Nrf-2-driven long noncoding RNA ODRUL contributes to modulating silver nanoparticle-induced effects on erythroid cells

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ABSTRACT

The biosafety and biological effects of silver nanoparticles (AgNPs) on human health attract increasing concern. Although considerable studies have been performed to reveal the molecular mechanisms responsible for AgNP-induced effects, the current understanding mainly focuses on oxidative stress-associated signaling pathways activated by Ag particles and/or Ag ions. However, the molecular bases underlying the activation of these stress signaling pathways have not been thoroughly elucidated yet. In the current study, we aimed to shed light on the molecular bases of AgNP-induced effects on erythroid cells from the perspective of long noncoding RNAs. We identified a long-noncoding RNA molecule, ODRUL, which was substantially enhanced in K562 erythroid cells responding to AgNPs, coupled to accelerated cell death. Further, we uncovered oxidative stress-driven Nrf2 transcriptionally promoted ODRUL expression in K562 cells. Downstream of Nrf2-ODRUL activation by AgNPs, ODRUL was recognized to interact with PI4K α protein to modulate the activities of its targets AKT and JNK. As a result, the Bcl-2 level was negatively regulated by PI4K-AKT/JNK signaling under AgNP-induced stress, leading to enhanced cell death. Together, our findings unearthed that Nrf2-mediated lncRNA ODRUL was indispensable for AgNP-induced toxicity in erythroid cells through regulation of AKT/JNK-Bcl-2 signaling dependent on a physical interaction with PI4K α . Thus, this study would open a new path to depict the molecular bases of AgNP-induced effects on erythroid cells.

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1. Introduction

Increasing applications and usage of nanomaterials and

nanoproducts in a wide spectrum of fields would increase the potential risk to human health [1–3]. Among the nanomaterials, silver nanoparticles (AgNPs) represent the largest family of nanomaterials with the greatest applications thus far [4,5]. The toxicity and adverse impacts of AgNPs on human health have been greatly concerned. Numerous studies have documented that AgNPs incurred cytotoxicity to various cells by producing reactive oxygen species (ROS) and oxidative stress stemming from either Ag particles or Ag ions, leading to plasma membrane impairment, mitochondrial dysfunction, chromosomal abnormalities, DNA damage, and so on [6–8]. However, the detailed molecular bases underlying these AgNP-induced toxic events remain elusive. For example, the initiation of molecular signaling to trigger cell death has not been clearly elucidated.

Abbreviations: AKT, protein kinase B (PKB); Bcl-2, B-cell lymphoma-2; CAT, Catalase; ERK, extracellular regulated protein kinases; HO-1, hemeoxygenase; JNK, c-Jun N-terminal kinase; lncRNA, long non-coding RNA; Nrf2, nuclear factor erythroid 2-related factor 2; ODRUL, osteosarcoma doxorubicin-resistance related up-regulated lncRNA; PI4K α , phosphatidylinositol 4-kinase alpha; SOD, superoxide dismutase; STAT5, signal transducer and activator of transcription 5.

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Of non-coding RNAs, long noncoding RNAs (lncRNAs) are novel transcripts with the length greater than 200 nucleotides, but without a protein-coding function [9–11]. Recent studies have demonstrated that lncRNAs are in fact crucial regulators for gene expression and protein functions for fine-tuned cellular homeostasis. lncRNAs contribute to the regulation of cell survival, differentiation, metabolism, DNA repair and other cellular processes under normal physiological settings and in response to diverse endogenous and exotic stresses [12–14]. lncRNAs modulate the expression of a large number of genes at the post-transcriptional level through diverse mechanisms, including interaction with functional mRNAs to alter mRNA stability and adsorption of microRNAs (miRNAs) like a sponge to regulate the latter's actions [10,15,16]. Moreover, lncRNAs are also verified to interact with proteins, such as polycomb repressive complex 2 (PRC2) components, in order to orchestrate protein stability and functions [17,18]. Down-regulated expression of lncRNAs has been found to cause the occurrence and progression of a large array of disorders including cancers [13,19]. Additionally, emerging evidence suggests that lncRNAs are also involved in the regulation of nanomaterial-induced toxicity [20,21]. For example, lncRNA XLOC 010623 and linc-37 were dysregulated in response to self-assembled tetrahedral DNA nanostructures (TDNs) and graphene oxide (GO), respectively [20,21]. These findings implied a potentially important role of lncRNAs in nanomaterial-induced biological effects; however, the molecular mechanisms are still unclear. To the best of our knowledge, there are no studies to investigate the toxicological signaling regulated by lncRNAs upon AgNPs.

