

Silver Nanoparticles Exposure Impairs Cardiac Development by Suppressing the Focal Adhesion Pathway in Zebrafish

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Introduction: The potential toxic effects of wastewater discharges containing silver nanoparticles (AgNPs) and their release into aquatic ecosystems on aquatic organisms are becoming a major concern for environmental and human health. However, the potential risks of AgNPs to aquatic organisms, especially for cardiac development by Focal adhesion pathway, are still poorly understood.

Methods: The cardiac development of various concentrations of AgNPs in zebrafish were examined using stereoscopic microscope. The expression levels of cardiac development-related genes were analyzed by qRT-PCR and Whole-mount in situ hybridization (WISH). In addition, Illumina high-throughput global transcriptome analysis was performed to explore the potential signaling pathway involved in the treatment of zebrafish embryos by AgNPs after 72 h.

Results: We systematically investigated the cardiac developing toxicity of AgNPs on the embryos of zebrafish. The results demonstrated that 2 or 4 mg/L AgNPs exposure induces cardiac developmental malformations, such as the appearance of pericardial edema phenotype. In addition, after 72 h of exposure, the mRNA levels of cardiac development-related genes, such as *myh7*, *myh6*, *tpm1*, *nppa*, *tbx5*, *tbx20*, *myl7* and *cmlc1*, were significantly lower in AgNPs-treated zebrafish embryos than in control zebrafish embryos. Moreover, RNA sequencing, KEGG (Kyoto Encyclopedia of Genes) and Genomes and GSEA (gene set enrichment analysis) of the DEGs (differentially expressed genes) between the AgNPs-exposed and control groups indicated that the downregulated DEGs were mainly enriched in focal adhesion pathways. Further investigations demonstrated that the mRNA levels of focal adhesion pathway-related genes, such as *igf1ra*, *shc3*, *grb2b*, *ptk2aa*, *akt1*, *itga4*, *parvaa*, *akt3b* and *vcl*, were significantly decreased after AgNPs treatment in zebrafish.

Conclusion: Thus, our findings illustrated that AgNPs could impair cardiac development by regulating the focal adhesion pathway in zebrafish.

Keywords: AgNPs, focal adhesion, cardiac development, zebrafish

Introduction

Nanoparticles (NPs) have a wide range of uses, because of their distinctive characteristics, including size, shape, structure, and surface area.¹ Concerns concerning the consequences of unintentional NP exposure on human health have been raised by the extensive usage of NPs.² NPs can enter organisms through a variety of routes, including through the skin, mouth and respiratory tract, and cause toxic effects.³ The adverse effects of NPs are strongly related to cytotoxicity, oxidative stress, gene expression, proinflammatory responses, alterations in calcium homeostasis and cellular signaling events.^{3,4}

AgNPs are a type of labile metal-based nanoparticle (MeNP), and the metal ions present in AgNPs have the ability to separate from the nanoparticle surface in aqueous solutions and may undergo a variety of physicochemical alterations.⁵⁻⁸ In

particular, AgNPs have several attractive properties, such as chemical stability and antifungal and bactericidal properties.⁹ Therefore, AgNPs are widely used in health products, cosmetics, wound dressings, food packaging films and so on.^{9,10}

The production and extensive application of AgNPs has resulted in their elevated discharge into aquatic ecosystems, and AgNPs can contaminate the groundwater environment.^{10–13} After entering freshwater, AgNPs typically undergo oxidation, transforming into silver ions (Ag^+), which possess toxicity towards aquatic organisms.¹¹ AgNPs can also enter the body via the food chain and accumulate in aquatic organisms.⁹ In addition, long-term exposure to silver through oral and inhalation routes can result in the development of argyria or argyrosis.^{9,11} AgNPs can damage many organs and systems in vivo, such as the skin, eyes, hepatobiliary system, nervous system, urinary system, respiratory system, reproductive system and immune system,¹⁰ and may cause cell death through necrosis or apoptosis, as well as genetic mutations, and result in toxicity to tissues and organs.^{14–17}

Focal adhesion is an important mechanism of cell communication and plays a key role in cell growth, differentiation, motility, migration, proliferation and survival.¹⁸ Renshaw et al indicated that normal cell growth involves the induction of cell adhesion in the presence of growth factors.¹⁹ Many focal adhesion proteins, including $\alpha 4$ integrin, $\alpha 5$ integrin, β 1 integrin, paxillin, vinculin, talin, integrin-like kinase and FAK (focal adhesion kinase), play essential roles in embryonic development.¹⁸ In this context, the focal adhesion kinase protein is situated at the cortex of notochord cells and on the notochord-somite boundary. Throughout the process of somitogenesis, FAK accumulates in the basal region of epithelial cells at intersomitic boundaries, and phosphorylated FAK protein is detected at both the notochord-somite boundary and intersomite boundaries.²⁰ FAK plays instrumental roles in cell survival, cell proliferation, cell migration, and the integration of multiple cues in the extracellular environment, and FAK activity can be stimulated by several types of extracellular ligands.²¹ In postnatal or adult mice, focal adhesion proteins are essential for the functionality of specific tissues and organs.²² FAK phosphorylation is required for somite boundary formation during the somitogenesis of zebrafish embryos.²⁰ FAK deficiency can induce vascular development dysfunction, leading to embryonic lethality.^{23,24} Endothelial cell-specific deletion of focal adhesion kinase induces vascular defects associated with apoptosis.^{25,26} Paxillin is a focal adhesion-associated adaptor protein that can recruit FAK and vinculin to focal adhesions to guarantee regular signal reception and transduction.²⁷ Paxillin is very important for the recruitment of vinculin, a pivotal structural component in cardiomyocytes that plays essential part in mechanotransduction, to focal adhesions.^{27–30} The knockout of the paxillin gene in mice results to an early embryonic lethal phenotype.³¹ Paxillin and FAK play important roles in the vertebrate heart, and are both required to recruit and stabilize vinculin at sites of force transmission to regulate cardiac contractility.²⁹

Since zebrafish is an excellent animal model for studying the potential ecotoxicity of water pollutants, especially for investigating their biological effects on neurological and cardiovascular development. At early developmental stage, zebrafish embryos are transparent, enabling more easy observation of morphological abnormalities and developmental characteristics.³² In addition, it has been shown that the accumulation of AgNPs in the liver, spleen and lung can cause toxic effects.^{33,34} AgNPs are toxic to zebrafish embryos and cause death, delayed hatching, physical deformities and heart rate decreases.³⁵ Herein, our study explored the effects of AgNPs on cardiac developmental toxicity and the underlying molecular mechanisms. In addition, the DEGs after exposure to 2 and 4 mg/L AgNPs were analyzed by using transcriptome sequencing techniques to elucidate the potential mechanisms of cardiac developmental toxicity caused by AgNPs exposure. KEGG signaling pathway enrichment analysis of the DEGs were used to further explore the potential downstream signaling pathways in zebrafish with or without AgNPs treatment. qRT-PCR and Western blotting were also utilized to further analyze the mechanism underlying the potential toxicity of AgNPs exposure in zebrafish cardiac development. Thus, our findings illustrate the mechanism of AgNPs-induced cardiac disruption via regulating the focal adhesion pathway in zebrafish, which provide new insights into the potential risks of the toxic effects of AgNPs exposure to human health.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Shantou University Medical College. The protocol was approved by the Shantou University Medical College Animal Committee (No: SUMC-2020-015).

