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ORIGINAL PAPER

Gene expression in zebrafish embryos following exposure to TiO_2 nanoparticles

Min-Kyeong Yeo[1] **& Hyo-Eun Kim**[1]

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Abstract In this study, we investigated the effects of TiO_2 nanoparticles, potential biological toxins, on zebrafish (*Danio rerio*) embryogenesis. We exposed zebrafish embryos to TiO_2 particles of three different diameters (12-14 nm, 80-100 nm, 150-200 nm) and compared the acute responses of the embryos during embryogenesis. Scanning electron microscope (SEM) images demonstrated that 12-14 nm TiO_2 particles were small ball types, while 80-100 nm TiO_2 particles were square and ball types. Square layers were observed in the 150-200 nm TiO_2 particles. The zebrafish morphants survived that exposure to the TiO_2 nanoparticles exhibited incomplete notochord formation, with epidermal injuries observed in larvae exposed to 12-14 nm and 150-200 nm particles. In microarray analysis, several genes involved in immune response, tumor necrosis factor, and endocytosis and its regulation were differentially expressed in accordance to the nanoparticle size. Gene expression in embryos exposed to 12-14 nm particles was significantly upregulated in comparison to the control group and embryos exposed to other particle sizes. The results of the present study suggest that TiO_2 nanoparticles 12-14 nm in size have toxic effects on zebraish development. TiO_2 nanoparticles of larger sizes (80-100 nm, 150-200 nm) exhibit different types of genetic effects.

Keywords Nanometer-sized TiO_2, Zebrafish, Gene expression, Regulation endocytosis, Immune response

[1]Department of Environmental Science and Environmental Research Center, College of Engineering, Kyung Hee University, 1 Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-701, Korea
Correspondence and requests for materials should be addressed to M. K. Yeo (✉bioclass@khu.ac.kr)

Nanometer-sized TiO_2 photocatalytic particles may be used to disinfect microorganisms and eliminate environmental pollutants such as volatile organic compounds (VOCs) and bisphenol A[1]. However, some researchers question the environmental safety of TiO_2 in natural ecosystems. Reactive oxygen species (ROS) cause DNA damage during the TiO_2 photocatalysis reaction[1-4]. The potential toxicities of nanoparticles including titanium dioxide (TiO_2), carbon nanotubes, and fullerenes have drawn significant attention in recent studies[5,6]. Investigations involving TiO_2 have been particularly prominent, reflecting the extensive commercial use of TiO_2 nanoparticles in cosmetics, sunscreen, pharmaceuticals, and implant debris. Even so, there is insufficient knowledge about the potential toxicity of TiO_2, particularly regarding the relationship between biological toxicity and TiO_2 particle size.

Microarrays are powerful tools that enable the synchronous comparative analysis of gene expression for a large number of genes that may cover a significant portion of the genome[7,8]. Microarray methods are both qualitative and quantitative because they are able to detect changes in the expression levels of treated cells as compared with control samples[9,10].

We used microarray analysis to investigate the effects of TiO_2 nanoparticles on zebrafish (*Danio rerio*, wild-type) gene expression. We first assessed the characteristics of TiO_2 nanoparticles using high resolution transmission electron microscopy (HR-TEM) and scanning electron microscopy (SEM). We then exposed developing zebrafish embryos to three different sizes of TiO_2 nanoparticles (12-14 nm, 80-100 nm, 150-200 nm) at 20 ppt concentrations during embryogenesis. Finally, we used microarray analysis to investigate the genetic effects of different sizes of TiO_2 nanoparticles on zebrafish larvae.