Upon various exposure routes, nanomaterials are transported to distant organs with the aid of red blood cells (RBCs), and finally accumulate in liver, spleen, bone marrow and other organs [22,23]. Of these distant sites, bone marrow and spleen (only under stresses) are responsible for hematopoiesis including RBC formation [24]. Thus, RBCs and their progenitors are severely subjected to nanomaterial-induced stress during transportation, circulation and accumulation in erythropoietic sites. We recently demonstrated that AgNPs repressed RNA transcriptional activity through a direct reciprocal interaction with RNA polymerase in erythroid progenitor cells [25]. Furthermore, we reported that AgNPs inhibited globin transcription through reducing the methylation status of histone in erythroid progenitor cells, highlighting an important role of epigenetic mechanisms in nanotoxicity [26]. In the current study, we aimed to uncover the lncRNA-initiated signaling pathways in erythroid cells after AgNP exposure. Our combined results revealed that lncRNA ODRUL was a novel coordinator for AgNP-induced cytotoxicity in erythroid cells, which linked the Nrf2 stress signaling with the AKT/JNK-Bcl-2 survival signaling through its partner PI4K α .

2. Materials and methods

2.1. Nanoparticle characterization

AgNPs coated with PVP were purchased from Cold stones company (Suzhou, China). AgNPs were characterized by transmission electron microscopy (TEM, Hitachi H-7500, Japan). Hydrodynamic diameter and zeta-potential were assayed using dynamic light scattering (DLS) (Malvern, U.K).

2.2. Cell culture

Human chronic myeloid leukemia cell line K562 was obtained from the Cell Resource Center of the Institute of Basic Medical Sciences (CAMS, China). Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, CA, USA) supplemented with 10% bovine

calf serum (Hyclone), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Hyclone) at 37 °C with 5% CO₂.

2.3. Cytotoxicity assessment

Cell viability was determined by the Alamar Blue assay. Cells were seeded in 96-well plates and then treated with AgNPs at various concentrations for 24 h. Then, resazurin (Sigma) was added into culture media at a final concentration of 10% (v/v), and cells were cultured for another 2 h. After incubation, fluorescence intensity was detected at an excitation wavelength of 530 nm with an emission wavelength of 590 nm.

2.4. LDH release assay

Cell membrane permeability was evaluated with a commercial kit of lactate dehydrogenase (LDH) release. After treatment with AgNPs for 24 h, culture medium was collected and was then centrifuged at 12,000 g, followed by the analysis of LDH leakage according to the manufacture's instruction (Jiancheng Institute of Biotechnology, Nanjing, China). Levels of LDH were reflected by the changes of NAD⁺ reduction at 490 nm.

2.5. Localization of silver nanoparticles inside cells by TEM

K562 cells were treated with AgNP-S, AgNP-M and AgNP-L at 4, 40 and 200 μ g/mL for 24 h, respectively, and then washed with phosphate-buffered saline (PBS) 3 times. The harvest cells were fixed with 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide. After dehydrated in graded ethanol, cells were cleaned with propylene oxide, treated with a 1:1 mixture of propylene oxide and Spurr's low viscosity resin, and embedded in epoxy resin. Thereafter, the blocks were sectioned and the ultrathin sections (70 nm) were stained with 1% lead citrate and 0.5% uranyl acetate, followed by examination under a high-resolution transmission electron microscope (JEOL JEM 2010F).

2.6. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of intracellular Ag mass

After treatment, K562 cells were washed with PBS and then collected. The harvested cells were digested with mixed acid using a microwave on a MARS machine (CEM Corp., Matthews, NC). Digested samples were finally subjected to ICP-MS assessment using an Agilent 7500 machine (Agilent, Tokyo, Japan), as previously described [26,27].

2.7. ShRNA knock-down assay

Full length ODRUL was amplified from human cDNA by PCR and then cloned into the pGEM-T vector. Human ODRUL, PI4K α and Nrf2 shRNA sequences were cloned into shRNA vector PLKO.1, respectively, according to manufacturer's instructions. The primer sequences are shown in [Supplementary Table 1](#). Plasmid transfection was performed, as described in our recent study [28].

2.8. Cell death determination by flow cytometry

Cells were collected and washed twice with PBS after treatment with AgNPs for 24 h. Then, cells were stained with 5 μ L PI solution (20 μ g/mL) for 30 min following the instructions from the manufacturer (BD Biosciences, USA). Dead cells were analyzed by flow cytometry as described previously [28]. To figure out the roles of targeted proteins in cell death, K562 cells were first pre-incubated with specific inhibitors for 1 h before AgNP treatment. The

inhibitors were LY 294002 (Sigma-Aldrich, USA), SP600125 (MedChem express, USA), PD98059 (MedChem express, USA) and Stat5 inhibitor (MedChem express, USA).

2.9. ROS determination

The generation of intracellular ROS was assessed by fluorescent staining assay. K562 cells were cultured into 96-well plates and exposed to AgNPs at 20 and 40 $\mu\text{g/mL}$ for 6 h. Dichlorofluorescein diacetate (DCF-DA, Sigma) was then added at a final concentration of 10 μM . After incubation for 30 min, cells were subjected to fluorescence analysis at 525 nm with excitation at 488 nm.

2.10. SOD and CAT activities measurement

Cells after treatment were harvested and then lysed with RIPA buffer for 1 h at 4 °C. After centrifugation, supernatants were separated for further analysis. The measurements of SOD and CAT activities were performed using assay kits according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China).

