Prediction and Validation of Regulatory Role of microRNAs in Zebrafish (*Danio rerio*) Responses to Nanoparticle Exposure with *in silico* and *in vitro* toxicological approaches

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Dedicated to my Family

And all the people I love

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ABBREVIATIONS

Abbreviation	Full name
8-OHdG	8-hydroxy-2' -deoxyguanosine
AP	Acid phosphatase activities
APAF-1	Apoptotic protease activating factor 1
ARS	Alizarin Red S
AuNPs	Gold nanoparticles
BRSNs	Biological response-specific
CAT	Catalase
CCP	Clathrin-coated Pits
CLP	Classification, labelling and packaging
DDR	DNA damage repair
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DSB	DNA double-strand breaks
DSB	Double-strand breaks
dsDNA	Double strand DNA

ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EtBr	Ethidiumbromide
GPx	Glutathione peroxidase
GSH	Glutathione
HBSS	Hank's balanced salt solution
hpe	Hours post exposure
HR	Homologous recombination
JNK	c-*Jun* N-terminal protein kinase
LPO	Membrane lipid peroxidation
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
miRNAs	MicroRNAs
mRNA	Messenger RNA
mu-GST	Mutagenic glutathione S-tranferase
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NO	Nitric oxide

NPs	Nanoparticles
PAHs	Polycyclic aromatic hydrocarbons
PAMP	Pathogen associated molecular pattern
PC	Protein carbonyl
PDCD4	Programmed cell death 4 protein
$PM_{2.5}$	Particulate matter with a diameter of 2.5 micrometer
PMA	Phorbol myristate acetate
PS	Polystyrene
QPCR	Quantitative real-time PCR
ROS	Reactive Oxygen Species
SMADs	Sma and Mad proteins
SOD	Superoxide dismutase
SSB	Single Strand Break
ssDNA	Single Strand DNA
TAOK1	Thousand and one kinase 1
TBE	Tris-borate-EDTA
TGF	Transforming growth factor
TiO_2	Titanium dioxide

TLR	Toll-like receptor
TSNs	Tissue-specific networks

ABSTRACT AND KEY WORDS

The release of engineered nanoparticles as by-product of human activities in the environment can interfere with normal biology and health of the exposed organisms. MicroRNAs have been suggested as potential toxicology biomarkers, however the information about expression and role of microRNA in regulation of signaling pathways in organisms exposed to nanoparticles (NP) is limited. Summary of reported biological and pathological outcomes of NP induced toxicity in zebrafish was followed with *in silico* analysis of the genes potentially responsible for observed toxicological effects. After identifying relevant genes, we constructed six miRNA-mRNA regulatory networks involved in nanoparticle induced toxicological responses in zebrafish. Based on our prediction and selection criteria, we identified six miRNAs that overlapped in networks with high prediction scores, and were validated by previous mammalian and zebrafish microRNA profiling studies: dre-miR-124, -144, -148, -155, -19a, -223.

As the next step, we validated the expression of these six miRNAs in THP-1 human monocytic cell line after the exposure to Polystyrene (PS NPs) and ARS labeled Titanium dioxide nanoparticles (TiO_2-ARS NPs). Also, identification of miRNAs expression post exposure to PLGA nanoparticles and *E. coli* BioParticles was used to exclude potential activation and engagement of miRNAs through phagocytosis or pro-inflammatory specific responses. In our study, miR-155-5p showed the most promise as biomarker for PS NPs and TiO_2-ARS NPs induced adverse effects.

To determine potential for PS NPs and TiO_2-ARS NPs for genotoxicity, time and dose dependent DNA damage profile induced by PS NPs or TiO_2-ARS NPs was established by comet assay. Results indicated the severe DNA damage was triggered by both PS NPs and

TiO_2-ARS NPs. However, we observed that the expression of DNA damage repairing genes was elevated post TiO_2-ARS NPs but not post PS NPs exposure, questioning the utility of the comet assay as universal assessment tool for genotoxicity induced by nanoparticles in general. It was observed that after PS NPs exposure the successful transfection of miR-155-5p mimic induced the expression of *ATM*, *TAOK1*, *TRIP13*, and *APAF-1* while the expression of *ERCC1* was attenuated. The *ATM*, *APAF-1* and *RAD51* were strongly activated post TiO_2-ARS NPs stimulation in mimic-transfected cells. These observations suggest there is significant involvement of miR-155-5p in PS NPs and TiO_2-ARS NPs induced adverse effects.

Keywords: Nanoparticles; MicroRNA; Zebrafish; Titanium dioxide; Polystyrene; THP-1; Genotoxicity

ZUSAMMENFASSUNG

Für den menschlichen Gebrauch entwickelte Nanopartikel geraten auf Grund ihrer Verwendung in die Umwelt und gehen dort unter Umständen adverse Wechselwirkungen mit den biologischen Abläufen und der Gesundheit der angetroffenen Organismen ein. MicroRNAs sind bereits mögliche Biomarker für ähnliche toxikologische Fragestellungen. Es ist jedoch ungeklärt, wie sich microRNA Expressionsmuster und deren Regulation von Signalwegen nach einer Nanopartikel (NP) Exposition verhalten.

Nachstehende Untersuchungen wurden daher angestellt: Die biologischen und pathologischen Folgen einer NP induzierten Intoxikation im Zebrafischmodell und daraufhin eine *in silico* Analyse von relevanten Genen, die potentiell mit toxikologischen Effekten im Zusammenhang stehen könnten. Nach einer Bestimmung dieser relevanten Gene, konnten wir sechs miRNA-mRNA Regelnetzwerke auffinden, die im Zusammenhang mit einer Nanopartikel induzierten Reaktion stehen können. Basierend auf dieser Vorhersage und der so getroffenen Auswahlkriterien war es möglich, diese miRNAs: dre-miR-124, -144, -148, -155, -19a, -223 zu identifizieren, die mit hohen Vorhersagewerten in den Regelnetzwerken auftreten und bereits in vorangegangen Profiling Studien bestätigt wurden.

Im nächsten Schritt validierten wir die Expression dieser sechs miRNAs in THP-1 Monozyten nach einer Exposition mit Polystyrol (PS NP) und ARS markierten Titandioxid Nanopartikeln (TiO2-ARS NP). Ebenfalls wurde die miRNA Expression nach einer PLGA Nanopartikel und *E. coli* Bio-Partikel Exposition untersucht, um eine potentielle Verbindung der miRNA Aktivierung zu Phagozytose Vorgängen oder pro-inflammatorischen Reaktionen ausschließen zu können. Die microRNA miR-155-5p zeigte in dieser Studie das vielversprechendste Potential, um als Biomarker für PS NP und TiO2-ARS NP induzierte negative Effekte nutzbar

zu sein. Zur Bestimmung des genotoxischen Potentials einer PS NP und TiO2-ARS NP Exposition wurde zusätzlich ein zeit- und dosisabhängiges DNA Schadensprofil durch einen Comet Assay erstellt. Die Ergebnisse zeigten, dass beide Nanopartikelarten in diesem erhebliche DNA Schäden provozieren. Ebenso konnten wir aber feststellen, dass auf eine TiO2-ARS NP Exposition hin, entsprechende DNA-Reparatur-Gene verstärkt exprimiert wurden, diese Reaktion blieb auf PS NP aber aus. Dieser Widerspruch stellt den Nutzen des Comet Assays als universelles Bewertungswerkzeug für genotoxische Geschehnisse durch Nanopartikel generell in Frage.

Nach einer PS NP Exposition von THP 1 Zellen, bei den vorher eine erfolgreiche Transfektion mit einem miR-155-5p Mimic stattgefunden hat, wurde eine vermehrte induzierte Expression von *ATM, TAOK1, TRIP13 und APAF-1* festgestellt, während die Expression von *ERCC1* gedämpft wurde. Die Gene *ATM*, *APAF-1* und *RAD51* wurden auch stark in Zellen aktiviert, die das Mimic enthielten aber TiO2-ARS NP ausgesetzt wurden. Diese Beobachtungen lassen vermuten, dass die mircroRNA miR-155-5p eine signifikante Rolle bei den adversen Auswirkungen spielt, die durch PS und TiO2-ARS Nanopartikel in Organismen provoziert werden.

Keywords: Nanopartikel; microRNA; Zebrafisch; Titandioxid; Polystyrol; THP-1; Genotoxizität

1. LITERATURE REVIEW AND DATA SUMMARY

1.1. Nano-hazards and MicroRNA in Nano-toxicology

Use of natural and engineered nanoparticles (NPs) is becoming widespread in various products including building and construction materials, electronics, clothes, personal hygiene, sunscreens, drugs, or food additives (Aitken et al. 2006; Maier and Korting 2005). The NPs from many of these products can be directly (as waste) or indirectly (via material degradation) released to the environment (Aitken et al. 2006). Nanoparticles are moving into environment from urban areas after household and industrial use to landfills or wastewater effluents, including rain or snow runoff from affected surfaces. As a consequence, a rise in NP loading burden into aquatic ecosystems has been observed (Buzea et al. 2007), leading to increased exposure of aquatic life to various NP and higher risk of bioaccumulation and/or biomagnification of NPs in the food chain. Release of NPs from primary products is making them bioavailable to human beings, and potential health consequences for both animals and human beings have only recently been recognized (Chen et al. 2004; Daughton and Ternes 1999). Evidence that NPs can be involved in adverse effects on fish and mammalian health has been recently presented (Bouwmeester et al. 2011; Jovanović et al. 2011a; Nagano et al. 2013; Sharma et al. 2012). Therefore, efforts to determine mechanisms of NP toxicity, including molecular and cellular regulatory processes that are affected by the NPs, can assist in identification of biomarker indicators (such as microRNAs) of NPs exposure and toxicity.