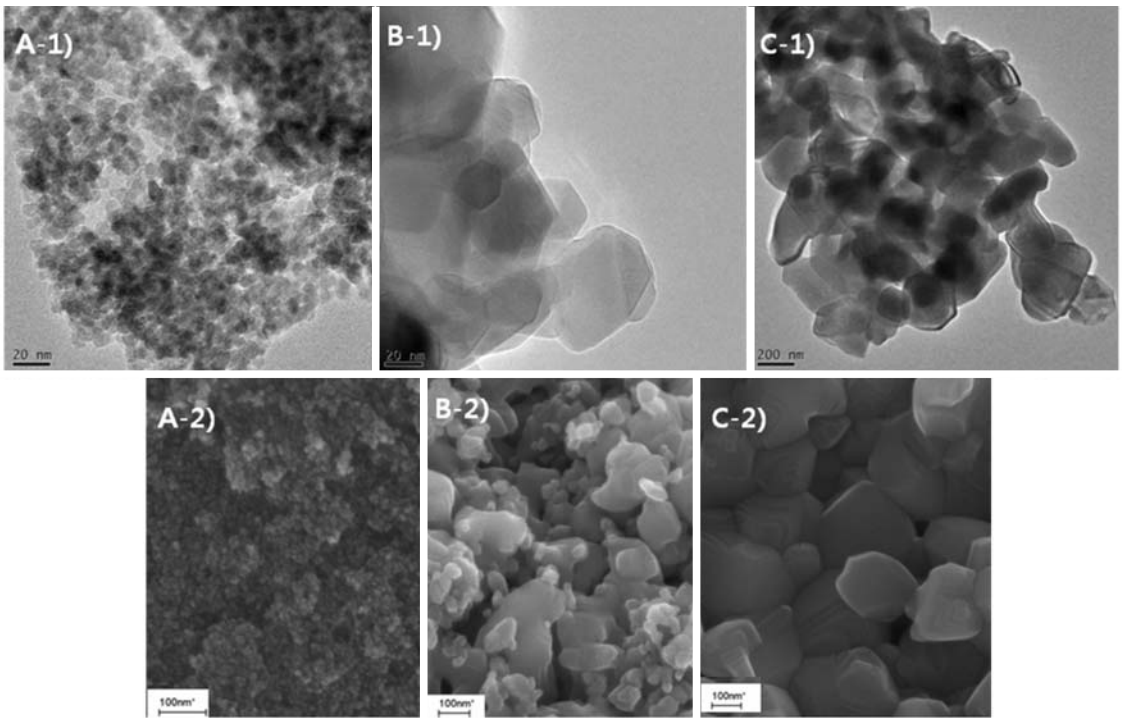

Figure 1. HR-TEM analysis and FE-SEM images of differently sized TiO_2 nanoparticles. HR-TEM analysis: A-1) 12-14 nm, B-1) 80-100 nm, C-1) 150-200 nm. FE-SEM images: A-2) 12-14 nm, B-2) 80-100 nm, C-2) 150-200 nm.