2.11. RNA-protein pull-down assay and protein identification

The pGEMT-ODRUL was linearized with restriction enzyme Spe1 (New England Biolabs), and then used to synthesize RNA *in vitro* by T7 polymerase according to manufacturer's instruction (Invitrogen, USA). RNA-pull down assay was performed using a commercial RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, USA). Thereafter, RNA-pulled down components were subjected to SDS-PAGE to separate precipitated proteins. Selected protein bands in the gel were identified by mass spectrometry analysis at Beijing Protein Institute. Protein concentrations in the pellets were assessed by Western blot analysis.

2.12. Real-time qRT-PCR analysis

Total RNAs from cells were extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). Two micrograms of total RNAs from different samples were reverse-transcribed into cDNA with M-MLV reverse transcriptase (Promega). Expression levels of various lncRNAs and different genes were determined using a standard SYBR Green System. GAPDH was used as an internal control. The primer sequences are shown in [Supplementary Table 1](#).

2.13. Western blot analysis

Western blot analysis was performed as described previously [26,28]. Cells were washed with cold PBS and then lysed in RIPA lysis buffer supplemented with PMSF and protease inhibitor cocktail. Equal amounts of proteins for each lysate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The primary antibodies were against Nrf2 (Proteintech, China), HO-1 (Proteintech, China), PI4K α (Proteintech, China), Bcl-2 (Proteintech, China), p-AKT (Cell Signaling Technology, USA), p-JNK (Cell Signaling Technology, USA), p-ERK (Cell Signaling Technology, USA), p-Stat5 (Cell Signaling Technology, USA), and GAPDH (Santa Cruz Biotechnology, USA).

2.14. Statistical analysis

All data are expressed as means $\pm$ SD, and statistical analysis was determined by independent *t*-test or One-Way ANOVA test. A

p-value less than 0.05 (*) or 0.001 (#) indicated significant differences.

3. Results

3.1. Characterization of AgNPs

We here employed PVP-coated AgNPs with 3 different sizes, termed AgNP-S, AgNP-M and AgNP-L. Prior to cellular treatment, the physicochemical properties of AgNPs were thoroughly characterized. The morphology and size distribution of PVP-coated AgNPs were assessed by TEM. All AgNPs appeared as sphere-like shape without agglomerations (Fig. 1A), and their average diameters calculated from the TEM images were approximately 11 nm, 27 nm and 95 nm, respectively (Fig. 1B). The hydrodynamic diameters were 16.9, 66.5 and 149.9 nm for AgNP-S, AgNP-M and AgNP-L, respectively, suggesting that the formation of protein corona on AgNP surface [25,26]. The zeta-potential analysis showed that all AgNPs were similarly negatively charged (Fig. 1B), in analogy to previous studies [25,26].

3.2. Determination of cytotoxicity of K562 erythroid cells in response to AgNPs

To screen the concentrations at which AgNPs would not cause significant cell death, Alamar Blue assay was performed to determine the cell viability of K562 erythroid cells at various concentrations of AgNPs. K562 cells were initially derived from a patient with chronic myelogenous leukemia, and these cells are pluripotent in differentiating into mature erythroid cells, megakaryocytes and monocytes, dependent on differential induction methods [29,30]. Thus, K562 cells have been widely used as an *in vitro* model for the study of normal hematopoiesis, especially for erythroid differentiation, at transcriptional, posttranscriptional, translational and posttranslational levels [31–34]. Moreover, K562 cells are recognized as a desirable model to study the molecular bases underlying stressed erythropoiesis in response to various exogenous stimuli, such as arsenic, UV and anthracyclines [35–37]. Meanwhile, reticulocytes (red blood cells) are often used for the investigation on the translational control of cellular signaling [38]. To this end, K562 cells would be an ideal platform to interrogate genetic and epigenetic regulations on cellular signaling under stressed erythropoiesis [39–41].

As shown in Fig. 2A–C, AgNP-S was more toxic to K562 cells than AgNP-M and AgNP-L, as their calculated IC₅₀ were about 8, 100 and 500 $\mu\text{g/mL}$, respectively. To avoid dramatic cytotoxicity that would mess up mechanistic studies, we thus endeavored to use relatively low concentrations in the following cell treatments: 2 and 4 $\mu\text{g/mL}$ for AgNP-S, 20 and 40 $\mu\text{g/mL}$ for AgNP-M and 100 and 200 $\mu\text{g/mL}$ for AgNP-L, respectively. Corroborated with the cytotoxicity data, AgNPs at lower concentrations did not massively increase the extracellular LDH release, compared to untreated control (Fig. 2D).