AgNPs Solution

Silver nanoparticles powder (CAS No. 7440-22-4, Sigma-Aldrich, Merck, Germany) was suspended in ultrapure water (Millipore, Sigma-Aldrich) and then sonicated for 50 min at 50 W/L and 40 kHz to prepare a 100 mg/L stock solution.³⁶ The morphology and diameter of AgNPs were characterized by transmission electron microscope (TEM) and dynamic light scattering (DLS) according to our previously described.³⁷ The percentages of different diameters of AgNPs particles were 96% (< 62 nm) and 4% (62–100 nm). The AgNPs exposure solutions were prepared every 24 h to maintain fresh and consistent levels of exposure.

Zebrafish Husbandry and Treatment

Wild-type (AB) zebrafish were reared at 28 °C under a constant cycle of 14/10 h light/dark and fed decapsulated freshly hatched brine shrimp twice daily. Healthy adult zebrafish were selected for breeding, and healthy embryos for the exposure experiment were collected at 4 hours postfertilization (hpf).³⁸ A total of 150 embryos (4 hpf) were transferred to 10 cm Petri dishes and supplemented with aqueous solutions of AgNPs at different levels of concentration (0, 1, 2 and 4 mg/L).^{37,39,40} Three replicates were performed for each concentration group.

For AgNPs treatment, base on our previous report, the median lethal concentration (LC₅₀) assay was performed to evaluate the potential toxic effect of AgNPs in zebrafish embryos.³⁷ Briefly, After treating AgNPs solution with different concentrations in 4 hpf zebrafish embryos for 72 h, the survival rate of 8 mg/L group is 32% with severe developmental disruption, whereas 67% and 74% survival rates in 4 and 6 mg/L groups. Thus, the selected exposure concentrations (0, 1, 2 and 4 mg/L) were utilized in the following experiments.

Zebrafish Morphology

After AgNPs exposure, the phenotypes of the zebrafish embryos at 24–72 h were observed using a stereoscopic microscope. Zebrafish embryo developmental abnormalities, delayed yolk sac absorption and including pericardial edema, were determined at 48–72 h.^{41,42}

Gene Expression Analysis

After zebrafish embryos were exposed to AgNPs for 72 h, 30 larvae were collected for RNA extraction using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. The first-strand cDNAs were transcribed with a PrimeScriptTM RT reagent kit with a gRNA eraser (Takara Bio, Japan) following the manufacturer's instructions, and 1 µg of total mRNA was utilized for cDNA synthesis process. qRT-PCR was then performed using an RT-PCR Kit (Top Green qPCR Super Mix, TransGen, China) in an ABI7500 real-time PCR system (PerkinElmer Applied Biosystems, Foster City, CA). The primers used for the selected genes and their sequences are listed in [Table S1](#). The housekeeping gene *β-actin* was designated as the internal control. All the samples were run in triplicate. The mRNA expressions of genes were standardized against to those of *β-actin* utilizing the 2^{-ΔΔCT} method.

Whole-Mount In situ Hybridization (WISH)

Zebrafish larvae were collected for WISH experiments after exposure to the highest concentration of AgNPs (4 mg/L) for 72 h as our previously described.⁴³ The ISH antisense probes were designed to target the 3'UTRs of the *myh7* (myosin heavy chain 7), *myh6* (myosin, heavy chain 6, cardiac muscle, alpha), *tpm1* (tropomyosin 1), *tnnt2a* (troponin T type 2a), *nppa* (natriuretic peptide A), *myl7* (myosin, light chain 7) and *cmlc1* (cardiac myosin light chain-1) transcript sequences. The T7 RNA polymerase promoters were included in the probe template. The probe templates were purified with a TAINquick Midi Purification Kit (TIANGEN, China) and then transcribed with a MAXIscript T7 Kit (Ambion, Thermo Fisher Scientific). The sequences of the WISH probes are presented in [Table S2](#).

Global Transcriptome Analysis

After 72 h of AgNPs exposure, zebrafish larvae were sent to the BGI Company (Shenzhen, China) for transcriptome sequencing. The downregulated DEGs were selected and subjected to functional enrichment analysis. KEGG pathway

analysis was performed with DAVID (<https://david.ncicrf.gov/gene2gene.jsp>). The top 4 pathway with the lowest *p* values were selected for plotting. The GSEA results were analyzed by Dr. TOM software (<https://biosys.bgi.com/#/report/login>). The Chiplot website (<https://www.chiplot.online>) was used to construct a heatmap of the down-regulated DEGs with $\text{Log}_2\text{FC} \leq -0.5$, and the KEGG pathway database (<https://www.kegg.jp/kegg/pathway.html>) was used to analyze the signaling pathways associated with the enriched DEGs.

Western Blot Analysis

After exposure to the AgNPs solution for 72 h, total protein was obtained from the zebrafish larvae using a total protein extraction kit (C500005, Sangon Biotech). Forty micrograms of total protein extract were separated by 10% SDS–PAGE separation gel followed by transferred to a nitrocellulose membrane (Millipore). Subsequently, the membranes were placed in 5% bovine serum albumin (BSA) in TBST buffer (containing 0.1% Tween-20) for 1 h of incubation. The membranes were then incubated with primary antibodies in the refrigerator at 4 °C overnight. The following day, the primary antibody was removed and after washing with TBST three times, the membranes were incubated with secondary antibodies for 1 h at room temperature. The results were visualized using a Bio-Rad ChemiDoc XRS system. The pixel intensities were measured with ImageJ software (NIH, Bethesda, MA). The sources of the antibodies used are listed in [Table S3](#).

Data Analysis

Statistical analyses were performed using analysis of variance (ANOVA), student's-*t* test, and Tukey's multiple range tests. A value of *p* < 0.05 was regarded as statistically significant. All values are expressed as the means ± standard errors (SEMs). GraphPad 9.0 software (GraphPad Software, San Diego, CA), and ChiPlot (<https://www.chiplot.online/>) were used for plotting.