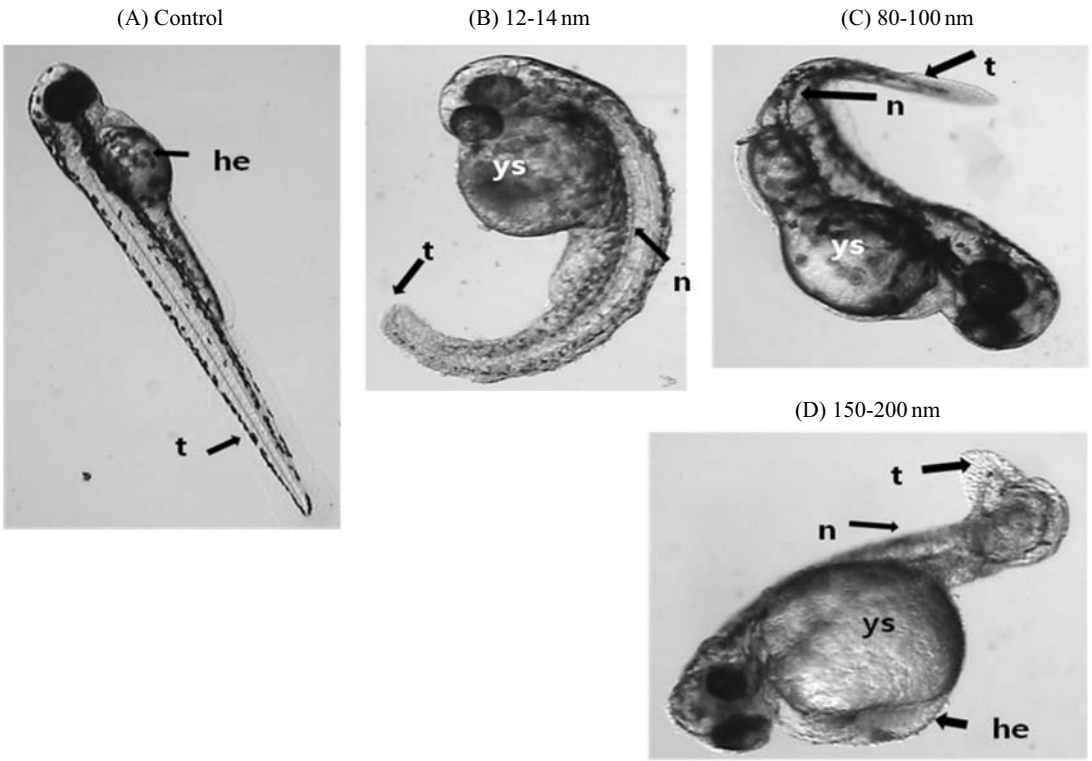

Figure 2. The morphologies of surviving embryos after exposure to TiO_2 nanoparticles. Embryos were exposed to three different nanoparticle sizes (12-14 nm, 80-100 nm, and 150-200 nm) at 20 ppt. These images show zebrafish embryos exposed to each treatment at 72 hours post-fertilization. Abbreviations: he, heart; ys, yolk sac; n, notochord; t, tail.