MicroRNAs (miRNAs) belong to a class of a single-strand noncoding RNAs with typically 21-23 nucleotides in length, engaged in a range of biological processes such as cell

differentiation, disease development and response to the toxicant exposure. miRNA regulate the expression of many protein-coding genes through interaction with messenger RNAs (mRNA) (Bartel 2004). For example, pol II promoter often contains toxicologically significant enhancer regions and is associated with the biogenesis processes of miRNAs (Ha and Kim 2014). This indicates potential for miRNAs to perform critical role in the cellular responses to xenobiotics (including nanoparticles), further supported by detected changes in expression of miRNAs after exposure to particulate matter (PM), that contains variable amount of nano-sized particles or aggregates of NPs. Hsa-miR-222 and hsa-miR-21 were overexpressed in the blood leukocytes of workers in a working environment rich in heavy-metal PM after three work days (Bollati et al. 2010). miR-21 was involved in the reactive oxygen species (ROS) triggered cellular injury protection via the apoptotic pathway, by negatively regulating PDCD4 (programmed cell death 4 protein). Further, miR-21 positively correlated with 8-OHdG (8-hydroxy-2' -deoxyguanosine) expression in carcinogenesis which at the same time brings ROS level enhancement (Cheng et al. 2009; Tu et al. 2014). Meanwhile, miR‑222 expression in post-exposure samples was positively associated with the mean lead exposure levels measured in the PM mass indicating an activation of leukocytes and inflammation in response to environmental stimuli. Moreover, miR-21 expression in humans is also influenced by inhalation exposure to diesel exhaust particles ($PM_{2.5}$, black carbon and organic carbon) (Fossati et al. 2014). Results from this study suggested that miR-21 may be involved in the "HMGB1/RAGE signaling pathway" through the modification of transcription factor NF-kappa B, thus playing a role in mechanisms related to particulate matter toxicological responses such as inflammation and endothelial dysfunction. Moreover, a most recent study indicated that the expression of miR-21-5p was negatively associated with the quantity of $PM_{2.5}$ (Particulate matter with a diameter of 2.5 micrometer) exposure in human serum (Chen et al. 2018). Therefore, miR-21 could be an important mechanistic link

demonstrating the association between environmental particulate contamination and disease. Recent research focused on the miRNA expression pattern under the exposure of particulate matter based on Zebrafish model, results from which screened out 8 microRNAs that were potentially related to $PM_{2.5}$ induced damage in zebrafish embryos supporting that miRNAs play a critical role (Duan et al. 2017).

In addition to particulate matter, miRNAs expression can be altered by inorganic metalloid or organic polycyclic aromatic hydrocarbons (PAHs). After arsenic exposure, expression of miRNA can be altered in mammals (Li et al. 2012b; Sturchio et al. 2014). In human Jurkat leukemic T cell line, the inorganic arsenite (iAs) treatment resulted in the up-regulation of hsa-miR-222 and the down-regulation of hsa-miR-181a (Sturchio et al. 2014). Observations from this research suggested that hsa-miR-222 and hsa-miR-181a could be involved in DNA demethylation and ROS related pathways respectively, by combining with their target genes in the protective response to iAs induced toxicity. On the other hand, hsa-miR-181 miRNA family (miR-181a, -181b, and -181d) was significantly upregulated under the exposure to PAHs, in human hepatocellular carcinoma cells (HepG2 cell line) (Samanta et al. 2002; Song et al. 2013). Their finding illustrated the direct regulation of miR-181 family toward MKP-5 (MAPK phosphatase-5) activated p38-MAPK pathway and further induced carcinogenesis process under the PAHs treatment.

With increasing number of studies indicating miRNAs integral involvement in the toxicological processes induced by a variety of environmental pollutants, this class of noncoding RNA has the potential to be used as a novel biomarker of NPs exposure. As a matter of fact, several studies are already investigating the association between the NPs exposure and miRNAs expression pattern (Grogg et al. 2016; Huang et al. 2015). However, relatively few studies illustrated the connection between miRNAs and NPs exposure in aquatic organisms-models that were frequently utilized in toxicity assessment (e.g. zebrafish),

which represents knowledge gap that needs to be addressed. In response to this knowledge gap, we were the first to summarize the biological adverse outcomes of NPs induced toxicity while collecting the genes that were proven responsible for this toxicological effect in a frequently used and standardized fish model in aquatic toxicology: Zebrafish.

1.2. MicroRNA in nanoparticles induced toxicity and toxicological pathways

MicroRNA, a class of gene regulatory molecules in various organisms, plays a pivotal role in diverse biological processes including responses to toxicants via targeting mRNA for cleavage or translational repression. While the critical role of miRNAs in the oncogenesis has been described, the importance of miRNAs in nanoparticle induced toxicological processes has not been investigated until recent years (Calin and Croce 2006). The increasing evidence that the expression of miRNAs is affected by nanoparticles certainly suggests an important role of miRNAs in nanotoxicology. Here, we summarized the validated miRNAs that were altered during NPs exposure in vertebrates (mostly in mammalian models) or in invertebrates from current existing articles and categorized them into toxicology-related signaling cascades for a better understanding of the critical role that miRNAs play in these signaling pathways and nano-toxicological processes (Table 1). Based on the table, we also calculated the frequency of pathways that engaged in these processes and are involved in four most relevant pathways regulated by microRNA during nanoparticles induced toxicity responses: p53 pathway, Wnt pathway, MAPK pathway and TGF-beta pathway.

1.2.1. p53 signaling pathway

When the cellular stress or DNA damage occurs, protein p53 triggers cell-cycle arrest followed by either repair of the damage or apoptosis process through signaling with other proteins. This signaling cascade response was defined as the p53 pathway (Farnebo et al. 2010). Upon the exposure to silver nanoparticles (AgNPs), DNA damage and apoptosis increased in human Jurkat T cell line, while the expression of hsa-miR-504 significantly decreased with a corresponding rise of p53 protein level (Eom et al. 2014). Moreover, another study demonstrated that hsa-miR-504 was a direct negative regulator of the transcriptional activity of p53 in human cell line while the overexpression of miR-504 significantly reduced p53-meditated cell-cycle arrest and apoptosis (Hu et al. 2010). So hsa-miR-504 is involved in AgNPs induced toxicity effects through mediating p53 expression regulated cell-cycle and apoptosis in human cell line. In addition to regulating p53 negatively, miRNAs were also trans-activated by p53 during the NPs exposure (Chang et al. 2007a). Research by S. Li et al (Li et al. 2011b) demonstrated that exposure of NIH/3T3 mouse embryonic fibroblast line to CdTe quantum dots (CdTe QDs), resulted in apoptosis-like cell death during increase of the p53 protein level. CdTe QDs also induced p53 post-translational modification by phosphorylation at Ser-15 (Li et al. 2011b). Interestingly, the transcriptional level of mmu-miR-34s was up-regulated instead of down-regulated. The stated facts, as well as miR-34 family direct trans-activation by p53 protein may lead to a conclusion that p53 is involved in the transcription of pri-miR-34s, which therefore supplemented the p53 function in apoptosis and cell-cycle induced by NPs (Chang et al. 2007b).

1.2.2. MAPK signaling pathway

Mitogen-activated protein kinase (MAPK) signaling pathway is well known for its function in the cellular stress response, apoptosis and inflammation responses through manipulation of a variety of transcription factors (DiDonato et al. 2012). MAPK signaling is frequently activated in the NPs induced toxicity such as endoplasmic reticulum stress, mitochondrial damage and inflammation process (Christen et al. 2014; Eom and Choi 2010). hsa-miR-155 has been reported to be up-regulated during the gold NPs treatment in human MRC5 lung fibroblast line as well as by manganese NPs in human neuronal CATH cell line (Grogg et al. 2016; Ng et al. 2011). In both experiments, cells suffered cellular damage, had increased generation of ROS, and decreased viability. Moreover, the transfection of miR-155 mimics was followed by significant reduction of metallic NPs' ability to trigger *TNF-α* and *IL-6* gene expression in CATH cell line, demonstrating miR-155 involvement in MAPK pathway and having predictive value in consequences of cellular stress (Ceppi et al. 2009). This data support regulatory role of miR-155 in the transcriptional activation of cytokines by mediating MAPK signaling in response to NPs toxicity. The application of gold nanoparticles, (AuNPs) to human dermal fibroblasts suppressed their proliferation and significantly changed the expression of hsa-miR-20, -30, and -9 (Huang et al. 2015). For miR-30 and miR-9, the targeting mRNA was validated to be *HSPA5* and *PAK2* involved in the MAPK signaling pathway (Yang et al. 2010). PAK2 can be activated during caspase-meditated apoptosis through combining with Rac1, a classic signaling cascade for the MAPK pathway (Déléris et al. 2011). Therefore, it appears that miRNAs is acting as a functional mediator in the MAPK pathway response to the cellular toxicity caused by the exposure to NPs, however, the precise mechanism remains elusive in all of the studied model organisms.

1.2.3. Wnt signaling pathway

Wnt signaling pathway is involved in a series of cellular processes including cell apoptosis, DNA damage repair, and inflammatory responses as protective mechanisms against NPs toxicity (Almeida et al. 2005; Gordon and Nusse 2006; Zhang et al. 2011). Emerging experimental evidence illustrates that the Wnt pathway is affected after the exposure to NPs. Silica NPs were shown to specifically attenuate Wnt signaling by targeting the Dvl protein, a key component of Wnt signaling cascade, thus influencing Wnt-mediated physiological or pathological processes (Yi et al. 2016). Moreover, TiO_2 NPs suppression of dendritic development correlated with inhibition of the canonical Wnt signaling pathway (Hong et al. 2017). miRNAs were assumed to act as a mediator in the Wnt signaling response to NPs in mammals. Multiple studies confirmed that the expression of miR-135 family is significantly altered by the exposure to different categories of NPs while targeting genes involved in Wnt pathway, such as *runx2* (Bourdon et al. 2012; Halappanavar et al. 2011). Moreover, nano-TiO_2 treatment downregulated miR-449a abundance and predicted targeting *lef1* in this study as a downstream effector of Wnt signaling (Halappanavar et al. 2011). On the other hand, miR-34 family links p53 activity with the canonical Wnt pathway and the expression profile of miR-34 was proven to fluctuate under the treatment of NPs in mammalian cell line indicating miR-34 family may be bridging or co-activating apoptotic and Wnt signaling pathways during nanoparticle exposures.