Results

AgNPs Exposure Impairs Cardiac Development in Zebrafish

To explore the potential effects of AgNPs on the development of zebrafish, we first examined the morphological phenotypes of zebrafish treated with or without AgNPs. The results indicated that developmental malformations, such as pericardial edema (red broken arrows) and delayed yolk sac absorption (blue broken arrows) ([Figure 1A and B](#)), appeared at 48 and 72 h after the embryos were exposed to 2 mg/L AgNPs. Compared with those in the control group, the proportion of embryos with pericardial edema and delayed yolk sac absorption were significantly up-regulated after treatment with 2 or 4 mg/L AgNPs for 48–72 h ([Figure 1C–F](#)). Exposure of zebrafish embryos to 2 or 4 mg/L AgNPs affected heart development.

AgNPs Exposure Interferes with the Transcript Levels of Cardiac Development-Related Genes

To study the regulation of cardiac development-related genes after AgNPs exposure, we performed a qPCR assay in zebrafish treated with or without AgNPs at different concentrations (2 and 4 mg/L) and exposure times (24, 48, and 72 h). The results indicated that the mRNA levels of the *tnnt2a*, *nppa* and *nkx2.5* genes were increased significantly by 1.68-, 1.27- and 2.69-fold, respectively, after treatment with a high concentration (4 mg/L) of AgNPs for 24 h (*P* < 0.05), whereas the level of *tbx20* was relatively decreased compared with that in the control group ([Figure 2A](#)). Furthermore, after exposure to 4 mg/L AgNPs for 48 h, the transcript levels of *myh7*, *myh6*, *nkx2.5*, *tbx5* and *cmlc1* were increased by 1.32-, 1.75-, 2.47-, 1.32- and 1.38-fold, respectively (*P* < 0.05, [Figure 2B](#)). Interestingly, in the 72-h treatment groups, the mRNA levels of *myh7*, *myh6*, *tpm1*, *tbx5* and *cmlc1* were significantly reduced by 0.87-, 0.63-, 0.74-, 0.88- and 0.85-fold, respectively, in 2 mg/L AgNP-treated zebrafish embryos (*P* < 0.05, [Figure 2C](#)). In the 4 mg/L AgNPs-treated group, the mRNA levels of most cardiac development-related genes, including *myh7*, *myh6*, *tpm1*, *nppa*, *tbx5*, *tbx20*, *myl7* and *cmlc1*, were significantly decreased by 0.87-, 0.61-, 0.70-, 0.46-, 0.77-, 0.76-, 0.77- and 0.77-fold, respectively (*P* < 0.05, [Figure 2C](#)), except for the mRNA level of the *nkx2.5* gene ([Figure 2C](#)). Subsequently, we performed WISH analysis

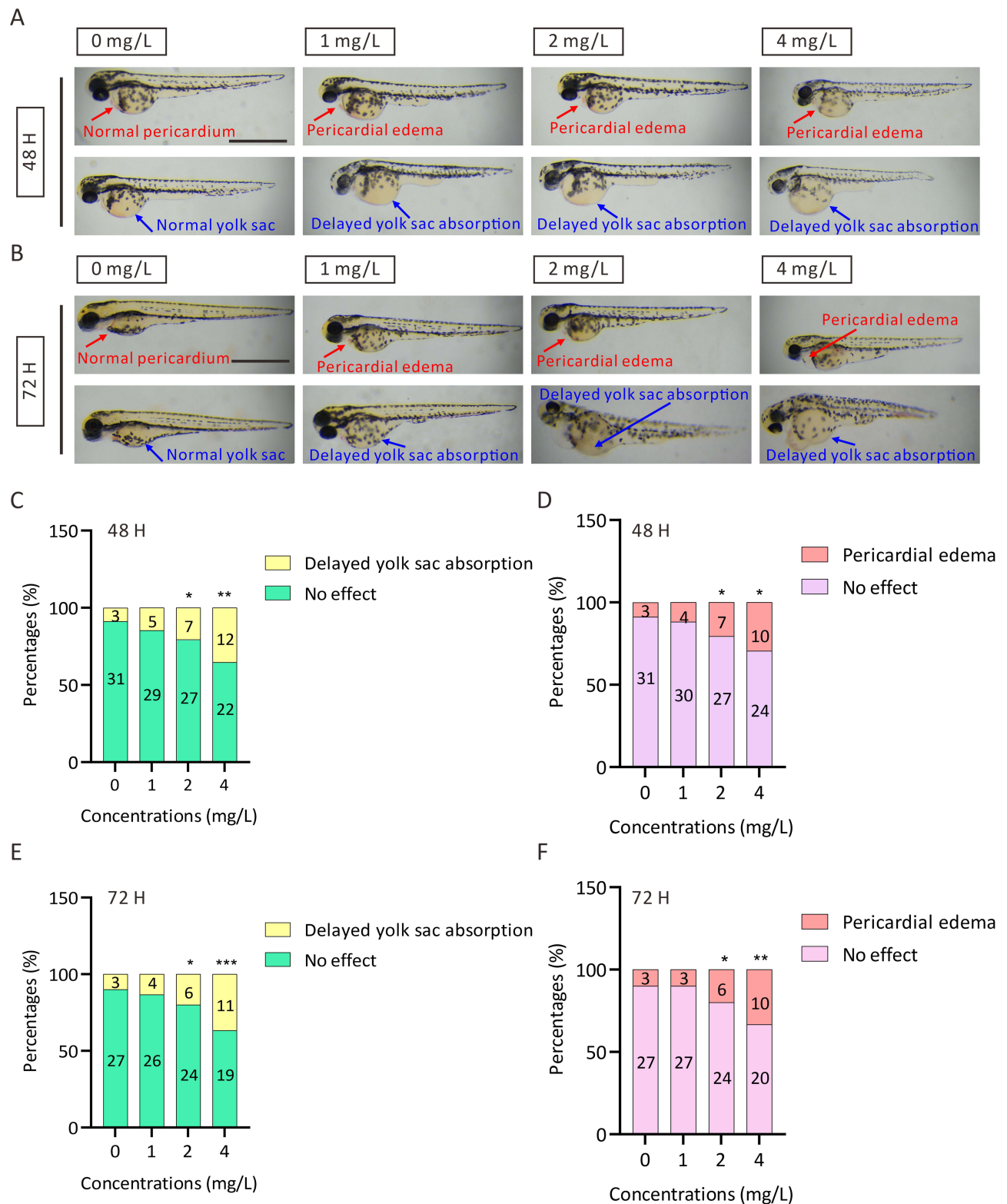


Figure I Phenotypic analysis of zebrafish embryos after exposure to AgNPs. (A, B) Representative images of zebrafish embryos after 48 and 72 h of AgNP treatment. Scale bar, 1 mm. **(C, D)** The percentage of malformed zebrafish embryos after exposure to AgNPs for 48 h ($n = 34$ for each group). **(E, F)** The percentage of malformed zebrafish embryos after exposure to AgNPs for 72 h ($n = 30$ for each group). All values are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Pericardial edema, red Broken Arrows; yolk sac, blue broken arrows.