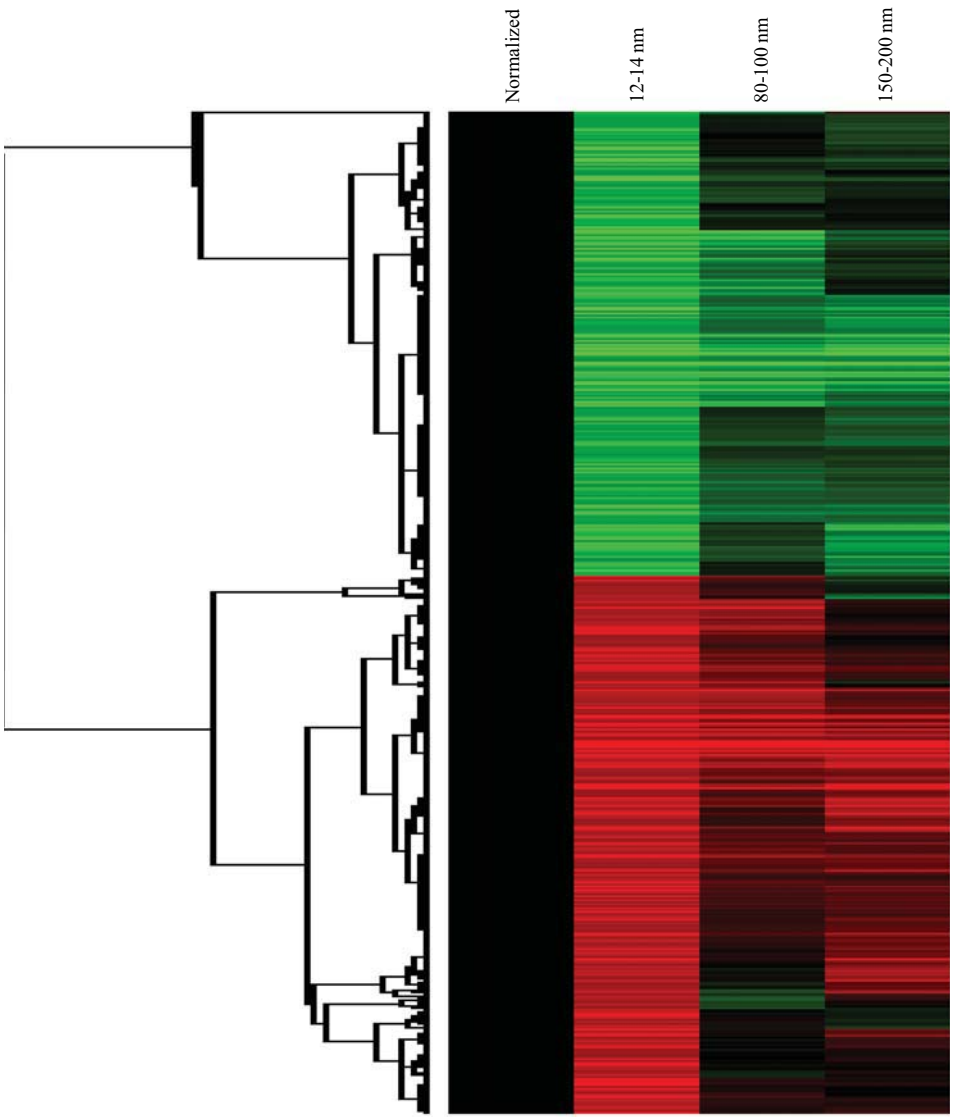

Figure 3. Hierarchical cluster image showing the differential gene expression profiles for zebrafish embryos exposed to TiO_2 nanoparticles.

Characterization of TiO_2 nanoparticles

We observed TiO_2 nanoparticles of three different sizes using HR-TEM analysis: A-1) 12-14 nm, B-1) 80-100 nm, and C-1) 150-200 nm (Figure 1). Scanning electron microscope images showed that 12-14 nm TiO_2 particles (Figure 1A-2) exhibited a small ball type shape, while 80-100 nm TiO_2 particles demonstrated square and ball type shapes (Figure 1B-2). Square layers were observed in 150-200 nm TiO_2 particles (Figure 1C-2).

Phenotypes of zebrafish larvae exposed to TiO_2 nanoparticles

Heart defects were observed in zebrafish larvae exposed to TiO_2 nanoparticles 72 h post-fertilization, including the heart muscle atrophied, pericardial edema, and blood pooling (Figure 2B, C & D). Inflammation and epidermal malformations were observed in the tails of larvae exposed to TiO_2 nanoparticles 12-14 nm and 150-200 nm in size (Figure 2B & D). The remainder of the surviving zebrafish morphants exposed to all three sizes of TiO_2 nanoparticles demonstrated incomplete notochord formation (Figure 2B, C & D).

Altered gene expression in zebrafish embryos exposed to TiO_2

Gene expression profiles were significantly up- or downregulated in embryos exposed to TiO_2 nanoparticles when compared to control embryos that were not

Table 1. Analysis of genes with altered expression in zebrafish embryos exposed to TiO_2 nanoparticles.