1.2.4. TGF-beta signaling pathway

The TGF (Transforming growth factor)-beta signaling pathway is a signaling cascade involved in many cellular processes including cell growth, apoptosis, and immune responses (Derynck and Zhang 2003; Letterio and Roberts 1998). This pathway is functionally

activated through a signaling response mediated by type II and type I SMADs (Sma and Mad proteins from *Caenorhabditis elegans* and *Drosophila*, respectively) and resulting in transcription induction in a variety of cell types (Attisano and Wrana 2002). The miRNA 17-92 cluster comprised of miR-17, -18a, -19a, -20a, -19b and -92a has been identified as a pivotal regulator towards TGF-beta signaling by either directly targeting the TGF-beta responsive genes (*CDKN1A* or *BCL2L11*) or repressing the SMADs in both up-stream and down-stream signaling (Li et al. 2012a; Mestdagh et al. 2010). After exposure to gold nanoparticles (AuNPs) with primary size of 100nm, one of the miR17-92 cluster members (mmu-miR-17) was significantly altered in mice liver (Balansky et al. 2013). In human dermal fibroblasts, the AuNPs induced the expression change in has-miR-20a, another member of miR 17-92 cluster (Huang et al. 2015). This indicates that the miRNA 17-92 cluster could be important in regulating detoxifying processes in mammals. Apart from NPs, in zebrafish model, the introduction of $PM_{2.5}$ down regulated the expression of dre-miR-19a. Taken together, miR 17-92 cluster may regulate NPs as well as PM induced organism responses across species, one of the affected pathways likely being the TGF-beta signaling cascade.

1.3. Adverse outcomes of nanoparticles induced toxicity in Zebrafish

1.3.1. Biological adverse responses and responsible genes

Zebrafish is an aquatic vertebrate model organism frequently used in toxicological and genetic studies and valued for its short reproductive cycle, transparent embryos, and a high degree of homology with human genome. In the field of nano-toxicology, zebrafish are

frequently used as research animal model for detecting the NPs induced toxicological endpoints, including histopathological and biochemical aspects, as well as underlying molecular mechanisms. Here we summarized the adverse outcomes of zebrafish NPs exposures and grouped them in three major NPs induced toxic responses: oxidative stress, DNA damage, and inflammation, including targeted changes in gene expression that are deemed responsible for these adverse outcomes in zebrafish.

1.3.1.1. Oxidative stress

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the ability of the organism to contain their harmful effects. Surplus of ROS will lead to the damage of all components of the cell and can alter cell signaling. NPs can induce a dose, size and exposure time dependent oxidative stress response in a vast variety of species including mammals and aquatic organisms (Choi et al. 2010; Greven et al. 2016; Lu et al. 2017; Xiong et al. 2011; Zhao et al. 2016). The ROS-scavenging antioxidant defense system in fish is similar to mammalian, as several major antioxidant enzymes, SOD, CAT, GSH and GPx, neutralize the deleterious toxic effects caused by ROS in both animal classes. Apoptosis has been implicated as a major mechanism of cell death caused by NP-induced oxidative stress which generally involves the activation of JNK and p53 pathway (Hsin et al. 2008; Piao et al. 2011). In zebrafish exposed to different NPs, the expression of anti-apoptotic genes (*bcl-2*) and pro-apoptotic genes (*bax*) are consistently altered, and comes along with significant increase in number of apoptotic cells and levels of superoxide dismutase (Zhao et al. 2016; Zhao et al. 2013a). Moreover, since mitochondria are one of the major target organelles for NPs induced oxidative stress, the intrinsic mitochondrial apoptotic pathway can be of significant importance during NPs triggered apoptosis processes (Xia et al. 2008).

Reduction of mitochondrial membrane potential initiates the release of cytochrome c into the cytosol and translocation of BAX to mitochondria, thus promoting apoptosis, a phenomenon that was commonly occurring in mammals and zebrafish exposed to NPs (Hsin et al. 2008; Zhao et al. 2013a).

1.3.1.2. DNA damage and repair

Generation of ROS by nanoparticles can subsequently lead to DNA damage in a variety of forms (Bar-Ilan et al. 2013). In the case of oxidative stress causing oxidized lesions of the DNA, one frequently activated repairing mechanism is the Nucleotide excision repair (NER). Nucleotide excision repair pathway is known to be responsible for the NPs induced DNA damage and repairing in zebrafish since the critical genes involved in NER (such as *ercc5* and *baxa*) were altered in zebrafish treated with NPs (Hanawalt and Spivak 2008; Kim et al. 2013; Park and Yeo 2013a). More interestingly, γ-H2AX, the DNA double-strand breaks (DSB) biomarker was detected in zebrafish after the treatment with silver NPs. This may indicate that the non-homologous end joining (NHEJ) or homologous recombination (HR), responsible for DSB repairing, is also involved in the DNA damaging and repairing mechanisms in zebrafish (Choi et al. 2010).

Transition metal-based NPs can dissolve into metal ions. Metal ions can covalently bind with DNA thus causing DNA damage which is consisted with the fact that PBR 322 plasmid was injured under the exposure of Ag^{+} (Onuki et al. 1994; Yeo and Kang 2008). We summarized validated genes reported in relation to DNA damage and repair in zebrafish in Table 2, and used this information to construct a predicted molecular network responsible for NPs induced DNA damage in zebrafish.

1.3.1.3. Inflammatory response

Inflammation is a complex and protective response of an organism against different noxic agents, involving cellular and humoral responses such as the release of chemokines, cytokines, etc. Universal sign of inflammation at biochemical/molecular level is the up-regulation of pro-inflammatory cytokines such as IL-1β and TNFα. Under the stimulation of zebrafish embryos with silica NPs (SiNPs), a genome-wide screening of transcriptome was performed (Hu et al. 2016), revealing that critical pathway for pathogen associated molecular pattern (PAMP) recognition, the toll-like receptor (TLR) complex was activated, indicating that NPs could be recognized as a stressor by the innate immune effectors of zebrafish. Furthermore, activation of *myd88* suggested that SiNPs could induce myd88-dependent classic signaling cascade of the TLR pathway followed by the up-regulation of pro-inflammatory cytokines, and initiation of the zebrafish inflammatory response. This result is consistent with the finding that in mouse cell line, zinc oxide NPs and quantum dots raised cytokine expression through MyD88-dependent TLR pathway, suggesting it's a fundamental mechanism for NPs induced inflammation in both mammals and zebrafish (Chang et al. 2013; Ho et al. 2013). Moreover, the reported study (Hu et al. 2016) that the IL-6 dependent jak1/stat3 pathway was also activated by SiNPs in zebrafish, leading to further activation of pro-inflammatory mechanisms such as mobilization of responsive cells, and increased vascular permeability during acute inflammatory responses in mammals (Elsabahy and Wooley 2013). Furthermore, Zebrafish embryos exposure to zinc NPs resulted in an up-regulation of c-*Jun* N-terminal protein kinase (*jnk*) and activation of pro-inflammatory responses (Brun et al. 2014).

Inflammatory processes induce excessive generation of ROS in various forms, including superoxide anions and nitric oxides, and this is no different in nanoparticle induced defense or inflammatory reactions in either mammals or fish (Alinovi et al. 2015; Fialkow et

al. 2007; Larsen et al. 2010) . The inflammation phenotype was directly observed by confocal laser microscopy in zebrafish larvae treated with titanium dioxide NPs (TiO_2 NPs) as *cxcl12* was upregulated via NF-κB signaling (Maroni et al. 2006) suggesting that other NPs would likely be able to induce inflammation in zebrafish (Yeo and Kang 2012; Yeo and Kim 2010). As TiO_2 NPs can enter mitochondria, it is also possible that respiratory burst may be increased causing increased damage of the organelles, and contributing to pro-inflammatory responses (Aguilera-Aguirre et al. 2009).

Innate immune cells including neutrophils, macrophages, and dendritic cells play key roles in host defenses and inflammatory responses (Dallegri and Ottonello 1997). Neutrophils are usually the first cell type to encounter and react with the potentially noxic particles, releasing pro-inflammatory mediators in both mammals and fish models (Mathias et al. 2006). In fathead minnow (*Pimephales promelas*), neutrophil function was however significantly inhibited by a hydroxylated fullerene NPs exposure (Jovanović et al. 2011a). A corresponding histopathological research in the same model clearly observed a sign of neutrophil congestion upon hydroxylated fullerene NPs treatment (Jovanović et al. 2014). Other NPs (e.g. nano-TiO_2) can also interfere with neutrophil function in fish (Jovanović et al. 2011b). However, the response of neutrophil during NP induced inflammatory processes was rarely investigated in zebrafish. Recent study by Duan et al. tracked a recruitment of neutrophils during low-dose exposure of SiNPs and suggesting that neutrophil mediated cardiac inflammation could induce cardiac dysfunction in zebrafish (Duan et al. 2016).

1.3.2 Histopathological adverse outcomes and related genes

1.3.2.1. Gills

The teleost gills are multifunctional organs involved in gas exchange, ion exchange, and endocrine regulation and are considered as the first line of non-specific fish defenses against invading pathogens (Dos Santos et al. 2001; Evans et al. 2005; Rombough 2007). The direct exposure of gills to the external environment makes them a primary target for interaction with NPs in water, and also to possible damages.

In zebrafish, histopathological abnormalities typically emerged in the gill filaments and lamellas, were induced by different nanoparticles, and appear size and dose dependent. Gills exposed to citrate-coated silver NPs displayed a severe hyperplasia and fusion with a decreased number of erythrocytes and clustered mucoid cells in the secondary filaments. Two-fold increase in thickness of gill filaments was observed in zebrafish exposed to nano-silver. Treatment with nano-copper caused gill lamellae edema and changes in gills on molecular level that include changed expression of genes responsible for bacterial recognition, apoptosis, structural molecule activity, and cell division cycle regulation, therefore indicating that nanoparticles can also affecting functional responses as well as structural integrity of the gills (Griffitt et al. 2009; Griffitt et al. 2007; Osborne et al. 2015). Exposure to NPs inhibited Na^+/K^+ ATPase channel in epithelial cells of gills (Katuli et al. 2014), with a corresponding expression change in the sodium channel complex-related genes, such as *scn1a* and *slc31a1* (Table 2) (Griffitt et al. 2007; Osborne et al. 2015). According to previous studies, NPs can induce histopathological abnormalities in zebrafish gills, inhibition of ion exchange, structural damages of the ion-channels, over-production of mucus and particle aggregation in mucus layer. However, the mechanisms of the changes, as

well as their possible regulation are not completely understood, especially from molecular perspective.