Our previous study suggested that AgNPs could translocate into erythroid cells through the “Trojan Horse effect” [25]. The TEM images revealed that AgNPs could localize inside k562 cells, mostly in phagosomes (Fig. 2E), similar to previous findings [25,42]. In addition, we quantified the Ag contents in K562 cells upon AgNP treatment for 24 h through ICP-MS analysis, and the results are shown in Fig. 2F. A greater Ag accumulation was found in cells upon exposure to AgNP-M and AgNP-L, especially for the latter, compared to AgNP-S (Fig. 2F, *P* < 0.001). The elevated cellular uptake of Ag particles by cells upon AgNPs of larger sizes may be attributed to higher concentrations used for them, although they induced comparable toxicity to K562 cells. These results

Fig. 1. Characterization of AgNPs. (A) Representative TEM images of AgNPs used in the current study. The scale bar was 100 nm. The size distribution was shown in the lower panel. (B) DLS data and zeta potentials of AgNPs. AgNPs were first added into cell culture medium with 10% FBS for 24 h, and then isolated and re-dispersed in ultrapure water for the analysis of hydrodynamic diameters and zeta potentials ($n = 3$).

demonstrated that AgNPs readily intruded into K562 cells, giving rise to potential cellular damage.

3.3. lncRNA ODRUL regulates AgNP-induced cytotoxicity

To investigate whether lncRNAs contribute to the regulation of AgNP-induced cytotoxicity of erythroid cells, we deliberately looked into the expression of lncRNAs in K562 cells in response to AgNPs. First, 27 lncRNAs were selected based on their reported functions with relevance to erythroid cell survival or differentiation. As shown in Fig. 3A, a few lncRNAs were significantly elevated or repressed in K562 cells treated with AgNP-M at 40 $\mu\text{g/mL}$ for 24 h to a differential extent, including ODRUL, PTENP, lncRNA-ROR, PVTI and H19 ($P < 0.05$). Among these altered lncRNAs, the greatest change was observed for ODRUL (osteosarcoma doxorubicin-resistance related up-regulated lncRNA), with nearly a 10-fold induction in cells upon AgNP-M exposure ($P < 0.001$). To substantiate this observation, AgNP-M was subjected to cellular treatment at different time points. As shown in Fig. 3B, the level of ODRUL was significantly increased in K562 cells upon AgNP-M treatment for 3 h, 12 h and 24 h ($P < 0.05$), displaying that AgNP-M quickly initiated ODRUL induction. To look into size-

dependency, K562 cells were exposed to AgNP-S and AgNP-L separately. As shown in Fig. 3C and D, a great induction of ODRUL expression was observed in K562 cells responding to both AgNP-S and AgNP-L ($P < 0.001$), and a dose-dependent increase was found in AgNP-L-treated cells, relative to untreated cells ($P < 0.05$). These data indicated that all AgNPs at a comparable toxicity level induced ODRUL expression, but without showing significant size dependence. Previous studies have demonstrated the crucial role of particle size in determining the AgNP-induced cytotoxicity [7,43]. Therefore, more efforts are needed to address the exact dependence of ODRUL expression on size in response to AgNPs. Since all 3 sized AgNPs could robustly promote the increase of ODRUL and they manifested differential IC_{50} values, we here used AgNP-M (abbreviated as AgNPs) as the representative material for detailed mechanistic studies in the following experiments.

To validate the hypothesis that lncRNA ODRUL plays a crucial role in dictating AgNP-induced toxicity, stable ODRUL knockdown transfectants (here named ODRUL-low cells) were obtained through the lentiviral shRNA approach. As shown in Fig. 4A, the endogenous ODRUL level was diminished by 75% in ODRUL-low cells, relative to that in scrambled control cells ($P < 0.05$). Accordingly, the ODRUL levels were much lower in ODRUL-low cells upon

Fig. 2. Cytotoxicity evaluation upon AgNP treatment in K562 cells. Cell viability was assessed with the Alamar Blue assay upon exposure to AgNP-S (A), AgNP-M (B) and AgNP-L (C) for 24 h at indicated concentrations ($n = 6$). (D) Relative extracellular LDH levels in response to AgNP treatment at various concentrations for 24 h. The changes of NAD^+ reduction reflecting extracellular LDH concentration were detected at 490 nm ($n = 4$). (E) TEM images of cells treated with 4 $\mu\text{g/mL}$ AgNP-S, 40 $\mu\text{g/mL}$ AgNP-M and 200 $\mu\text{g/mL}$ AgNP-L for 24 h, respectively. Localizations of Ag nanoparticles inside phagosomes (red arrows) were denoted in the enlarged images (lower panel) from the dash line squares. (F) The intracellular Ag contents in K562 cells treated with 4 $\mu\text{g/mL}$ AgNP-S, 40 $\mu\text{g/mL}$ AgNP-M and 200 $\mu\text{g/mL}$ AgNP-L for 24 h, respectively. The cellular Ag contents were determined by ICP-MS assay ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Elevated levels of lncRNA ODRUL in response to AgNPs. (A) Expression changes of lncRNAs involved in cell survival or differentiation in K562 cells exposed to 40 µg/mL AgNP-M for 24 h, as characterized by qRT-PCR analysis ($n = 3$). (B) Relative levels of lncRNA ODRUL in K562 cells after treatment with 40 µg/mL AgNP-M for 3, 12 and 24 h ($n = 3$). ODRUL mRNA levels upon exposure to AgNP-S (C) and AgNP-L (D) at indicated concentrations for 24 h, as determined by qRT-PCR assay ($n = 3$).