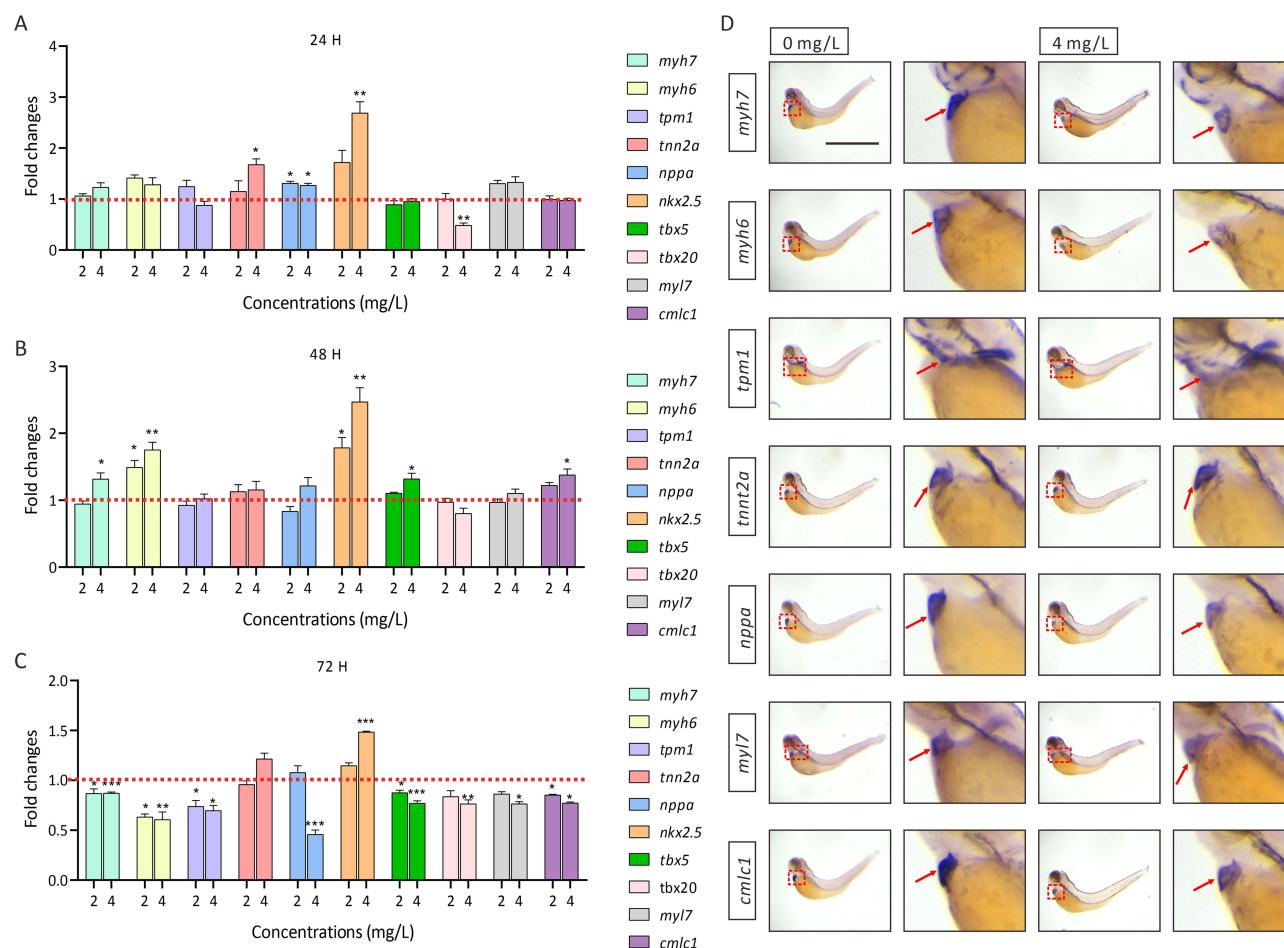


Figure 2 The mRNA levels of cardiac development-related genes in zebrafish with or without AgNP exposure. (A–C) The mRNA levels of cardiac development-related genes in AgNP-treated zebrafish at different concentrations and after different exposure times ($n = 3$ replicates). **(D)** Representative images of whole-mount in situ hybridization of zebrafish embryos treated with or without AgNPs for 72 h. Scale bar, 1 mm. All values are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Expression regions of cardiac development maker, red dashed boxes; positive signal zones of WISH analysis, red arrows.

on zebrafish larvae treated with 4 mg/L AgNPs for 72 h. We found that the expression trends of cardiac development-related genes, such as *myh7*, *myh6*, *nppa*, *myl7* and *cmlc1*, were broadly consistent with the qPCR analyses (Figure 2D). Taken together, the WISH and qPCR results revealed that AgNPs exposure might induce cardiac abnormalities by interfering with the transcript levels of genes related to cardiac development in zebrafish.

Identification of the Downstream Signaling Pathway Involved in AgNPs-Induced Cardiac Developmental Disruption in Zebrafish

To gain insight into the downstream signaling pathways after AgNPs (2 or 4 mg/L) exposure in zebrafish, KEGG pathway analysis was performed using DAVID. The results indicated that the downregulated DEGs in zebrafish exposed to AgNPs were enriched in the focal adhesion pathway (Figure 3A and B). In addition, GSEA revealed that the most of the DEGs enriched in the focal adhesion pathway were downregulated after AgNPs treatment in zebrafish (Figure 3C and D), suggesting that the expression of genes associated with the focal adhesion pathway was dramatically altered after exposure to AgNPs.

To further investigate the potential roles of 2 or 4 mg/L AgNPs exposure, we next analyzed the downregulated DEGs ($\text{Log}_2\text{FC} \leq -0.5$) involved in the focal adhesion pathway using heatmap (<https://www.chipshot.online/>). The results indicated that the number of DEGs increased after AgNPs treatment in a dose-dependent manner (Figure 4A and B). KEGG signaling pathway website (<https://www.kegg.jp/>) was used to analyses the downregulated DEGs ($\text{Log}_2\text{FC} \leq -0.5$) in the focal adhesion signaling pathway and to explore the underlying mechanisms (Figure 4C). Specifically, the