1.3.2.2. Intestine

In teleost fishes, primary route of nanoparticle uptake is considered to be through feed, as mucous layers and tight intercellular junctions in gills and skin may decrease ability of NPs to penetrate the organism. After exposure to silver NPs, zebrafish intestine showed goblet cell hyperplasia, vacuolization and partial loss of microvilli (Osborne et al. 2015), and the Na^+/K^+ ATPase pump was inhibited. No previous studies, that we are aware off, investigated transcriptomic gene expression profiles under the NPs induced toxicity in zebrafish intestine. Therefore we had to assume that the target genes would be likely similar to mammalian studies (Bouwmeester et al. 2011). After an alignment analysis between the zebrafish transcriptome, a variety of related genes was identified, and listed in Table 3.

1.3.2.3. Liver

NPs induced histopathology in the liver is a widely observed phenomenon in fishes including fathead minnow (Jovanović et al. 2014), rainbow trout (Smith et al. 2007) and zebrafish (Choi et al. 2010) having apoptosis as a common endpoint (Choi et al. 2010). Additionally, mRNA expression of several apoptotic-critical genes such as *p53* (tp53), *Bax1* and *Noxa1* have been altered after the NPs treatments (Choi et al. 2010). Transcriptome studies of fish liver after exposure to NPs are not widely available until 2018, a transcriptomic study conducted by Gao et al. indicating that 33 genes were commonly differentially expressed after three types of cadmium nanoparticles exposure in zebrafish

liver (Gao et al. 2018). Meanwhile, we are also using data from studies that had already screened the transcriptomes of mammalian hepatocytes after exposure to NPs, which are sharing similar histopathology outcomes, such as with that of a zebrafish liver (Balasubramanian et al. 2010; Sharma et al. 2012). Therefore, we performed an alignment analysis of those target mammalian genes and the corresponding genes in zebrafish.

1.4. Genotoxicity induced by Nanoparticles

1.4.1. General introduction of Nanoparticles triggered genotoxicity and mode of actions

Increase in various industrial applications of nanoparticles is accompanied by acceleration in quantity and variety of NPs produced annually (Hendren et al. 2011). Such rapid changes in nanoparticle use are raising awareness about their potential adverse effects on environmental fate and public health. Major mechanisms underlying the concern for NPs induced toxicity are their inflammatory potency, oxidative stress induction, and ability to trigger genotoxicity (Fadeel et al. 2017).

Among the listed mechanisms, genotoxicity presents itself as the major concern, since such inducible mutagenesis by NPs could be related to increased risk of carcinogenicity. Regulatory toxicologists are taking a more serious consideration regarding the mutagenic carcinogens because the exposure thresholds for this type of toxic agents doesn’t exist, and only “Benchmark Doses” that are derived from reference exposure values are applicable according to EFSA (European Food Safety Authority) guidelines (Authority 2005). Furthermore, the ECHA (European Chemicals Agency) updated the guidance for classification, labelling and packaging (CLP) of substances and mixtures with an emphasis on

genotoxic carcinogens by classifying and labeling this kind of toxins separately (ECHA 2015). Even as number of research papers regarding the NPs genotoxicity is increasing in recent years, contradicting statements from those reports do not yet appear to be resolving (Chen et al. 2014; Paget et al. 2015).

Generally speaking, the mechanisms that are involved in the NPs genotoxicity are not fully understood, and two major processes that could trigger the genotoxicity of NPs have been frequently observed: 1) direct interaction between NPs and DNA or 2) indirect DNA damage caused by NPs generated ROS or other toxic ions (Colognato et al. 2008; Kisin et al. 2007). Direct DNA damage induced by NPs starts after crossing of cellular membranes and reaching of nucleus. This characteristic of NP translocating to nucleus is generally shared by various NPs, and is being widely used in Nanoparticle-based drug delivery and transfection systems (Ghosh et al. 2008; Sokolova and Epple 2008). Several NPs were found in the cell nuclei area after *in vitro* exposure, including gold NPs (Au NPs) and ZnO NPs (Gu et al. 2009; Hackenberg et al. 2011). When NPs persisted in the nucleus for extended periods, they showed tendency to aggregate in larger particles causing mechanical damage to chromosomes or even deformations of the nucleus (Di Virgilio et al. 2010).

NPs can also induce genotoxicity without direct contact with DNA. For example, different free radicals appear to be increasingly generated on the surface of NPs due to their high surface to mass ratio, and this phenomenon was reported by several studies (Barillet et al. 2010; Shukla et al. 2011). If these free radicals interact with DNA, they can cause single strand DNA damages such as oxidized base lesions, or DNA double strand breaks (Karanjawala et al. 2002). Moreover, some soluble NPs such as Au NPs or silver NPs could release transition metal ions. These ions could covalently bind with DNA or induce the generation of intracellular ROS by Fenton type reactions (Murata-Kamiya et al. 1997; Yeo and Kang 2008).

1.4.2. Titanium dioxide and Polystyrene Nanoparticles induced genotoxicity

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in sunscreens and pigments worldwide. However, the potential adverse effects of TiO_2 NPs have also been reported and are a cause for concern. TiO_2 nanoparticles can interfere with inflammatory responses, environmental biofilms, and can also cause DNA damage in experimental animals (Jovanović et al. 2011b; Jovanović and Guzmán 2014; Park et al. 2009; Woodruff et al. 2012). Comet assay is used as standard toxicological assay for evaluating DNA strand breaks *in vitro* (Fairbairn et al. 1995), including measuring the DNA damage induced by TiO_2 NPs. (Chen et al. 2014). It was observed that DNA strands damage is triggered by presence of TiO_2 NPs with lowest reported concentration that could induce this damage of 1 $\mu g\ ml^{-1}$ (Shukla et al. 2013). Since the crystalline structure is strongly related to the toxicity of TiO_2 NPs, a general consideration is that TiO_2 NPs in its anatase form exhibits higher toxicity than the rutile phase due to different surface properties (Jin et al. 2011). However, it also appears that a mix of crystalline forms of TiO_2 NPs (anatase, and also 80% anatase with 20% rutile) induced comparable DNA strand damage and breaks (Valdiglesias et al. 2013).

Polystyrene is known to be one of the most frequently used organic polymers in our daily life. Multiple studies addressing risks of polystyrene contamination or pollution focused on the environmental perspective because of the long half-life and its persistence in the environment (Pruter 1987). Polystyrene microspheres recently prompted further research, as their potential for adverse effects of oxidative stress and genotoxicity was discovered in the aquatic models (Avio et al. 2015; Jeong et al. 2016). However, risk of genotoxicity induced by nano-sized polystyrene particles (PS NPs) remains unclear as there is limited information available (Liu et al. 2011c; Paget et al. 2015). An intracellular dynamic imaging study

indicated that cationic functionalized PS NPs could result in a prolonged G0/G1 phase in the cell cycle during mitosis in NIH 3T3 cells, therefore indicating potential for DNA damage and the interference with checkpoint control activation (Liu et al. 2011c). Paget et al. indicated that non-functionalized PS NPs did not induce a general genotoxicity except at the highest tested dose of after 8.1 μg/cm^2 exposure for one hour (Paget et al. 2015). Therefore, the potential for genotoxicity of PS NPs regardless of their surface modification, requires further investigation. We are not aware of any studies that reported activation of molecular regulatory mechanisms post exposure of PS NPs at transcriptional level. Therefore, we also conducted a study to investigate transcriptional level of molecular regulation underlying potential PS NPs induced genotoxicity.

1.4.3. MicroRNAs in DNA damage repairing

MicroRNAs are endogenous gene regulatory molecules in various organisms playing an important role in diverse biological processes including DNA damage repair. miRNAs are involved in multiple regulatory pathways of DNA damage repair (DDR) and it is frequently observed that a single miRNA can regulate multiple DDR mechanisms. For example, one of miR-155 targets, *WEE*1, is involved in cell cycle checkpoint regulation (Pouliot et al. 2012), and it was up-regulated after exposure to with gold NPs and reported to be involved in MAPK pathway upon the cellular stress (Ceppi et al. 2009; Grogg et al. 2016). These studies indicate that use of miRNAs as potential tools and biomarkers in the risk assessment for nanoparticles has significant potential. Also, regarding the complexity of the miRNA-mRNA regulations in the DNA damage, the intricacy of miRNA regulations underlying nano-toxicity is expected. Therefore, further research is needed to elucidate multiple possible correlations between miRNAs and nanoparticle-induced DNA damage.

1.5. Objectives of the study

Based on the literature review and identification of existing gaps in information related to role of miRNA regulatory function during toxicological responses of an organism to nanoparticle exposure, we have developed two major study objectives:

1.5.1. Objective 1

The first objective was to summarize the biological adverse outcomes of NPs induced toxicity and determine which genes are responsible for this toxicological effect in a standardized vertebrate model used in aquatic toxicology: Zebrafish (*Danio rerio*). As part of this objective, we depicted six different miRNA-mRNA regulation networks, both biological-specific and tissue-specific, by targeting validated genes with miRNAs in the zebrafish miRNAs database. We also highlighted top predicted miRNAs and entitled them as the potential biomarkers in zebrafish nano-toxicology.

1.5.2. Objective 2

Our second objective was to verify the time and dose dependent DNA damage induced by Polystyrene and Titanium dioxide Nanoparticles in human monocytic THP-1 cell line. As part of this objective, the expressions of top predicted miRNAs in the previous *in silico* prediction were validated, and to select the miRNA biomarker with highest potential for Polystyrene nanoparticles and ARS labeled Titanium dioxide nanoparticles exposure. The miRNA functional investigation regarding the selected potential miRNA biomarker and its regulation towards DNA damage responsible genes was performed in the final step of this objective.

1.6. Data and prediction summarizing tables

Table 1. Validated microRNAs and their relevant functioning molecular pathways influenced by encounters with nanoparticles and induced toxicity with their Orthologues in Zebrafish

The summary of the validated miRNAs that were altered during Nanoparticle exposure in vertebrates (mostly mammals), these miRNAs were categorized into toxicology-related signaling pathways.