AgNP treatment, with approximately 57%–78% reduction in comparison to AgNP-treated scrambled control cells (Fig. 4A, $P < 0.05$). We then evaluated the cytotoxicity of AgNPs to ODRUL-low cells. As shown in Fig. 4B, the reduction of cell viability was greatly reversed in ODRUL-low cells upon AgNP treatment at various concentrations ($P < 0.05$). The viability of ODRUL-low cells upon AgNPs was comparable to that of untreated control, in contrast to 27–42% reduction of cell viability in scrambled control cells. This finding suggested that AgNPs did not induce sufficient toxicity to cells without a comparable level of ODRUL. In support of this finding, cell death examination also revealed similar reversion of PI-positive proportion in ODRUL-low cells in comparison to scrambled control cells upon AgNP treatments (Fig. 4C, $P < 0.05$). The proportion of PI-positive cells in ODRUL-low cells was reduced by approximately 50%, compared to scrambled control cells after 24 and 48 h-treatment of AgNPs, and a bigger difference was found at 48 h than that at 24 h (Fig. 4C, $P < 0.05$). Together, these findings demonstrated that AgNPs elicited toxicity to K562 cells, at least partially, through inducing lncRNA ODRUL expression.

3.4. Relatively specific response of ODRUL induction by AgNPs

To look into the possible induction of ODRUL by other nanoparticles, we detected the ODRUL expression levels in K562 erythroid cells upon treatment with ZnO and TiO₂ nanoparticles (here named nZnO and nTiO₂). First, the cell viability and ROS production were determined in cells treated with nZnO and nTiO₂. Our results showed that nZnO incurred more reduction of cell

viability, compared to nTiO₂ at the same mass concentrations (Supplementary Figs. 1A and B, $P < 0.05$), consistent with previous reports [44,45]. Therefore, we chose the concentrations that elicited a similar degree of toxicity to K562 cells for nZnO and nTiO₂ for further study. At these defined concentrations, both nZnO and nTiO₂ greatly induced the production of ROS by approximately 2 fold (Supplementary Fig. 1C, $P < 0.001$). Meanwhile, nZnO and nTiO₂ exerted no effect on the levels of ODRUL (Supplementary Fig. 1D, $P > 0.05$). Moreover, Ag ions are believed to contribute to ROS generation and the overall cytotoxicity induced by AgNPs [46,47]. To determine the possible stimulating effects of Ag ions on ODRUL expression, we also looked at ODRUL expression in K562 cells treated with Ag ions. Since the proportion of Ag ions dissolved from AgNPs was small [25], Ag ions at low concentrations were used to treat K562 cells. As shown in Supplementary Fig. 2, Ag ions induced lncRNA ODRUL expression ($P < 0.05$), suggesting that dissolved Ag ions also contributed to ODRUL induction under AgNP-stimulated oxidative stress. Collectively, these findings uncovered a relatively specific response of ODRUL induction upon AgNPs relative to other nanoparticles, although more experiments are needed to elucidate the underlying molecular mechanisms, such as implications of Ag ions and protein coronas.

3.5. ODRUL expression is regulated by Nrf2 upon AgNP-induced oxidative stress

Thereafter, we looked into the molecular mechanisms responsible for ODRUL induction in K562 cells upon AgNP treatment. Since

Fig. 4. ODRUL promotes erythroid cell death upon AgNP exposure. The endogenous ODRUL was stably knocked down in K562 cells, and then ODRUL levels (A) and cell viability (B) were detected upon AgNP exposure. (C) The percentage of cell death for scrambled-shRNA control cells and ODRUL-low cells responding to AgNP treatment at 80 µg/mL for 24 and 48 h. Cell death was determined through flow cytometry analysis with PI staining ($n = 3$).

oxidative stress has been demonstrated to be the most important mechanism underlying AgNP-associated cytotoxicity [48,49], we assayed the generation of ROS in K562 cells responding to AgNP exposure. As shown in Fig. 5A, a 30–40% increase of intracellular ROS was found in K562 cells upon AgNP treatment at 20 and 40 µg/mL ($P < 0.05$). Consistent with this result, the activity of superoxide dismutase (SOD) was largely elevated, while the activity of catalase (CAT) was repressed in treated cells (Supplementary Figs. 3A and B, $P < 0.05$). These data collectively demonstrated that AgNPs induced significant cellular oxidative stress. In view of the results of cell viability (Figs. 2B and 4B), we hypothesized that AgNP-induced oxidative stress promoted the ODRUL induction. Herein the effect of *N*-acetyl-L-cysteine (NAC), a potent ROS scavenger, on ODRUL induction was evaluated. As shown in Fig. 5B, the induction of ODRUL expression by AgNPs was greatly reversed by more than 50% with NAC pre-treatment ($P < 0.05$).

