also missed many potential AgNP targets, including the 17 additional target genes associated with GO terms similar to those of the top 10 sensitive target genes, which were revealed by the cross-sensitivity hierarchical clustering analysis (Figure 2). However, the genome-wide screening system remains a useful tool for primary screening because the 35 randomly added genes (except 3 genes) were proven to be insensitive to AgNPs. One possible explanation is that the microarray analysis is not yet perfect due to an erroneous mismatch between the array chip and the bar code probe due to mutations. To improve the genome-wide screening method, we are in the progress of utilizing next-generation sequencing technology. This cutting-edge technology would better enable screenings by a direct counting of signals rather than the indirect hybridization of a microarray.

An accumulating body of evidence has shown that metal nanoparticles are absorbed into cells through a transport system called the “Trojan horse-type mechanism” (Limbach et al., 2007). The intracellular AgNPs that interact with cellular components, such as proteins and thiol, become oxidized (Henglein, 1998; Lok et al., 2007) and release Ag⁺ (Liu and Hurt, 2010; Limbach et al., 2007). In accordance with the previous reports, we also showed that AgNPs could penetrate yeast cells and release Ag⁺, thereby resulting in cytotoxicity (Figure 1). Since all engineered nanoparticles have their own unique physical, chemical, and biological properties, they act as auxiliary factors for inducing cytotoxicity by themselves. However, AgNP-induced cytotoxicity is basically attributed to Ag⁺ release via the generation of ROS, resulting in the depletion of reductant potential levels, reduction of mitochondrial membrane potential, and subsequently damage of DNA and proteins (Kang et al., 2012). Accordingly, AgNP targets should present a variety of genes related to reducing ROS levels. It was not surprising that the stress most similar to AgNPs was that induced by Ag⁺, followed by that induced by the typical ROS inducer H₂O₂ (Figure 2). Like H₂O₂, metals such as As and Cd also have been previously described to induce ROS in systems from yeast strains to cell lines (Guo et al., 2016; Kennedy et al., 2008). In this regard, the 33 targets screened contain 9 genes related to antioxidant function, including 6 and 3 genes in the GO term of sulfur compound metabolism and signal transduction, respectively (Table 2). In the category of sulfur compound metabolism, our screening results revealed targets such as gcs1 and gcs2 related to the synthesis of GSH, which requires NADPH for the regeneration of its oxidized form GSSG (Birben et al., 2012). In addition to the important reducing power molecule GSH (Pluskal et al., 2016), the sulfide-quinone system is well known as an ROS defense system in a variety of organisms (Hildebrandt and Grieshaber, 2008) via the reduction of the antioxidant CoQ10 in the lipid fraction of the mitochondrial membrane (Bentinger et al., 2007). The mitochondrial sulfide-quinone oxidoreductase, hmt2, has been screened, which we have previously reported as a defense enzyme against Cd in fission yeast (Kennedy et al., 2008). Indeed, the human ortholog of hmt2, the SQORD (sulfide-quinone oxidoreductase) gene, has been described to detoxify against hydrogen sulfide (Hourihan et al., 2013). The other mitochondrial thiocyste sulfurdtransferase rd21 also has been screened (Hildebrandt and Grieshaber, 2008; Melideo et al., 2014). Since the deletion of rd21 has been described to become sensitive to a sodium selenide exposure in budding yeast (Feyroche et al., 2012), it would be related to AgNP-induced ROS. Also, phytochelatin synthase (pcs2) has been screened, which has been reported to be critical for Cd detoxification by producing the low-molecular-weight sulfur-containing peptide phytochelatin in plants (Cobbett, 2000). Oxidized phytochelatin gets reduced by the reducing power of GSH (Guo et al., 2016). Another important GO term for the detoxification of AgNPs should be the signaling pathway to respond to oxidative stress. The msc4 and wiso genes screened have been previously reported to be key factors of the stress-activated protein kinase (SAPK) signaling cascade as an upstream regulator of Sty1 (also known as Spc1), which is important for the detoxification of oxidative stress (Rodriguez-Gabriel and Russell, 2005) and Cd (Kennedy et al., 2008). For example, the PI3K and p38 MAPK signaling cascades have been described to engage in AgNP-induced cytotoxicity in human cells (Eom and Choi, 2010). However, microarray screening has missed the transcription factors in the p38 MAPK signaling cascade, including Pap1 and Atf1 (Table 1). They play a key role in concert for the transcriptional regulation of antioxidant genes such as sod1 and gpx1 against ROS-inducing stresses (Lee et al., 2002; Yamada et al., 1999), as shown in Figure 5. Intriguingly, we have found the SPCC1827.07c gene as an AgNP target, whose function is not yet clear in the signaling GO term. Since the SYG1 mutant in the budding yeast, the ortholog of fission yeast SPCC1827.07c has been reported to increase sulfur (1⁺) accumulation with abnormal mitochondrial morphology against a variety of ion stresses (Yu et al., 2012), such as excess manganese (Chesi et al., 2012), the function of the unknown gene is likely to be related to a signaling pathway involved in ROS stresses.