Organism	Toxicant	Representative altered miRNAs	Valid / potential target mRNA	Corresponding miRNAs in Zebrafish	Orthologues in Zebrafish	Targeting statues in Zebrafish (Predicted)	Biologic effect	Relevant pathways	Study
Human Jurkat T Cell line	Silver nanoparticles (AgNPs)	hsa-miR-219-5p, -654-3p, -504	*MT1F*, *TRIB3*, *ENDOGL1*	dre-miR-219-5p	*trib3*	miR-219-5p: *trib3*	DNA damage, apoptosis	p53 signaling pathway, Cell cycle, HIF-1 signaling pathway	(Eom et al. 2014)

Mice (Transgenerational toxicity)	Gold nanoparticles (AuNPs)	mmu-let-7a, mmu-miR-183, -16, -17, -196a, -467, -185	*Ras, Myc, Pten, Tgf-beta*	dre-let-7a, dre-miR-183-5p	*mal2, cep97, slain1a,*	N/A	Chromosome damage, mitochondrial damage	TGF-beta signaling pathway, c-Myc pathway	(Balansky et al. 2013)
Mice	TiO2 NPs	mmu-miR-449a, -1, -135b, -144, -133, -21	*Runx2, Phosphodiesterase 8b, Lef1*	dre-miR-1, -135a, -144-5p, -133, -21	*runx2a, pde8b, lef1,*	miR-135a, -144-5p: *runx2a*; miR-1, -135a, -144-5p: *pde8b;* miR-135a: *lef1*	Altered expression in genes associated with acute phase and immune response	WNT signaling, Cytokine-cytokine receptor interaction	(Halappanavar et al. 2011)

NIH/3T3 mouse embryonic fibroblast line	CdTe quantum dots (CdTe QDs)	mmu-miR-29a, -93, -145, -214	*Znfx1, Fam45a, Epha4, Col4a5*	dre-miR-29a, -93, -145-5p, -145-3p, -214	*znfx1, fam45a, epha4a, col4a5,*	miR-29a: *znfx1;* miR-145-5p, -145-3p, -214: *fam45a;* miR-145-5p, -3p: *epha4a;* miR-29a, -93: *col4a5*	Apoptosis-like cell death	p53 signaling pathway	(Li et al. 2011b)
Human embryonic stem cell (hESC)-derived neural stem cells (NPCs)	AgNPs	hsa-miR-297, -132, -22, -27b, -196b, -1226	*HMOX1, GSTA4, FTL, HERPUD1, HSP40*	dre-miR-132-3p, -22a-3p, -27b-3p, -196a-5p	*hmox1a, gsta.1, FTL, herpud1*	miR-132-3p, -27b-3p: *FTL;* miR-22a-3p, -27b-3p:*herpud1*	Oxidative stress, cellular apoptosis, cell population in sub-G1 increased	NRF2-mediated oxidative stress response, eNOS signaling, Rho family GTPase signaling	(Oh et al. 2016)

Human MRC5 lung fibroblast line	AuNPs	hsa-miR-155	*PROS1*	dre-miR-155	*pros1*	N/A	Chromatin condensate	MAPK signaling pathway, PI3K/Akt pathway	(Ng et al. 2011)
NIH/3T3 mouse embryonic fibroblast line	Multi-walled carbon nanotubes (MW-CNTs)	mmu-miR-34a, -21, -29a	N/A	dre-miR-34a, -21, -29a	N/A	N/A	Cellular viability decreased	Wnt signaling pathway, MAPK pathway, TGF-beta signaling pathway	(Li et al. 2011a)
Mice	Carbon black nanoparticles (CBNPs)	mmu-miR-135a, -146b, -21, -146a	*Adamts9, Bmper, Klf4, Cxcl12, Rrbp1, Cxcl10*	dre-miR-135a, -146b, -21, -146a	*adamts9, bmper, klf4, cxcl12a, rrbp1a*	dre-miR-146b, -21: *adamts9* dre-miR-21: *bmper*	Strong and persistent pulmonary inflammation	Wnt signaling pathway, Leukocyte transendothelial migration	(Bourdon et al. 2012)

Human dermal fibroblasts (HDFs)	AuNPs	hsa-miR-30b, -148a, -181a, -20a, -19b, -20b, -222	*FH, HNRPK, ATP5H, HSPA5, HSPA8*	dre-miR-30b, -148, -181a-5p, -20a-5p, -19b-3p, -19c-3p, -20b-5p, -222a-3p	*fh, hnrnpk, atp5h, hspa5, hspa8,*	dre-miR-181a-5p, -222a-3p: *fh* dre-miR-30b, -19b-3p, -19c-3p: *hspa8*	Cell proliferation rate decreased	MAPK signaling pathway, mRNA processing pathway	(Huang et al. 2015)
Human neuronal CATH / microglia C8-B4 cell line	Metallic NPs (MnNPs)	hsa-miR-124-3p, -1-3p, -16-5p, -155-5p, let-7a-5p	*TNF, IL-6R, IL-1B, IFNG*	dre-miR-124-3p, -1, -16a, -155, let-7a	*tnfa, il6r, il1b*	dre-miR-124-3p, -let-7a: *tnfa* dre-miR- let-7a: *il6r* dre-miR-16a: *il1b*	Cell viability decreased, ROS generation increased	IL-6 signaling, NF kappa B signaling, IL-8 signaling	(Grogg et al. 2016)
Mice	AuNPs	mmu-miR-140-5p, -29b-3p, 327	*Txnrd1, Canx, Syn1, IL-1α*	dre-miR-140-5p, -29b,	*canx, syn1*	dre-miR-140-5p: *canx*	Mild systemic inflammation, proinflammatory cytokine expression increased	N/A	(Ng et al. 2016)

Human osteoblast cells MC3T3-E1 cell line	AgNPs	hsa-miR-374, -124, -325, -16, -503, -130	*BMP2*, *BMP3*, *BMPR1A*, *BMPR1B*, *BMPR2*, *CRIM1*, *BMP6*, *BMP7*, *BMP8A*, *BMP8B*	dre-miR-124-5p, -16a, -16b, -130a	*bmp2a*, *bmp3*, *bmpr1aa*, *bmpr2a*, *crim1*, *bmp6*, *bmp7b*, *bmp8a*	dre-miR-124-5p, -16a: *bmpr1aa* dre-miR-130a: *bmpr2a* dre-miR-16a, -16b: *crim1* dre-miR-124-5p, -16a: *bmp6*	Cellular mineralization enhanced	Wnt/β-catenin pathway	(Mahmood et al. 2011)
Human germ cell GC-2 cell line	Silica NPs	hsa-miR-98	*HUWE1*, *CASPASE-3*	dre-let-7h	*huwe1*, *casp3a*	N/A	Cellular permeabilization increased and mitochondrial membrane potential decreased	Caspase mediated apoptosis pathway	(Xu et al. 2015)

Mice	Silica NPs	mmu-miR-122, -192,- 194	N/A	dre-miR-122, -192, -194a	N/A	N/A	Severe liver damage	N/A	(Nagano et al. 2013)

Table 2. The three major biological toxic responses of NPs exposure: oxidative stress, DNA damage and inflammation and the altered genes that responsible for these three adverse outcomes in zebrafish.

The summary of the adverse outcomes after nanoparticle exposure in zebrafish. Adverse outcomes were categorized as three major NPs induced biological toxic responses: oxidative stress, DNA damage, and inflammation. The respective altered genes in zebrafish during NPs exposure were summarized as well.

Nanoparticles	Exposure dose	Exposure time	Species	Biochemical alternation	Major Biological response	Altered genes	Additional toxicity endpoints	Study
TiO_2 NPs	50 mg/l	96h	Zebrafish adult	SOD activity decreased in liver, increased in gut. CAT activity reduced in liver. GSH concentration decreased in liver, increased in gut.	Oxidative stress			(Xiong et al. 2011)
ZnO NPs	5 mg/l	96h	Zebrafish adult					(Xiong et al. 2011)

Multiwall carbon nanotubes (MWCNTs)	100 ul/well (96-wells plate)	12,24,36, 48 hpf	Zebrafish embryo				Hatching delay, MWCNTs entered the embryo through the chorion	(Lu et al. 2017)
ZnO NPs	10,30,60,90 ,120 mg/l	96 hpf	Zebrafish embryo	SOD and MDA activity increased, caspase3 and 9 activities increased, ROS exceedingly generated		*cat, gpx1a, sod1, ppara alpha, bcl-2a, bcl2b, bax1, apaf-1, bbc3*	Decreased hatching rate, apoptotic cells increased	(Zhao et al. 2016)
Cobalt ferrite NPs	0,10,63,125 ,250,500 uM	7 dpf	Zebrafish larvae	ROS generated, MDA content, *mu*-GST and AP activities increased			Hatching rate decreased up to 90%, heart beat rate increased	(Ahmad et al. 2015)
CuO NPs	5,20,40,60, 80 mg/l	96 hpf	Zebrafish embryo	Dose-dependent ROS generation, LPO levels, PC content, NO levels increasing	Oxidative stress		Dose depended lethality increasing, heart beat rate retardation, hatching rate decreasing	(Ganesan et al. 2016)
C60 NPs/Micro Particles	7.5, 15, 30 mg/kg	24 h	Zebrafish adult	AChE activity enhanced lipid peroxidation augmented		*ache*		(Dal Forno et al. 2013)

ZnO NPs	1, 5, 10 ,20 ,50 ,100 mg/l	144 hpf	Zebrafish embryo	SOD activity, MDA content and CAT activity increased, GPx content not altered, ROS formation increased		*gstp2, ucp2, nqo1*	Hatch rate decreasing, malformation rate increasing	(Zhao et al. 2013b)
Ag NPs	500 mg/l	2, 5, 8, 22, 27, 32, 48, 52, 72 hpf	Zebrafish embryo		DNA damage	*sels, h2afx*	Notochord abnormality and curved tail	(Yeo and Kang 2008)
TiO_2 NPs (Citrate coated)	500 µg/mL	120 hpf		8-hydroxydeoxyguanosine (8-OHdG) level increased	DNA damage		Pericardial edema, craniofacial malformation, and opaque yolk	(Kim et al. 2014)
AuNPs	30 mg/L	120 hpf	Zebrafish embryo		DNA damage	*tp53, baxa, pax6a, rx1, sox10, mitfa, otx*	Developmental eye defected and cell death	(Kim et al. 2013)
TiO_2 NPs (Light illuminated)	0.01-10000 ng/mL	0-23 dpf	Zebrafish embryo-larva	8-OHdG level increased	DNA damage		Stunted growth, delayed metamorphosis, malformations, organ pathology	(Bar-Ilan et al. 2013)