Nrf2 is a master transcription factor in regulating the expression of oxidative stress-related genes against stress-associated toxicity [50]. We then assessed the concentration of Nrf2 in K562 cells upon AgNP treatment at different concentrations. As shown in Fig. 5C, a pronounced elevation of Nrf2 concentration was found in cells in a dose-dependent manner. As a downstream target of Nrf2, the level of heme oxygenase-1 (HO-1) was similarly enhanced in the presence of AgNPs (Fig. 5C). In addition, Nrf2-HO-1 pathway was activated over time after exposure to 40 µg/mL AgNPs (Supplementary Fig. 4). To scrutinize the role of Nrf2 in ODRUL-determined signaling pathway, we further stably knocked down endogenous Nrf2 expression (here termed Nrf2-low cells) (Supplementary Fig. 5

and 5D). Nrf2 concentration was remarkably diminished in Nrf2-low cells in comparison with scrambled control cells upon AgNP treatment (Fig. 5D). ODRUL induction by AgNPs was also inhibited to the baseline level in Nrf2-low cells (Fig. 5E, $P < 0.05$), and cell death was greatly diminished after 24 and 48 h-treatment of AgNPs, especially for 48 h-treatment (Fig. 5F, $P < 0.05$). These results indicated that Nrf2 might directly regulate the expression of ODRUL, similar to other lncRNAs such as ROR and SCAL1 [51,52].

3.6. Phosphatidylinositol (PI) 4-kinase alpha (PI4Kα) is a partner of ODRUL in bridging the signaling pathways responsible for AgNP-induced toxicity

Previous studies have revealed that lncRNAs interact with partner proteins (e.g., chromatin-modifying enzymes and transcriptional regulators) to fine-tune gene expressions [18,53]. We thus surveyed the partners that were necessary for the signaling transduction in AgNP-induced cytotoxicity. As shown in Fig. 6A, the biotin-labeled full length ODRUL RNA probes pulled down much more proteins than biotin-nonsense RNA probes in erythroid cell lysates. Mass spectrometry analysis identified these proteins, and, among them, PI4Kα was recognized (Supplementary Table 2). Western blot analysis further substantiated the interaction between ODRUL and PI4Kα (Fig. 6B). To understand the role of this interaction in AgNP-induced cytotoxicity, stable PI4Kα-knockdown (termed as PI4Kα-low) cells were obtained through lentiviral shRNA (Supplementary Fig. 6, $P < 0.05$). Reduced PI4Kα was demonstrated before and after AgNP treatment (Fig. 6C). AgNP-

Fig. 5. Oxidative stress-driven Nrf2 regulates ODRUL expression upon AgNPs. (A) Relative intracellular ROS levels were reflected by DCF fluorescence intensities in K562 cells after treatment ($n = 4$). (B) Relative expression levels of ODRUL in cells with or without NAC pretreatment ($n = 3$). (C) Western blot analysis of Nrf2 and its target HO-1 levels in cells exposed to various concentrations of AgNPs for 24 h. After knocking down the endogenous Nrf2 expression, Nrf2 protein levels (D) and ODRUL mRNA levels (E) were assessed in scrambled-shRNA control cells and Nrf2-low cells. (F) Evaluation of cell death for scrambled-shRNA control cells and Nrf2-low cells in response to AgNP treatment at 80 μg/mL for 24 and 48 h through flow cytometry analysis with PI staining ($n = 3$).

induced cytotoxicity was exacerbated by approximately 80% in PI4K α -low cells, compared to that in scrambled-shRNA control cells (Fig. 6D), demonstrating the protective role of PI4K α against stress [54,55]. Together, these results revealed that AgNP-induced ODRUL interacted with PI4K α , which led to the inhibition of PI4K α activity and consequently a great increase in cellular death.

3.7. AKT and JNK are the downstream targets of the ODRUL/PI4K α complex

To explore the downstream signaling events of ODRUL/PI4K α

complex, we further determined PI4K α 's targets. As shown in Fig. 7A, the phosphorylation of AKT, JNK, Stat5 and ERK were enhanced in a dose-dependent manner. The increases of the phosphorylation for these kinases were also found in K562 cells upon AgNP treatment over the course of time (Fig. 7B). To figure out the roles of these proteins in AgNP-induced cytotoxicity, selective inhibitors were used. As shown in Fig. 7C, the inhibition of AKT phosphorylation increased AgNP-induced cell death by 1.8 folds, compared to the vehicle control ($P < 0.05$). In contrast, the blockage of JNK pathway abrogated cell death from 9.7% in AgNP-treated cells without the JNK inhibitor to 5.5% in AgNP-treated cells with

Fig. 6. ODRUL interacts with PI4K α to enhance cell death upon AgNP treatment. (A) The ODRUL partners were analyzed by RNA-pull down assay and mass spectrometry. (B) Western blot analysis of PI4K α protein levels in biotin-ODRIL and biotin-NS pull-downed complexes. Biotin-NS: biotin-nonsense RNA probes. (C) Protein levels of PI4K α in scrambled-shRNA control cells and stable PI4K α -low cells after treatment. (D) Flow cytometry analysis of cell death in AgNP-treated scrambled-shRNA control cells and PI4K α -low cells ($n = 3$).

the JNK inhibitor (Fig. 7C, $P < 0.05$). However, this abrogation conducted by the JNK inhibitor did not reverse cell death to the baseline level, as 2.9% cell death in vehicle control group and 3.5% in cells treated with the inhibitor only (Fig. 7C, $P < 0.05$). These data suggested that blocking the JNK pathway only partially suppressed AgNP-induced cell death. Nevertheless, inhibitions on ERK and Stat5 signaling did not alter the toxicity (Fig. 7C). Therefore, AKT and JNK pathways were more important in modulating cell survival upon AgNP treatment.