Of particular interest are the 3 essential target genes related to the GO term of one-carbon metabolism by folate (met9) or cellular component organization (peg1 and sfh1). The sfh1 gene encoding the chromatin structure remodeling (RSC) complex subunit would be required for the effective transcription regulation of a series of defense genes against AgNPs (Uffenbeck and Krebs, 2006). The peg1 gene encoding the CLASP (cytoplasmic linker associated protein) family microtubule-associated protein would be critical for maintaining microtubule structure, as AgNPs have been reported to disrupt cytoskeleton components (Xu et al., 2013). The met9 gene encoding MTHFR would be necessary for NADPH production, which supplies a variety of antioxidant enzymes with reducing power. Notably, the folate pathway has been suggested to be a pathway for producing cytosolic NADPH in addition to the canonical pentose-phosphate pathway (Fan et al., 2014).

Next, we aimed to find the corresponding human orthologs of the 3 novel essential targets and determine their functional conservation in a human cell line. The met9 gene has been well conserved throughout evolution from bacteria to humans (Naula et al., 2002; Yamada et al., 2001), as all living organisms use NADPH as the basic reducing power molecule against ROS. Indeed, MTHFR has been described to be involved in many diseases when mutated (Frost et al., 1995; Ma et al., 1996; van der Put et al., 2001). For example, MTHFR deficiency causes a decreased level of folate, resulting in excess oxidative stress and increased plasma levels of homocysteine, called hyperhomocysteinemia (Ma et al., 1996). A genetic variation in MTHFR (C677T) leads to vascular disease and neural tube defects via hyperhomocysteinemia (Frost et al., 1995; van der Put et al., 2001) as well as mitochondrial dysfunction (Visalli et al., 2015). Human orthologs for the essential genes sfh1 and peg1 could not
be found. Likely, the cellular component organization is different between lower single-cell yeasts and higher multicellular organisms.

As far as we know, this is the first study claiming that the met9 essential gene is critical for the cellular defense of the AgNP-mediated toxicity in fission yeast via the regulation of NADPH and that its relevance is conserved in a human cell line. Also, the results support the recent discovery as a proof of concept saying that the folate cycle plays a key role for NADPH production along with the canonical oxidative pentose pathway (Fan et al., 2014). Since MTHFR may be a possible drug target for ROS-related diseases, further study is necessary for elucidation of the mechanism by which MTHFR plays a key role against ROS in human cells.

**SUPPLEMENTARY DATA**

Supplementary data are available at Toxicological Sciences online.

**FUNDING**

National Research Foundation (NRF) grants funded by the Korean Government, Ministry of Science and ICT (NRF-2012M3A9D1054667 and NRF-2017M3A9B5060880); National Research Council of Science and Technology (grant no. DRC-15-01-KRICT); Chungnam National University.

**AUTHORS’ CONTRIBUTIONS**

D.U.K. and K.L.H. conceived the project; A.R.L., S.J.L., M.N., S.L., J.C., and H.J.L. performed experiments and data analysis; M.L. performed bioinformatics; A.R.L., S.J.L., M.L., M.N., and K.L.H. wrote the paper.

**ACKNOWLEDGMENTS**

D.U.K. and S.J.L. were supported by KRRBB and Chonbuk National University, respectively. The remaining authors have no conflicts of interest to declare.

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