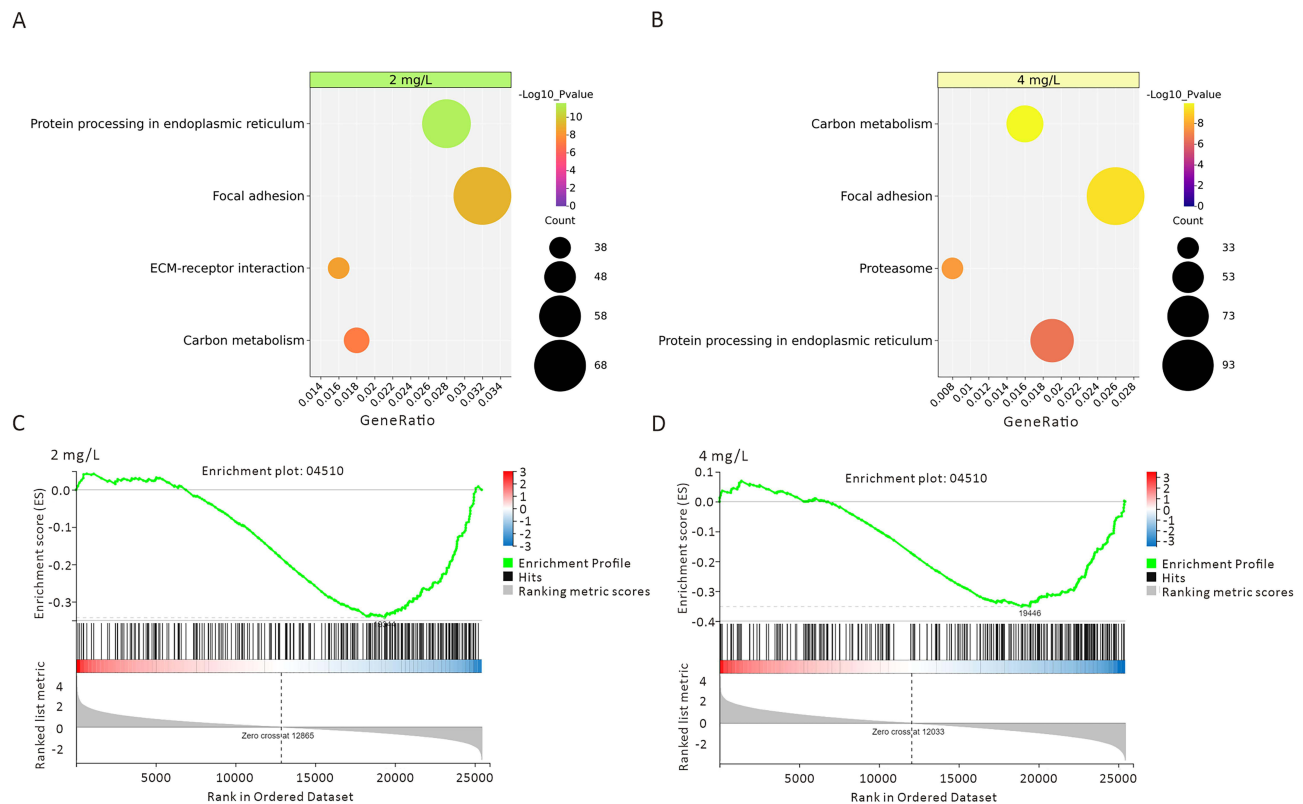


Figure 3 KEGG pathway enrichment and GSEA analyses of DEGs in zebrafish embryos with or without AgNP treatment. (A, B) KEGG pathway enrichment analysis of the down-regulated DEGs in AgNPs-treated zebrafish embryos. (C, D) The GSEA of the DEGs in focal adhesion pathway with AgNPs-treated zebrafish embryos at different concentrations.

downregulation of randomly selected DEGs, namely, *itgb3b*, *itgb1b*, *mylpfb*, *vaspa*, and *pak6b*, was determined by qRT-PCR (Figure 4D), which were also consistent with those of the KEGG enrichment analyses (Figure 4A and B).

We therefore performed a qPCR assay to analyze the transcript levels of the key genes in the focal adhesion pathway, which were identified by KEGG website (<https://www.kegg.jp/>), in zebrafish treated with or without AgNPs for 72 h (Figure 5). In this context, we noticed a noticeable alteration in the expression of most of the focal adhesion signaling pathway related genes under the higher concentration groups (2 and 4 mg/L) compared to the lower concentration group (1 mg/L). Specifically, after exposure to 2 mg/L AgNPs, the mRNA levels of *shc3*, *akt1*, *grb2b*, *itga4*, *vcl* and *parva* significantly decreased by 0.65-, 0.61-, 0.63-, 0.73-, 0.62- and 0.5-fold, respectively ($P < 0.05$). In addition, in the 4 mg/L AgNPs exposure group, the mRNA levels of *akt3b*, *ptk2aa*, *igf1ra*, *shc3*, *akt1*, *grb2b*, *itga4*, *vcl* and *parvaa* were decreased by 0.66-, 0.78-, 0.89-, 0.73-, 0.73-, 0.8-, 0.69-, 0.69- and 0.62-fold, respectively ($P < 0.05$). Notably, the mRNA level of *pik3cd* was unexpectedly increased by 1.31-, 1.38- and 1.39-fold after treatment with 1, 2 and 4 mg/L AgNPs, respectively, in zebrafish (Figure 5E), suggesting that AgNPs may affect zebrafish embryonic heart development by interfering with the mRNA expression levels of key genes involved in the focal adhesion signaling pathway.

AgNPs Exposure Suppresses the Expression Levels of Focal Adhesion Signaling Pathway-Related Genes

We then determined the expression levels of p-FAK, FAK, Paxillin and Vinculin, which are key focal adhesion pathway genes related to cardiac development, after 72 h of AgNPs exposure (Figure 6A). Western blot analyses indicated that a higher concentration of AgNPs suppressed the expression of genes related to this focal adhesion signaling pathway. Specifically, our findings indicated that the phosphorylation of FAK was dramatically inhibited after AgNPs treatment ($P < 0.01$, Figure 6B). In addition, the downregulation of paxillin was also detected in zebrafish embryos treated with the high-concentration groups of AgNPs (4 mg/L) ($P < 0.05$, Figure 6C). Moreover, although not statistically significant,