To further confirm the relationship between PI4K α and the activation of AKT and JNK signaling pathways, we investigated phosphorylation status of AKT and JNK in ODRUL-low cells and PI4K α -low cells. AKT phosphorylation was enhanced whereas JNK phosphorylation was repressed in ODRUL-low cells upon AgNP treatment, compared to scrambled-shRNA cells (Fig. 7D). Conversely, AKT phosphorylation was diminished and JNK phosphorylation was increased in PI4K α -low cells (Fig. 7E). These results signified that ODRUL/PI4K α complex reduced AKT activity and increased JNK activity, resulting in enhanced cell death in response to AgNP treatment.

3.8. Bcl-2 is the final executor of ODRUL/PI4K α complex in dictating cell death

To search the final executors of ODRUL/PI4K α complex, we screened the changes of the key targets of AKT and JNK signaling that are involved in cell survival regulation, including Bcl-2, BAX, Bcl-xL, Survivin, PTEN and CCND1. As shown in Fig. 8A–C, only Bcl-2 was significantly upregulated upon AgNP treatment at both mRNA and protein levels in a dose and time-dependent manner ($P < 0.05$). It should be noted that, although an increase of Bcl-xL at mRNA level was observed (Fig. 8A, $P < 0.001$), no increase was demonstrated at the protein level (Supplementary Figs. 7A and B). In addition, our results also showed that the protein levels of BAX were not altered responding to AgNPs (Supplementary Figs. 7A and B), indicating that BAX was not involved in AgNP-induced erythroid cell death. Therefore, we only focused on Bcl-2 for further investigation.

Since Bcl-2 is crucial in preventing cell death [56], the induction of ODRUL indeed antagonized the function of Bcl-2 under AgNP stress. We looked at the expression of Bcl-2 in ODRUL-low cells

Fig. 7. AKT and JNK are the downstream targets of ODRUL/PI4K α complex. (A) The phosphorylation levels of AKT, JNK, Stat5 and ERK were determined by Western blot analysis after treated with various concentrations of AgNPs. (B) Phosphorylation levels of AKT, JNK, Stat5 and ERK in K562 cells treated with 40 μ g/mL AgNPs at different time points. (C) The cell death proportions of AgNP-treated cells (at 80 μ g/mL for 24 h) with or without pretreatment of specific inhibitors against AKT (LY294002, 20 μ M), JNK (SP600125, 10 μ M), Stat5 (Stat5 inhibitor, 10 μ M) or ERK (PD98059, 50 μ M) for 1 h ($n = 3$). (D) Phosphorylation levels of AKT and JNK in scrambled-shRNA control and stable ODRUL-low cells upon treatment. (E) AKT and JNK activation in scrambled-shRNA control and PI4K α -low cells.

Fig. 8. Bcl-2 is the executor in promoting cell death upon AgNP treatment. (A) Expression changes of genes involved in AKT and JNK signaling in K562 cells treated with AgNPs for 24 h, as reflected by qRT-PCR analysis ($n = 3$). (B) Western blot analysis of Bcl-2 protein levels in K562 cells treated with different concentrations of AgNPs. (C) Bcl-2 protein levels in K562 cells exposed to 40 µg/mL AgNPs at different time points. (D) Levels of Bcl-2 protein in scrambled-shRNA control and ODRUL-low cells upon AgNP treatment for 24 h.

responding to AgNPs. A significant induction of Bcl-2 concentration was observed in ODRUL-low cells upon AgNP treatment, similar to scrambled-shRNA control cells (Fig. 8D). AKT and JNK are two important kinases that modulate cell death through regulating hundreds of kinases and transcription factors [57–59]. Previous reports have verified the activation of AKT by PI4K α [60]. Moreover, the activation of AKT signaling could inhibit JNK activity through interacting with its upstream kinases, such as ASK1 and MKK4/7 [61]. Therefore, based on our results, we assumed that ODRUL posed an inhibition on PI4K α -AKT-Bcl-2 signaling and also enforced the blockade of JNK on Bcl-2 through the AKT-JNK crosstalk in K562 cells upon AgNPs. These data together revealed that ODRUL/PI4K α -AKT/JNK signaling concertedly promoted erythroid cell death by sequestering the function of Bcl-2 in erythroid cells upon AgNP-conducted stress.