Cu_xTiO_y (Cu loaded TiO_2 NPs) NPs	10, 20 mg/L	2- 72 hpf	Zebrafish embryo	Glutathione increase, catalase activity increase, GST increase		Mutated embryos with abnormal notochord formation	(Yeo and Kang 2009)
ZnO NPs	50 mg/L	48-96 hpf	Zebrafish embryo			body length, heart rate and hatch rate were decreased	(Du et al. 2014)
Ag NPs	20 mg/L	2-72 hpf	Zebrafish embryo		DNA damage	314 genes in total, significantly altered genes: *ercc5*, *usp20*, *dapk*1, *slc11a2*, *cib1*, *dap3*, *gtf2h2*, *dab2*, *trip10a*, *dnaja3a*, *cxcl12b*, *amfr*, *dnaja2*, *hsf1*, *dlg5a*, *hif1ab*, *dhcr24*, *sh3bp4a*, *cdh24*, *usp20*, *enpp2*, *mark1*, *ahr1b*, *herpud1*, *dnajb11*	Embryo death rate increased	(Park and Yeo 2013a)
TiO_2 NPs	20 mg/L	2-72 hpf				360 genes in total, significantly altered genes : *f3b*, *gclc*, *bada*, *mcl1a*,	

					ypel3, *apoea*, *rhot1a*, *pycard*, *hdr*, *ripk2*, *dnajb12*, *foxa1*, *lcp1*, *sap30bp*, *agap2*, *wnt11r*, *spata4*, *apc*, *jmjd6*, *anxa3b*, *trip10a*, *usp33*, *grb2a*, *mib1*, *cfb*, *tnfb*, *mhc1laa*, *polr3b*, *fkbp5*, *dnajb9a*, *chordc1a*, *yod1*, *sec63*		
Ag nanotube	1 µg/L	72 hpf	Zebrafish embryo	Inflammation	*lgals3b*, *caspb*, *nt5c3a*, *thnsl2*, *map2k2a*, *slc45a3*, *mif4gdb*, *pvalb5*, *il15l*, *c6*	Genes required for embryogenesis are down-ragulated	(Park and Yeo 2015)
ZnO NPs	0.2, 1 and 5 mg/L	48-168 hpf	Zebrafish embryo and eleuthero-embryos	Pro-inflammatory cytokines alternated	*tnfa*, *il10*, *il-1β*, *c-jun*, *stat1a*, *mxA*	Hatching delay	(Brun et al. 2014)
Silica NPs (SiNPs)	Microinjected with 10 nL SiNPs	6, 24 hpf	Zebrafish embryo	Large scale of inflammatory responsible pathways activated	*cbx7a*, *fosl1a*, *cfb*, *timp2b*, *mmp9*, *junba*, *il13ra1*, *tnfrsf1a*, *cxcr4b*, *lepa*, *nfkbiaa*, *ctsk*, *tlr5b*, *myd88*, *ticam1*, *tlr4ba*, *cd40*		(Hu et al. 2016)

TiO_2 NPs	20 mg/L	2-52 hpf	Zebrafish embryo and larvae	Inflammation	*cxcl12b*, *ifngr1*, *tnfβ*, *elmod2*, *elmo1*, *dab2*, *lmbr1l*, *wnt11*, *mib1*, *jmjd6*, *tnfsf10l*, *tnfsf10l4*, *sh3bp4*, *il10*	Hatching rate decreased, abnormality emerged	(Yeo and Kang 2012; Yeo and Kim 2010)
SiNPs	Microinjected with 10 nL SiNPs	2-72 hpf	Zebrafish embryo	Inflammation	*atp2a1l*, *atp1b2b*, *atp1a3b*, *cacna1ab*, *cacna1da*, *tnnc1a*	Pericardial edema, accumulation of neutrophils	(Duan et al. 2016)

Table 3. Histopathological damage of three major organs and related genes upon nanoparticles induction in zebrafish

The summary of nanoparticles induced histopathological damage and genes that are altered during this damage in zebrafish.

Nano	Size diameter	Exposure dose	Exposure time	Species	Organ	Histopathological abnormality	Altered genes
Nano Copper (Griffitt et al. 2009)	26.6+-8.8 nm	1000ug/l	48 hours	Zebrafish adult	Gill	Gill filament edema	*aspn, ahcy, hey1, ctsla, sepp1a, per1b, pgam1a, cd74b, tmem47, ybx1, bcap31, ccnb1, atp1b1b, si:dkey-33i11.4, kcnj1a.1, tbcb, sepw1, ssb, ifi30*
Nano Silver (Griffitt et al. 2009)	26.7+-7.1	100 ug/l	48 hours	Zebrafish adult	Gill	Gill filament edema	*sparc, gnb1b, mxa, atp1b1b, spp1, pmp22a, mmp9, cad, sars, krt18, rpn1, phb, ncl, cdca7a, fbl, npm1a, aspn*
Nano Copper (Griffitt et al. 2007)	80nm	0.25 mg/l, 1.5mg/l	48 hours	Zebrafish adult	Gill	Edema emerged in gill lamellae	*hif1ab, hsp70.1, slc31a1, slc31a2, scn1a*

Citrate-coated Silver NPs (Osborne et al. 2015)	20nm, 110nm	1 mg/l, 2.5 mg/l, 5 mg/l	4h, 24h, 4 days	Zebrafish adult	Gill	Fusion and hyperplasia in secondary filaments, decreased number of erythrocytes and clustered mucoid cells	
Citrate-coated Silver NPs (Osborne et al. 2015)	20nm, 110nm	1 mg/l, 2.5 mg/l, 5 mg/l	4h, 24h, 4 days	Zebrafish adult	Intestine	Goblet cell hyperplasia, vacuolization and partial loss of microvilli	
Manganese dioxide-Cadmium NPs, Hydroxyapatite-$CdCl_2$ NPs (Gao et al. 2018)	20nm, 40nm	0.64 mg/L	96 hours	Zebrafish adult	Liver		*ela3l, acer, 1mmp13a, amy2a, cel.1, c6ast3, nos2a, cap1, zgc:112160, cela1, cpb1, si:dkey-14d8.6, zgc:136930, si:dkey-183i3.5, ela2, ela2l, apoda.2, ctrl, si:dkey-78l4.6, prss59.2 si:ch211-240l19.8, prss59.1, ctrb1, sycn, si:dkey-30j10.5, mfap4, cxl34c, tgm8, gstp2, sycn.2, cu855711.2, si:dkey-33b17.3, si:cabz01012857.1*
AgNPs (Choi et al. 2010)	5-20nm	30mg/l 60mg/l 120mg/l	24 hours	Zebrafish adult	Liver	Disruption of hepatic cell cords and apoptosis changes including chromatin condensation and pyknosis	*bax1, blp1, cat, gpx1a, mt2, noxa1, cdkn1a, tp53, sod1*

Table. 4. Targeting prediction results of six selected miRNAs in the network

In silico prediction result of the selected six critical miRNAs in zebrafish, following the selection criteria stated in section 2.1.2.

miRNAs	Predominantly regulated in Oxidative stress network	Predominantly regulated in DNA damage network	Predominantly regulated in Inflammation network	Predominantly regulated in Liver specific network	Predominantly regulated in Gill specific network	Predominantly regulated in Gut specific network	No. of genes regulated in all networks	No. of validation in mammalian studies	No. of validation in Zebrafish study(Duan et al. 2017)
dre-miR-124	No	No	Yes	No	No	No	35	2	0
dre-miR-144	Yes	Yes	Yes	No	Yes	No	63	1	0
dre-miR-148	Yes	Yes	Yes	No	No	Yes	52	2	0
dre-miR-155	Yes	No	No	No	No	No	15	2	0

dre-miR-19a	Yes	No	Yes	No	No	No	41	1	1
dre-miR-223	No	No	No	Yes	Yes	Yes	30	0	0

2. MATERIAL AND METHODS

2.1. Methodology: Prediction of MicroRNA-mRNA interacting nano-toxicological network in zebrafish

2.1.1. Data summary and *in silico* miRNA-mRNA targeting prediction

NPs are known to induce toxicity in zebrafish and alter gene expression profiles as described above and presented in Tables (1-3). The leading assumption of performed *in silico* analysis is that miRNAs may be involved in regulation of expression of these genes, serving as major mediator through their interaction with mRNAs. Here, we constructed six tissue-specific (TSNs) or biological response-specific (BRSNs) miRNA-mRNA regulation networks affected by nanoparticle exposure influence through miRNA-mRNA association predicting bioinformatics algorithms.

Sequences of all the miRNAs were obtained from miRbase (http://www.mirbase.org/), and the classifications of miRNA tissue distribution in zebrafish were as per Thatcher et al (Thatcher et al., 2008). The target genes in the tissue-specific predictions are listed in Table 3. In addition, for liver and intestine network depiction we collected microarray data from mammalian studies and summarized them in terms of NPs affected zebrafish genes in liver and intestine respectively, through an alignment analysis by clustalX (http://www.clustal.org/). These three lists of genes are referred to as "Tissue-specific summarized gene lists" (TSNs), and these TSNs were based on the prediction of interactions between tissue-specific miRNA and tissue-specific altered genes. For construction of the biological response-specific network, genes involved in: oxidative stress,

DNA damage and inflammation were used to make the corresponding gene lists, shown in table 2 and defined as "Summarized gene lists". These three networks were also supplemented with additional genes known to be involved in the following biological processes:

1. Zebrafish genes identified as peroxidases, peroxiredoxins and genes involved in ROS metabolism were provided by Qiagen zebrafish oxidative stress PCR array (http://www.sabiosciences.com/rt_pcr_product/HTML/PAZF-065Z.html). This list of genes is referred to as "Additional gene list for Oxidative stress" in this study;

2. Genes engaged in the human DNA damage signaling pathways while responsible for ATR signaling, DNA repair and cell cycle were obtained from http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-029Z.html. List of corresponding zebrafish orthologues was verified through alignment and named "Additional gene list for DNA damage";

3. Zebrafish genes in Gene Ontology terms of cytokine production (GO:0001816), cytokine-mediated signaling pathway (GO: 0019221), inflammatory response (GO:0006954), activation of innate immune response (GO:0002218) and inflammasome complex (GO:0061702) were retrieved by BiomaRt package embedded in the Bioconductor software (https://bioconductor.org/packages/release/bioc/html/biomaRt.html) and referred as "Additional gene list for Inflammatory response" after removing the redundant genes (Durinck et al., 2009).