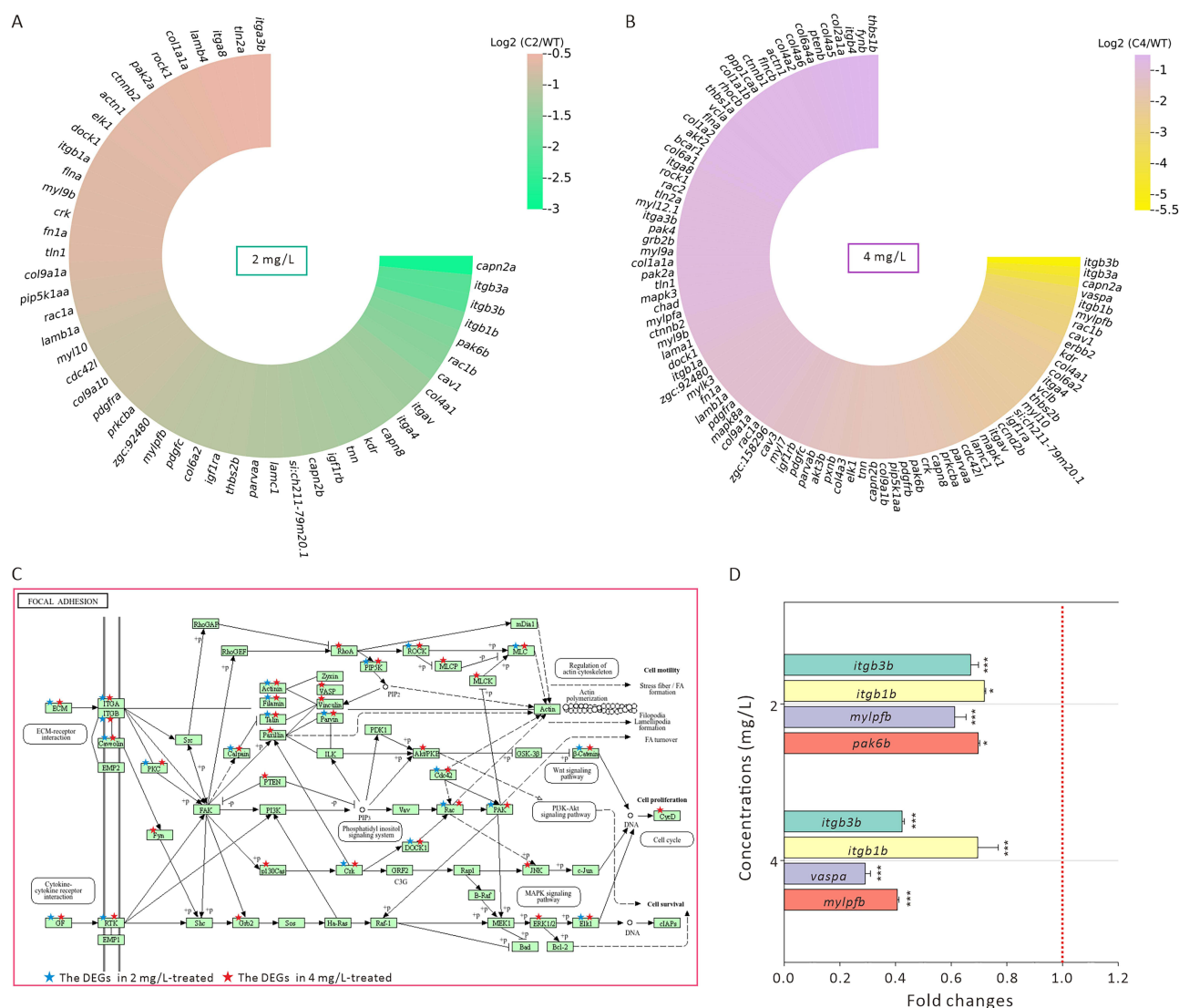


Figure 4 Transcriptional level analysis of focal adhesion pathway-related genes in zebrafish embryos after AgNP treatment. (A, B) Heatmap analysis of the DEGs ($\text{Log}_2\text{FC} \leq -0.5$) related to the focal adhesion signaling pathway in zebrafish after AgNPs treatment. **(C)** Schematic representation of focal adhesion pathway-related DEGs ($\text{Log}_2\text{FC} \leq -0.5$) in zebrafish embryos after 72 h of exposure to different concentrations of AgNPs. **(D)** Regulation of focal adhesion pathway-related genes (*itgb3b*, *itgb1b*, *mylpfb*, *vaspa*, and *pak6b*) in zebrafish embryos after AgNPs exposure ($n = 3$ replicates). All values are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

a trend towards the downregulation of Vinculin expression were observed in higher concentrations groups (Figure 6D). Notably, exposure to a lower concentration (1 mg/L) of AgNPs seemed to be insufficient to suppress the focal adhesion signaling pathway, which is consistent with the morphological phenotypes of zebrafish treated with AgNPs at lower concentrations (Figure 1A–F).

Discussion

AgNPs have been shown to have toxic effects, including oxidative stress induction, dysfunction of mitochondria, DNA damage, and apoptosis, both *in vivo* and *in vitro*.^{4,36} In our present study, it was found that the AgNPs exposed could cause cardiac toxicity in zebrafish embryos. The qPCR and WISH analyses revealed that AgNPs interfered with the expression levels of genes related to cardiac development. Transcriptome data showed that AgNPs affected the focal adhesion signaling pathway in zebrafish. The qRT–PCR analysis showed that the mRNA levels of genes relating to the focal adhesion signaling pathway were regulated after 72 h of AgNPs treatment of zebrafish, suggesting that the focal