Regulation profile and ratio			Gene symbol	Gene description
12-14 nm	80-100 nm	150-200 nm		
Immune response				
15.83904	3.856506	13.15453	cxcl12b	*Danio rerio* chemokine (C-X-C motif) ligand 12b (stromal cell-derived factor 1)
11.76992	1.576533	1.638738	il10	*Danio rerio* interleukin 10
8.725269	3.075582	2.603899	si:busm1-228j01.6	*Danio rerio* si:busm1-228j01.6 (si:busm1-228j01.6)
5.240796	0.381438	0.468547	ifn	*Danio rerio* interferon 1
Tumor necrosis factor				
6.959384	1.351233	3.057094	tnfβ	*Danio rerio* tumor necrosis factor beta (TNF superfamily, member 2) (tnfβ)
3.142931	0.509051	0.148055	tnfsf10l2	*Danio rerio* tumor necrosis factor (ligand) superfamily, member 10 like 2 (tnfsf10l2)
2.465645	0.66351	2.597969	tnfsf10l	*Danio rerio* tumor necrosis factor (ligand) superfamily, member 10 like (tnfsf10l)
0.393615	1.188472	0.159246	tnfsf10l4	*Danio rerio* tumor necrosis factor (ligand) superfamily, member 10 like 4 (tnfsf10l4)
Tumor				
8.291434	1.487481	3.262307	–	*Danio rerio* similar to CTCL tumor antigen se20-9
6.959384	0.76211	0.681195	–	Q6DG80_BRARE (Q6DG80) Suppression of tumorigenicity 5, partial (19%)
2.293102	1.368252	1.436215	tnfsf10l	*Danio rerio* tumor necrosis factor (ligand) superfamily, member 10 like
2.234918	1.351233	3.057094	tnfβ	*Danio rerio* tumor necrosis factor b (TNF superfamily, member 2)
2.141439	0.577169	0.897701	zgc:152947	*Danio rerio* suppression of tumorigenicity 14 (colon carcinoma) b (st14b), immune response, induction of apoptosis, tumor necrosis factor receptor binding
2.126618	0.509051	0.148055	tnfsf10l2	*Danio rerio* tumor necrosis factor (ligand) superfamily, immune response member 10 like 2
Endocytosis				
19.56718	11.28144	13.57432	elmod2	*Danio rerio* ELMO/CED-12 domain containing 2, partial cds.
8.263727	1.613844	1.63108	sh3bp4	*Danio rerio* SH3-domain binding protein 4 (sh3bp4) elmod2 protein biological process cellular component molecular function
3.841445	3.202933	5.046242	jmjd6	*Danio rerio* jumonji domain containing 6 SH3-domain binding protein 4 (phagocytosis) (cytoskeleton)
3.290803	0.510671	0.569197	sh3bp4	*Danio rerio* SH3-domain binding protein 4 (sh3bp4) cell differentiation, phagocytosis
3.00957	4.075308	2.032479	zgc:101777	*Danio rerio* zgc:101777 SH3-domain binding protein 4
2.182849	1.056814	1.093015	zgc:158733	*Danio rerio* zgc:158733 hypothetical protein, endocytosis, Cytoskeleton, lysosome, membrane, membrane attack complex, lipid binding
1.828063	2.621479	1.489759	elmo1	*Danio rerio* engulfment and cell motility 1 (ced-12 homolog, C. elegans) hypothetical protein LOC791199, endocytosis, cytoskeleton, lysosome, membrane, membrane attack complex, lipid binding
1.602451	2.594178	3.030434	dab2	*Danio rerio* disabled homolog 2 (Drosophila) (dab2), apoptosis, phagocytosis
1.402054	2.310328	6.933673	elmod2	*Danio rerio* ELMO/CED-12 domain containing 2 disabled homolog 2

Table 1. Continued.

Regulation profile and ratio			Gene symbol	Gene description
12-14 nm	80-100 nm	150-200 nm		
0.53868	0.898578	0.245703	lmbr1l	*Danio rerio* limb region 1 like Phagocytosis, cytoskeleton, lipocalin-interacting membrane receptor, endocytosis, integral to membrane, membrane, membrane attack complex, receptor activity
Regulation endocytosis				
2.999186	1.240782	2.27123	wnt11	*Danio rerio* wingless-type MMTV integration site family, member 11
0.493905	0.382599	0.633876	mib	*Danio rerio* KIAA1323-like protein mRNA, complete cds.

exposed to TiO_2 nanoparticles (Figure 3 & Table 1).

Several gene expressions were significantly altered in samples exposed to TiO_2 nanoparticles. TiO_2 nanoparticles of different sizes exhibited different impacts on several genes like ifn, tnfsf10l2, zgc:152947. Particularly, immune response related genes like cxcl12b and il10 were upregulated more than 10-fold in embryos exposed to 12-14 nm TiO_2 than control embryos. The embryos exposed to TiO_2 nanoparticles 12-14 nm in size exhibited the most altered gene expression. Moreover tumor necrosis factor superfamily genes (tnfβ, tnfsf10l2) related with tumor necrosis factor and tumors demonstrated significantly upregulation in embryos exposed to TiO_2 nanoparticles 12-14 in size (Table 1), as compared to the control. The remaining TiO_2 nanoparticle sizes, 80-100 nm and 150-200 nm, exhibited smaller effects on gene expression than the 12-14 nm nanoparticles. However, several genes (zgc:101777, elmo1, dab2, and elmod2) related to endocytosis were upregulated more in the embryos exposed to TiO_2 nanoparticles 80-100 nm and 150-200 nm in size than in embryos exposed to nanoparticles 12-14 nm in size.