Targetscan version 6.2 was used to make zebrafish miRNA target predictions http://www.targetscan.org/fish_62/. The whole miRNA-mRNA association prediction was based on the crossover prediction by Targetscan-zebrafish database and miRanda algorithms (http://www.microrna.org) (Betel et al., 2010; Grimson et al., 2007). Reliable predictions

between miRNA and mRNA were defined by the intersection thresholds as Targetscan (Context score < -0.2) and miRanda (Pairing score > 140 and energy score < -7). Throughout the manuscript, 3' untranslated region nucleotide sequences of all genes were retrieved from Ensembl-Gene 87 database (http://www.ensembl.org/index.html) unless otherwise stated. The depiction of TSN was based on the targeting of tissue specific miRNAs and objective genes while the construction of BRSN was based on the prediction between all the zebrafish miRNAs in miRbase towards "Summarized gene lists" plus "Additional gene lists".

2.1.2. Selection criteria for critical miRNAs

To emphasize the importance of genes which are altered after NPs exposure, based on the existing references, a selection criteria system was developed in order to determine potential biomarkers of nanotoxicity from the miRNA molecular database. Reliable predictions based on genes from summarized gene lists in BRSNs were assigned two prediction points while predictions targeting genes in the additional gene lists were assigned one prediction point (Figure 1). Then, the "TOP 15" miRNAs with the highest score from these three BRSNs were chosen and named as "miRNA candidates". The frequency of their participation was evaluated by counting the number of overlaps of these miRNA candidates in the three networks. miRNAs that overlapped in all three networks with highest overall prediction score were selected. Table 1 is a summary of all existing validated nanotoxicity related miRNAs from mammalian studies and it contains their alignment to the corresponding miRNAs in zebrafish. Zebrafish miRNAs orthologues that were confirmed by multiple mammalian studies were selected from "miRNA candidates" regardless of how many times they were overlapped in BRSNs. Moreover, a recent published study stated some of the influenced miRNAs upon the induction of particulate matter in zebrafish (Duan et al., 2017).

This publication was also utilized as a selection preference in our selection and referred to as "zebrafish study" in figure 1 and table 4. All miRNA candidates that were overlapped in more than two BRSNs while being validated in the mammalian study or zebrafish study were selected as the result. In each TSNs, miRNAs with top 15 prediction times were chosen and their overlapping times in three TSNs were counted. miRNAs that were predominately regulated in all TSNs were selected as the tissue-specific final potential biomarker for nano-toxicity in zebrafish. The flow diagram of whole selection process including selection criteria is shown in figure 1.

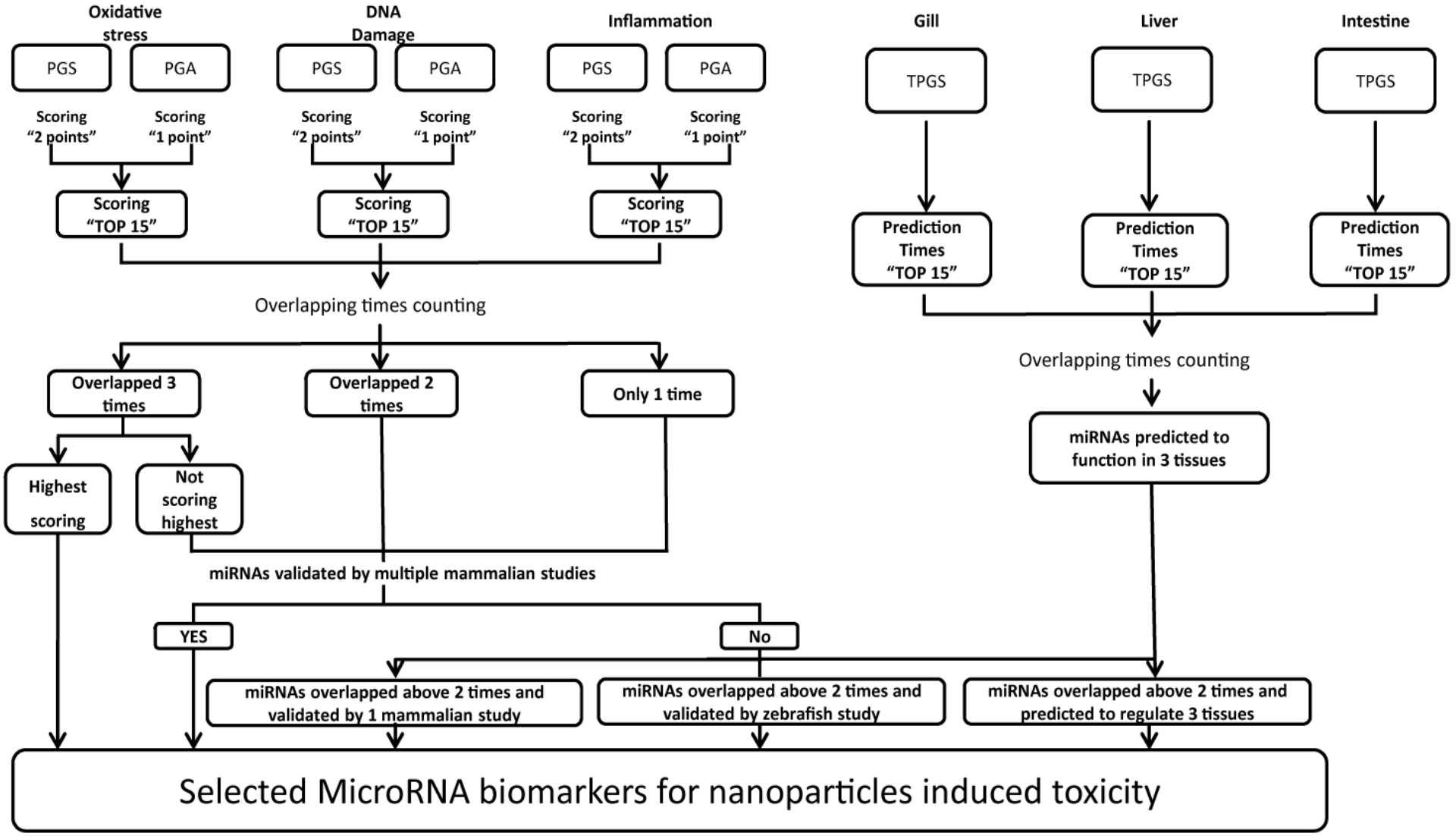

Oxidative stress
DNA Damage
Inflammation
PGS
PGA
PGS
PGA
PGS
PGA
Scoring "2 points"
Scoring "1 point"
Scoring "2 points"
Scoring "1 point"
Scoring "2 points"
Scoring "1 point"
Scoring "TOP 15"
Scoring "TOP 15"
Scoring "TOP 15"
Overlapping times counting
Overlapped 3 times
Overlapped 2 times
Only 1 time
Highest scoring
Not scoring highest
miRNAs validated by multiple mammalian studies
YES
No
Gill
Liver
Intestine
TPGS
TPGS
TPGS
Prediction Times "TOP 15"
Prediction Times "TOP 15"
Prediction Times "TOP 15"
Overlapping times counting
miRNAs predicted to function in 3 tissues
miRNAs overlapped above 2 times and validated by 1 mammalian study
miRNAs overlapped above 2 times and validated by zebrafish study
miRNAs overlapped above 2 times and predicted to regulate 3 tissues
Selected MicroRNA biomarkers for nanoparticles induced toxicity

Fig. 1. Critical MicroRNA selection criteria

Every reliable prediction based on genes from summarized gene lists in biological response-specific networks (BRSNs) was assigned 2 prediction points while predictions targeting genes in the additional gene lists were assigned 1 prediction point. Than "TOP 15" miRNAs with the highest score from these three BRSNs were chosen and named as "miRNA candidates". The frequency of their participation was evaluated by counting the number of overlaps of these miRNA candidates in the three networks. miRNAs that overlapped in all three networks with highest overall prediction score were selected. Zebrafish miRNAs orthologues that were confirmed by multiple existing mammalian studies were selected from "miRNA candidates" regardless how many times they were overlapped in BRSNs. The "Zebrafish study" in this figure refers to the study conducted by Duan J, *et al*. All miRNA candidates that were overlapped in more than two BRSNs while being validated in the mammalian study or zebrafish study were selected as the result. In each Tissue-specific Networks (TSNs), miRNAs with top 15 prediction times were chosen and counting their overlapping times in three TSNs; miRNAs that were predominately regulated in all TSNs were selected.