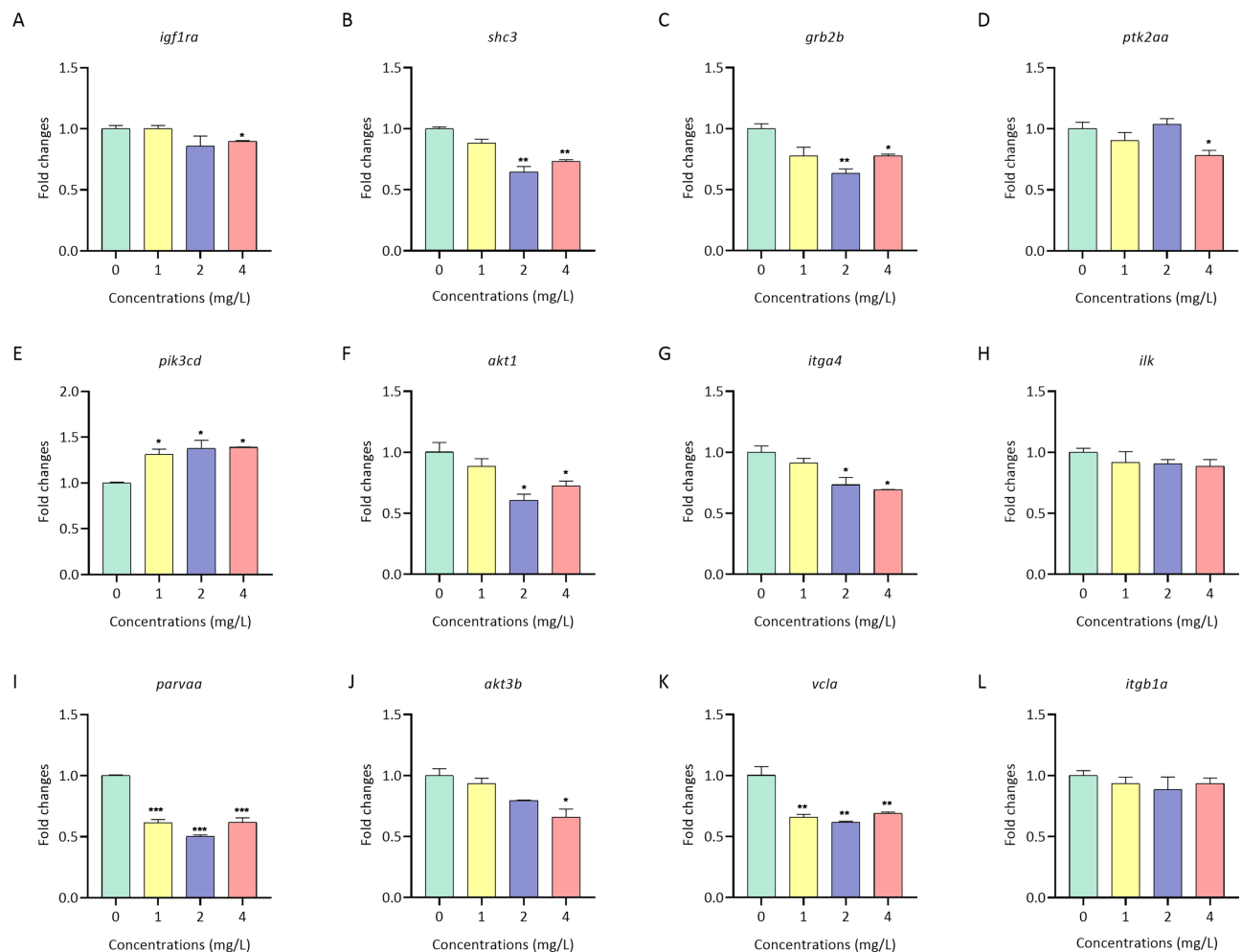


Figure 5 The mRNA levels of focal adhesion pathway-related genes in zebrafish after AgNP treatment. (A-L) The mRNA levels of focal adhesion pathway-related genes in zebrafish embryos after 72 h of exposure to different concentrations of AgNPs (n = 3 replicates). All values are presented as the mean $\pm$ SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

adhesion signaling pathway was disrupted. In addition, Western blot analysis revealed that AgNPs treatment can affect the protein expression levels of key focal adhesion genes in zebrafish after AgNPs exposure.

AgNPs exposure can cause developmental toxicity in zebrafish embryos. Studies have shown that AgNPs are taken up into embryos via the chorion and distributed throughout developing zebrafish tissues, which could cause an enlarged pore size in the chorion.⁴⁴ Zebrafish embryos were significantly affected after exposure to 4 nm AgNPs (0.963 mg/L) for 72 hpf, and significant *in vivo* uptake and delayed yolk sac absorption were evident.⁴¹ Our results showed that compared with control zebrafish embryos, exposed zebrafish embryos had growth abnormalities, delayed yolk sac absorption and induced pericardial edema (Figure 1A and B). After exposure to 2 or 4 mg/L AgNPs for 48–72 h, delayed yolk sac absorption and pericardial edema were observed and were significantly different from those in the control group (Figure 1C–F). According to previous studies, exposure to AgNPs can affect the heart rate of zebrafish larvae.³⁷ Xu et al reported that AgNPs could suppress Ca^{2+} signaling, decrease myogenic locus-specific DNA methylation and reduce heart rate.⁴⁵

Our results indicated that the transcript levels of zebrafish cardiac development-related genes, such as *tnnt2a*, *nppa* and *nkx2.5*, were increased after 24 h of acute exposure of zebrafish embryos to AgNPs (Figure 2A). As a cardiac contractility-related gene,⁴⁶ abnormal expression of *Tnnt2a* can cause hypertrophic cardiomyopathy.^{47,48} *Nppa* is known to play an essential part in cardiac development and disease, and has become an important marker for congenital heart defects.⁴⁸ A recent study indicated that *nppa* expression is upregulated upon cardiac stress.⁴⁸ *Nkx2.5* has been found to

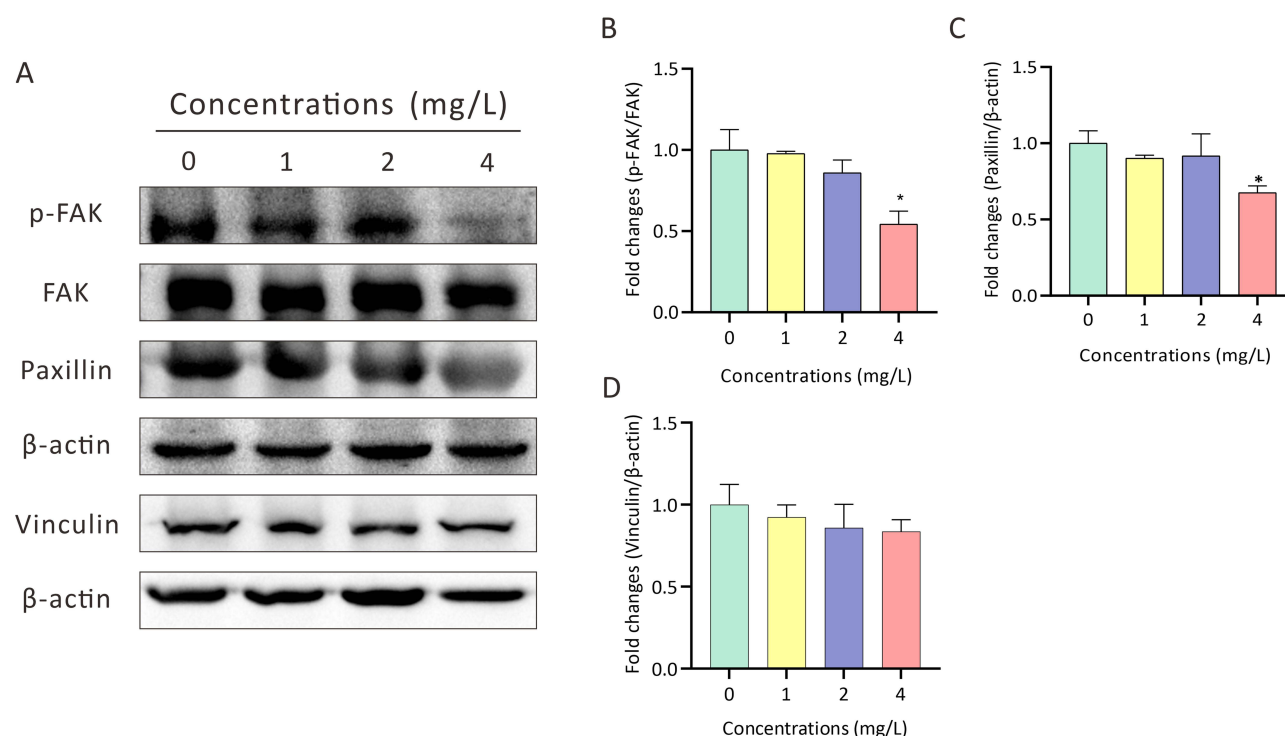


Figure 6 Determination of the expression of focal adhesion pathway-related genes in zebrafish embryos after AgNP treatment. (A–D) Representative images and quantification of the expression of p-FAK, FAK, Paxillin and Vinculin in zebrafish embryos treated with various concentrations of AgNPs for 72 h (n = 3 replicates). All values are presented as the mean ± SEM. *P < 0.05.