Discussion

Titanium dioxide nanoparticles were fabricated in three different sizes (12-14 nm, 80-100 nm, 150-200 nm) and characterized by transmission electron microscopy (Figure 1). There are numerous reports suggesting the toxicity of nanometer-sized materials, but the effects of nanoparticle size (i.e., materials of different sizes but the same chemical composition) on biotoxicity remain unknown. We investigated the phenotypes of zebrafish embryos exposed to TiO_2 nanoparticles of three different sizes that were observed to have different size-related shapes including ball (12-14 nm), square (150-200 nm), and mixed types (80-100 nm) in the results of SEM analysis (Figure 1).

The differing sizes and shapes of the TiO_2 particles tested demonstrated different effects on zebrafish embryogenesis throughout our experiments. Our results agree with those of a previous report demonstrating that TiO_2 particles possess cell-specific toxicity based on the concentrations and surface functionalities of specific particles[11].

Defects of the heart, including thinning of the heart and pericardial edema, were observed in zebrafish larvae 72 h following exposure to TiO_2 nanoparticles (Figure 2B, C & D). In the group exposed to TiO_2 nanoparticles, about 35% of the morphant defects proved lethal due to epidermal inflammation by 72 hours (Figure 2B & D), suggesting that nanometer-sized TiO_2 particles may function differently during different developmental stages, even though they cause similar immune system defects. These abnormal phenotypes are commonly associated with cxcl12b expression, and involve the modulation of immune response[12].

In our microarray analysis, *D. rerio* chemokine ligand 12b (CXC1l2b) expression exhibited greater upregulation in all groups exposed to TiO_2 than in controls. Moreover, the 12-14 nm and 150-200 nm TiO_2 nanoparticle groups were upregulated 15.839 and 13.154 times compared to control (Table 1). Chemokine CXCL12 is highly expressed throughout the central nervous system (CNS) by microendothelial cells under normal conditions, suggesting that it may play a role in maintaining the blood-brain barrier[12].

In this study, *D. rerio* interleukin 10 (IL-10) was observed to be upregulated. Since 1L-10 is an anti-inflammatory cytokine[13], this may indicate that nanometer-sized TiO_2 particles may increase inflammatory reactions. Furthermore, phenotypes related to inflammation were observed in the notochord during early developmental stages (Figure 2B & D), due to CNS inflammation in the spinal cord. The upregulation of spinal cord CXCL12 expression within the

active immune system suggests that this chemokine may be important in leukocyte migration during CNS inflammation[12,14].

We observed the upregulation of tumor necrosis factor beta (tnf beta) in embryos exposed to 12-14 nm and 150-200 nm TiO_2 nanoparticles. The cytokine, lymphotoxin [LT, tumor necrosis factor beta (tnf beta)], is a potent mediator of proinflammatory and tumoricidal activities[15].

The embryos exposed to TiO_2 nanoparticles 12-14 nm in size demonstrated upregulation of the zgc: 152947 gene resulting in expression that was 2.14 times greater than observed in the control group. This gene is also known as suppression of tumorigenicity 14 (colon carcinoma) b (st14b), and exerts its effects via induction of apoptosis or tumor necrosis factor receptor binding. Several genes related to endocytosis (elmod2, sh3bp4, jmjd6, sh3bp4, and zgc:101777) also demonstrated greater upregulation in this group than in embryos exposed to other particle sizes or control embryos. The nanoparticle penetration process in the cell suggests the endocytosis of non-self protein or phogocytosis[16].

The present study indicates that a particular size (12-14 nm) of TiO_2 nanoparticles exhibits uniquely toxic effects on zebrafish embryogenesis and development. Larger TiO_2 nanoparticles (80-100 nm, 150-200 nm) appear to demonstrate different genetic effects.

Materials & Methods

Characterization of TiO_2 nanoparticles

Titanium dioxide nanoparticles of three different sizes (12-14 nm, 80-100 nm, and 150-200 nm) were kindly donated by Dr. Misook Kang, Department of Chemistry, Youngnam University, Korea. The sizes, shapes, and compositions of the TiO_2 nanoparticles were observed by scanning electron microscopy (SEM, model JEOL-JSM35CF, Tokyo, Japan). The power and working distance of the microscope were set to 15 kV and 39 cm, respectively. In addition, a high-resolution transmission electron microscope (HR-TEM; JEOL, Tokyo, Japan), with an accelerating voltage of 300 kV, was used to study the structure and morphology of the TiO_2 nanoparticles.