4. Discussion

A large number of studies have described the toxicities of AgNPs *in vitro* and *in vivo* at multiple organism levels [6,7,62], and oxidative stress and direct or indirect activation of associated pathways are assumed to be predominantly responsible for AgNP-induced cytotoxicity [63]. However, the molecular bases that dictate the activation of the stress signaling pathways remain elusive. In the current study, we identified that lncRNA ODRUL linked the oxidative stress to downstream cytotoxic signaling to trigger AgNP-induced cell death in K562 erythroid cells. ODRUL was for the first time recognized as a novel target of Nrf2, and its expression was greatly induced by Nrf2 in erythroid cells responding to AgNPs. Nrf2 is the master regulator to combat oxidative stress in mammalian cells by targeting a number of anti-oxidant genes, such as HO-1, NQO1 and GST [64]. Different from

other targets, Nrf2-driven ODRUL induction was found to play an indispensable role in enhancing AgNP-induced cell death through antagonizing the function of Bcl-2. Thus, this is the first report on a pro-apoptotic role of Nrf2 through preventing the function of Bcl-2 dependent on lncRNA ODRUL.

ODRUL was first discovered to be a lncRNA upregulated in tumor specimens from osteosarcoma patients, and it was found to play a critical role in promoting doxorubicin resistance for osteosarcoma cells through regulating the expression of ATP-binding cassette, subfamily B, member 1 (ABCB1) gene [4]. In terms of drug resistance, lncRNA Linc-VLDLR also contributed to chemoresistance through regulating the expression of ATP-binding cassette, subfamily G member 2 (ABC-G2) [65], suggesting a similar action mode for lncRNAs to confer cancers cells with a capability of drug resistance. However, our results unearthed a novel function of ODRUL in promoting erythroid apoptosis upon AgNP exposure through regulation of AKT/JNK-Bcl-2 signaling that is dependent on a physical interaction with PI4K α . Therefore, our current data pinpointed that AgNP-induced ODRUL accounted for enhanced cell death, which is different from drug resistance, as previously discussed [4,65,66]. To this end, we here deciphered a mechanism responsible for ODRUL-modulated cell death under AgNP-induced stress. Although the detailed mechanism responsible for the interaction between ODRUL and PI4K α needs further investigation, our results demonstrated that the binding of ODRUL with PI4K α in fact impeded the pro-survival role of PI4K α . PI4Ks are the inositol lipid kinase that catalyzes phosphoinositide into phosphatidylinositol 4,5-bisphosphate [67,68]. PI4K α provides the substrate (PtdIns 4,5-P2) for PI3K and subsequently induces the activation of AKT [60]. Thus, the increase of ODRUL would prevent the activation of AKT by PI4K α , resulting in an inhibition on Bcl-2 in AgNP-treated K562 cells. In addition, AKT acts to suppress the activity of JNK

Fig. 9. A schematic depicting the proposed mechanism of ODRUL-provoked cell death upon AgNP treatment. Endocytosis and internalization of AgNPs by erythroid cell give rise to the increase of ROS, which contributes to the increase of Nrf2 protein. Thereafter, Nrf2 elevates the expression of lncRNA ODRUL at transcriptional level. Upregulated ODRUL interacts with PI4K α to inhibit its regulatory role in activating AKT and repressing JNK pathway. As a consequence, Bcl-2, a downstream target of AKT and JNK, is negatively regulated, leading to enhanced cell death.

[61], and JNK in return inhibits the pro-survival function of Bcl-2 [69]. To this end, the induction of ODRUL by AgNPs would also enhance the inhibitory effect of JNK on Bcl-2. Taken together, downstream of ODRUL/PI4K α complex, AKT and JNK converged on Bcl-2 to regulate cell death of K562 cells challenged by AgNPs. However, the detailed regulation between ODRUL/PI4K α complex and AKT/JNK-Bcl-2 needs further investigation.

Bcl-2 is a well-known anti-apoptotic protein, and its upregulation mostly contributes to the cytoprotective effect in many cell types including erythroid cells [70,71]. Bcl-2 exerts its anti-apoptotic function through sequestering the pro-apoptotic members within the Bcl-2 family, such as BID, BIM and Bax, to avoid their oligomerization and subsequent initiation of cell death [72,73]. Therefore, insufficient Bcl-2 induction under stress conditions would compromise the cellular capability against the initiation of apoptosis program, leading to elevated cell death [74,75]. Our current results showed that ODRUL contributed to enhanced cell death by negatively regulating the Bcl-2 level under AgNP-induced stress, highlighting the importance of ODRUL's regulation on Bcl-2 by undermining its pro-apoptotic function. Nonetheless, ODRUL may not be the single regulator of the AgNP

toxicity, and further studies are needed to shed more light on the complex network.

5. Conclusion

lncRNA ODRUL was recognized to be a novel stress regulator in enhancing AgNP-induced cytotoxicity. As illustrated in Fig. 9, the increase of ODRUL expression was attributed to the regulation of Nrf2 induced by oxidative stress. To modulate cell fate, ODRUL interacted with PI4K α to inhibit the pro-survival function of Bcl-2 by impeding the activation on PI4K α -AKT-Bcl-2 signaling and enhancing the inhibition of JNK on Bcl-2 through PI4K α -AKT-JNK signaling. Thus, AgNP-induced cell death in erythroid cells was accounted for (at least partially) by ODRUL-conducted inhibition on PI4K α and its downstream pro-survival signaling. Our study would offer more insights into the molecular bases underlying AgNP-induced toxicity in erythroid cells.

Competing interests

There is no potential conflict of interests to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2017.03.027>.

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