play a key role in determining myocardial cell fate and initiating cardiogenic differentiation.⁴⁹ Notably, a previous report showed that irregular heartbeats and arrhythmias were mainly due to increased mRNA expression levels of *ryr2*, *tbx5*, *atp2a2b* and *nkx2.5*.⁵⁰ Moreover, the upregulation of the transcription of *myh7*, *myh6*, *nkx2.5*, *tbx5* and *cmlc1* was detected after 48 h of acute AgNPs exposure (Figure 2B). Specifically, Tbx5 is required for heart development and for contacting cardiomyocyte differentiation.^{51,52} The atrium and the ventricle express the genes *myh6* and *myh7*, respectively; both chambers express *myl7*.^{53–55} The role of cardiac myosin light chain-1 (cMLC1) in cardiac development should not be neglected.⁵⁶ Previous studies indicated that once pathological stress occurs in the heart, the expression of genes related to cardiac development is induced, and this response is thought to play a role in the process of cardiac remodeling and compensation,^{57–61} which may be orchestrated by a stress-induced regulatory mechanism distinct from that of developmental regulatory programs.^{62,63} Furthermore, the transcript levels of *myh7*, *myh6*, *tpm1*, *nppa*, *tbx5*, *tbx20*, *myl7* and *cmlc1* were significantly downregulated after 72 h of AgNPs-treated (Figure 2C), which is similar to previous reports showing that the regulation of the mRNA levels of genes, including *myh6*, *myl7*, *tnnt2a*, *nkx2.5* and *tbx5*, is associated with cardiac developmental and functional defects.⁶⁴ WISH analysis of zebrafish embryos after 72 h of 4 mg/L AgNPs treatment found that the expression levels of *myh7*, *myh6*, *nppa*, *myl7* and *cmlc1* were decreased (Figure 2D).

Transcriptome analysis of zebrafish embryos after 72 h of AgNPs treatment revealed that the downregulated DEGs were mainly enriched in focal adhesion signaling pathways under high-concentration treatment (2 and 4 mg/L) (Figure 3A and B). GSEA revealed that the focal adhesion pathway was mainly enriched in the downregulated DEGs in the AgNPs treatment group (Figure 3C and D). In addition, transcriptome expression levels of genes associated with focal adhesion pathway were analyzed, and the DEGs were also validated and analyzed (Figure 4). It was found that the expression levels of focal adhesion pathway-related genes were mostly suppressed under high-concentration (2 and 4 mg/L) of AgNPs treatment (Figure 4). A previous study indicated that AgNPs exposure causes adverse effects on the cell adhesion dynamics and basement membrane.⁶⁵ Focal adhesion is necessary in cell survival, cell motility, and

proliferation.^{18,66} The lack of function of focal adhesion proteins during embryogenesis could impact the ECM adhesion, cytoskeletal organization, and embryonic survival.⁶⁷

In order to further investigate the genetic toxicity of AgNPs exposure in zebrafish, we have quantified the mRNA levels of several genes related to the focal adhesion pathway. The mRNA levels of *igf1ra*, *shc3*, *grb2b*, *ptk2aa*, *akt1*, *itga4*, *parvaa*, *akt3b* and *vcla* decreased after 72 h of AgNPs treatment (Figure 5). In this context, the knockdown of SHC expression can inhibit the PI3K-AKT-FoxO pathway through suppressing Akt and FoxO phosphorylation.⁶⁸ Akt is known to be an essential regulator of cell viability, cell death and the function of mitochondria.⁶⁹ An inactivating mutation in the *grb2b* gene can cause severe lymphatic damage.⁷⁰ Deficiencies of *itga5* and *itga4* can lead to serious myocardial defects and the morphological defects of the cardia bifida.⁷¹ Vela is known as a vinculin paralog and is associated with epicardial hyperplasia and coronary vessel disorganization.⁷² *Fak1a* and *fak1b* (*ptk2aa*) deficiency might result in heart failure.²⁹ Integrin β -1 (ITGB1), a member of the integrin family, mediates adhesion to extracellular matrices and facilitates cell-to-cell contact.^{73,74} The insulin-like growth factor (IGF) system is essential for growth regulation.⁷⁵ Parvin binds to PINCH proteins and integrin-linked kinases in a protein complex, and this complex is closely associated with heart failure in zebrafish.⁷⁶ Our findings suggested that AgNPs exposure could cause zebrafish cardiac developmental toxicity by interfering the mRNA levels of the focal adhesion pathway-related genes.

Notably, focal adhesion components also play important roles in cellular migration and differentiation during cardiac development,^{77,78} which is consistent with our finding that the protein expression levels of p-FAK and paxillin decreased in zebrafish after 72 h of 4 mg/L AgNPs exposure (Figure 6). FAK is a candidate enzyme and its phosphorylation is responsible for integrin-mediated mechanotransduction in cardiomyocyte focal adhesions and costameres.⁷⁸ In addition, the autophosphorylation site of FAK (Y397), which is a high affinity binding site for the SH2 domain or Src family protein tyrosine kinases,⁷⁹ can phosphorylate paxillin and other cytoskeletal proteins involved in myofibrillar assembly.⁷⁸ A previous report showed that inactivation of paxillin's target gene to progressive reduced cardiac contractility and heart failure in zebrafish.²⁹ FAK and paxillin are both essential for recruiting and stabilizing vinculin to regulate cardiac contractility.³⁷ In summary, our findings indicated that AgNPs exposure may result in cardiac dysgenesis by affecting the phosphorylation of FAK and the expression of paxillin in zebrafish.

Conclusion

In summary, our results demonstrated that AgNPs cause cardiac developmental toxicity in zebrafish embryos. Analysis of zebrafish morphology via qRT-PCR and WISH revealed that AgNPs can induce abnormal cardiac development and alter the mRNA levels of cardiac development-related genes. RNA-seq analysis revealed that AgNPs exposure disturbs the focal adhesion signaling pathway in zebrafish. Moreover, exposure to AgNPs for 72 h inhibited the focal adhesion pathway by regulating the mRNA levels of several focal adhesion pathway-related genes in zebrafish. Furthermore, the protein levels of focal adhesion pathway-related genes were also affected after AgNP treatment. AgNPs may cause cardiac developmental toxicity by affecting the focal adhesion signaling pathway.

Acknowledgment

This work is supported by the National Natural Science Foundation of China (81872070) and the Natural Science Foundation of Guangdong Province (2022A1515012424, 2023A1515011749), and GuangDong Basic and Applied Basic Research Foundation (2023A1515110234).

Author Contributions

Chunjiao Lu designed the experiment and acquired and analyzed the data. Xuwei Wu, Xin Meng, Yi Liu, Ting Yang, Yan Zeng, Yang Chen, Yishan Huang, Zhou Fang analysed the data. Juanjuan Luo and Xiaojun Yang supervised the project. All the authors reviewed and approved the final manuscript. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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