Experimental animals

The zebrafish (*Danio rerio*, wild-type) used in this study were bred in our laboratory and were approximately 7-8 months old. The zebrafish breeding conditions, developmental stages, morphologies, and hatching rates in our laboratory have been described previously[17]. Experimental animals were housed in a 60 L glass tank filtered with a carbon filter. The water temperature was maintained at $28 \pm 1^{\circ}C$ with a 14/10 h light/dark cycle. Adult fish were maintained on a diet of bloodworms, dry flake food, and brine shrimp. Eggs were laid and fertilized within 1 h of the beginning of the light cycle, which provided large samples of synchronously developing embryos. The embryos were collected, pooled, and rinsed several times. Embryonic staging was carried out according to the standardized staging series set forth by Kimmel *et al.*[18]. The embryos were immersed in exposure or vehicle control solutions at the 64- to 256-cell stage, and 2.5 hours post-fertilization. Dead embryos were removed to avoid contaminating the test solutions. Embryos were observed with a microscope (Olympus, SZ61, Japan) to determine the effects of TiO_2 nanoparticle exposure.

Chemical exposure during developmental stages

Nanometer-sized TiO_2 particles were diluted with tap water that had been allowed to stand for 24 hours to remove chlorine. The final nanometer-sized TiO_2 particle exposure concentration was 20 ppt. Each group of 300 viable embryos was placed in a 1 L glass beaker and maintained in a carbon-filtered water system at $28 \pm 1^{\circ}C$. Embryos were randomly divided into the following groups: Group 1 was the general control group, and Groups 2, 3, and 4 were exposed to the same concentrations of TiO_2 nanoparticles (20 ppt), but different particle sizes (Group 2: 12-14 nm; Group 3: 80-100 nm; Group 4: 150-200 nm).

Embryos were observed at 2, 5, 8, 22, 27, 32, 48, 52, and 72 hours post-fertilization, time points selected because they correspond with known developmental stages[17]. Dead embryos were removed throughout the experiment when necessary. Samples for microarray analysis were collected at 72 hours post-fertilization for experimental and control groups.

Microarray analysis

For control and test RNAs, the synthesis of target cRNA probes for hybridization was performed using Agilent's Low RNA Input Linear Amplification kit PLUS (Agilent Technology; USA) according to the manufacturer's instructions. Briefly, 1 μg total RNA and T7 promoter primer mix were incubated at 65°C for 10 min. The cDNA master mix (5X first strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mixer. Samples were incubated at 40°C for 2 hours and then the RT and dsDNA syntheses were terminated by incubating at 65°C for 15 min. The

transcription master mix was prepared according to the manufacturer's protocol (4X transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3-CTP). The transcription of dsDNA was performed by adding transcription master mix to the dsDNA reaction samples and incubating at 40˚C for 2 hours. Amplified and labeled cRNA was purified with the cRNA Cleanup Module (Agilent) according to the manufacturer's protocol. The labeled cRNA target was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc.; Wilmington, DE, USA). After checking the labeling efficiency, cRNA fragmentation was performed by adding 10X blocking agent and 25X fragmentation buffer and incubating at 60˚C for 30 min. The fragmented cRNA was resuspended with 2X hybridization buffer and directly pipetted onto an assembled Agilent's Zebrafish Oligo Microarray Kit V2 (44K). The arrays were hybridized at 65˚C for 17 hours using a hybridization oven (Agilent). The hybridized microarrays were washed according to the manufacturer's washing protocol.

Data acquisition and analysis

The hybridized images were scanned using an Agilent Microarray Scanner (Agilent #G2565BA) and quantified with Feature Extraction Software (Agilent). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent). Intensity-dependent normalization (LOWESS) was performed, and the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. The averages of normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity. Functional annotation of genes was performed according to the Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3. Gene classification was conducted based on searches done by BioCarta (http://www.biocarta.com/), GenMAPP (http://www.genmapp.org/), DAVID (http://david.abcc.ncifcrf.gov/), and Medline databases (http://www.ncbi.nlm.nih.gov/).

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