Abbreviations in this figure, PGS: Prediction based on Genes in the Summarized list, PGA: Prediction based on Genes in the Additional list, TPGS: Tissue specific prediction based on Genes in the Summarized list

2.2. Methodology: *in vitro* mutagenesis evaluation and the potential role of MicroRNA regulation induced by Polystyrene and Titanium dioxide Nanoparticles in THP-1 cell line

2.2.1. Nanoparticles preparation and characterization

Polystyrene Nanoparticle (Cat. #17149-10) was purchased from Polysciences, Inc. (Warrington, PA, USA). The stock solution of polystyrene nanoparticles (PS NPs) was in a form of 2.5% aqueous water suspension with internally fluorescent labeling (Fluoresbrite Yellow Green; ex./em. 480/520) and a nominal mean diameter of 0.05μm. Original PS NPs were centrifuged and resuspended with Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS) (HyClone Laboratories Inc, USA). Anatase Nano-TiO_2 nanopowder with a primary diameter below 25 nm (Sigma-Aldrich Corp, USA) was used in our experiment. Alizarin Red S (ARS) (Sigma-Aldrich Corp, USA) was used for TiO_2 nanoparticles (TiO_2 NPs) fluorescently labeling referring to the labeling method indicated by Thurn et al. (Thurn et al. 2009). ARS was diluted in distilled water until the concentration of 4 mM (pH=5.7-6) and mixed with TiO_2 NPs and stirred for 2 hours in room temperature. After stirring, the mixture was vortexed for 1 hour and centrifuged to eliminate the excessive staining. The HBSS was replenished for each time and the whole procedure repeated three times. The labeled TiO_2 NPs was referred as TiO_2-ARS NPs in our remaining context. Then, the characterization of TiO_2-ARS NPs and PS NPs were kindly performed by Dr. Frits Kamp by using dynamic light scattering (DLS) (Malvern Instruments, UK) for nanoparticle size distribution and zeta-potential. Unlabeled TiO_2 NPs were characterized as previously described (Jovanović et al. 2011b).

2.2.2. Cell culture

THP-1 monocytic cell (American Type Culture Collection, ATCC reference number TIB-202TM) was a kind gift from Prof. Peter Nelson. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich Corp, USA) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine-streptomycin-penicillin at 37 °C in 5% CO2. THP-1 monocytes were seeded in cell seeding plates and differentiated to macrophages by 24 h stimulation with 30 ng ml^{-1} phorbol myristate acetate (PMA) (Sigma-Aldrich Corp, USA). Adherent cells were washed three times with PBS before all subsequent experiments.

2.2.3. Single cell gel electrophoresis (Comet) assay

THP-1 cells were seeded and differentiated in 24-well plates (Thermo Scientific, USA) at a density of 5×10^4 cells each well for the comet assay. The adherent cells were either exposed to PS NPs or TiO_2-ARS NPs at a concentration of 10, 100, 500 μg ml^{-1} for 4, 8, 12 and 24 hours. Cells that were exposed by 10μM Hydrogen peroxide (H_2O_2) were referred as positive control groups, 20 μl HBSS added groups were referred as vehicle control groups and all control groups were exposed for an equal period of time as each NPs exposed groups. Every group of cells was harvested with 0.05% trypsin-EDTA (Thermo Scientific, USA) and re-suspended in the culture medium supplemented with 10% FBS. Slides were pre-coated with 1% Normal Melting point Agarose (Fisher Scientific, USA) and harvested cells were mixed with Low Melting point Agarose (Fisher Scientific, USA). For alkaline comet assay, one slide was prepared from each well (three well/concentration) and kept overnight at 4°C in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH10) with 1% Triton X-100 (Sigma-Aldrich Corp, MO, USA). The slides were subjected to freshly prepared electrophoresis buffer (1 mM EDTA sodium salt, 300 mM NaOH, pH>13) for DNA

unwinding and subsequently, electrophoresis was performed at 0.7 V/cm and 400 mA at 4°C for 30 min in the electrophoresis buffer. The excess alkali was neutralized with Tris buffer (400 mM, pH 7.5) and air dry in room temperature. Slides were then stained with 20 μg ml^{-1} ethidiumbromide (EtBr) and stored at 4°C in a slide box until microscopy imaging and scoring. For neutral comet assay, the preparation of slides with agarose and sample was similar to the alkaline assay as stated above. The slides were overnight incubated at 4°C in lysis solution followed by DNA unwinding and electrophoresis in 4°C TBE electrophoresis buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.5). Fifty Comets per slide were scored with CometScore 2.0 software (TriTek Corp, USA).

2.2.4. Nanoparticles exposure and MicroRNA Quantitative real-time PCR analysis

PS NPs and TiO_2-ARS NPs were prepared as previously described. THP-1 cells were seeded and differentiated in the 12-wells plate (Thermo Scientific, USA) at a density of 1×10^5 cells per well and incubated with either PS NPs or TiO_2-ARS NPs in a concentration of 10 μg ml^{-1} for 8 hours. Also, THP-1 cells were exposed with PLGA nanoparticles (Phosphorex Inc, USA) or *E. coli* BioParticles (Thermo Scientific, USA) respectively at 10 μg ml^{-1} for 8 hours for excluding miRNAs that are responsible for nanoparticle engulfment and PAMP specific pro-inflammation cascades. Cells were harvested with TRI-Reagent (Thermo Scientific, USA) and miRNA was isolated by mirVana™ miRNA Isolation Kit (Thermo Scientific, USA) subsequently following manufacturer's instruction. The quantity and purity of the miRNA was measured by using Spectra Max M5 microplate reader (Molecular Devices, USA). The reverse transcription of miRNA samples was performed by using Qiagen miScript II RT kit (Qiagen, Germany) following manufacturer's recommendation and 500 ng of miRNA was reverse transcribed into cDNA for each sample.

Primers for each miRNA Quantitative real-time PCR (QPCR) reaction was composed by a Universal Primer provided by Qiagen miScript SYBR Green PCR kit (Qiagen, Germany) and a miRNA specific primer that designed by using Premier Primer 6.0 software (PREMIER Biosoft, USA). In total 12 miRNA primers were designed for 12 miRNAs' expression detection, these miRNAs were selected by our previous work (Table 4). miRNA specific primer sequences are listed in Table 5.

miRNA QPCR was performed by Stratagene MX 3005 system (Thermo Scientific, USA), 2 μL miRNA reverse transcribed cDNA were added for a total volume of 25 μL and the following PCR protocol was started: denaturation step at 95 °C for 15 min, cycling program (95 °C, 15 s; 55 °C, 30 s; 70 °C, 30 s) for 40 cycles then goes melting curve analysis. U6 was selected as the housekeeping gene for each miRNA QPCR and the relative changes of miRNA expression level were analyzed by using the $2^{-\Delta\Delta Ct}$ method.

2.2.5. MicroRNA mimic transfection

The MicroRNA mimics for hsa-miR-155-5p and the Negative Control mimic were ordered from Sigma (Sigma-Aldrich Corp, USA) and diluted into a stock concentration of 10 μM. Prior to this experiment, a preliminary experiment was performed: THP-1 macrophages were transfected with different concentrations of miR-155-5p mimic (0, 1, 5, 10 nM) for 24 h for an optimization of mimic transfection concentration. Afterwards, 10 nM of miR-155-5p mimic or 10 nM of miRNA Negative control mimic was transfected to the differentiated THP-1 macrophages with Lipofectamine RNAiMAX reagent (Thermo Scientific, USA) respectively. Transfection reagent was replaced with FBS contained 1640 medium after 8 hours of incubation and after 24 hours of the whole transfection, cells were exposed with

either PS NPs or TiO2-ARS NPs in a concentration of 10 μg ml^{-1} for 8 hours. All samples were harvested with TRI-Reagent for downstream mRNA QPCR experiments.

2.2.6. Total RNA isolation and mRNA Quantitative real-time PCR analysis

Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol chloroform extraction protocol using TRI-Reagent (Chomczynski and Sacchi 2006). The concentration of the total RNA was identified by Spectra Max M5 microplate reader. Extracted mRNA was reverse transcribed into cDNA by reverse transcriptase and oligo-dT primer (Promega, Germany) subsequently. The expression of two DNA damage biomarker genes (*ATM, ERCC1*) and two hsa-miRNA-155-5p *in silico* predicted targeting (http://www.targetscan.org/vert_72/) while DNA damage-responsible genes (*TAOK1, TRIP13*) were identified by the mRNA QPCR. Moreover, three genes that were experimentally proven to be targeted by miRNA-155-5p while engaged in the DNA damage repair mechanism were selected additionally for expression measurement: *WEE1, APAF-1* and *RAD51* (Gasparini et al. 2014; Pouliot et al. 2012; Zang et al. 2012). The primers were designed by using Premier Primer 6.0 software and PrimerBank (https://pga.mgh.harvard.edu/primerbank/). Primers used were listed in Table 6. *β-actin* was selected as the internal reference control gene and QPCR was performed by following steps: one cycle (95°C, 10 min) and 40 cycles (95°C, 30 s) each followed by 1 min at the gene-specific annealing temperature. Fluorescence signals were read at the end of each cycle and melting curve analysis was performed subsequently. Completed QPCR datasets were analyzed by using the $2^{-\Delta\Delta Ct}$ method comparing to the control of each group.

2.2.7. Statistical analysis

All data were presented as the mean ± standard deviation (SD). All samples were randomly distributed to different groups. The Student t-test was used for paired or unpaired observations. One-way analysis of variance (ANOVA) was used for comparison between the mean values of groups. P-value of <0.05 was considered to be statistically significant unless specified differently. SPSS software (IBM Corp, USA), R language package (GGplot 2) and Origin version 8.0 software (OriginLab, USA) were used for data analysis and figure drawing.

Table 5. MicroRNA specific primers used in our study.

MicroRNA	miRBase Accession number	Primer sequence (5' – 3')
hsa-miR-124-3p	MIMAT0000422	TAAGGCACGCGGTGAATGCC
hsa-miR-124-5p	MIMAT0004591	CGTGTTCACAGCGGACCTTGAT
hsa-miR-144-5p	MIMAT0004600	GGATATCATCATATACTGTAAG
hsa-miR-144-3p	MIMAT0000436	TACAGTATAGATGATGTACTAAA
hsa-miR-148a-5p	MIMAT0004549	AAAGTTCTGAGACACTCCGACT
hsa-miR-148a-3p	MIMAT0000243	TCAGTGCACTACAGAACTTTGT
hsa-miR-155-5p	MIMAT0000646	TTAATGCTAATCGTGATAGGGGT
hsa-miR-155-3p	MIMAT0004658	CTCCTACATATTAGCATTAACAA
hsa-miR-19a-5p	MIMAT0004490	AGTTTTGCATAGTTGCACTACA
hsa-miR-19a-3p	MIMAT0000073	TGTGCAAATCTATGCAAAACTGA
hsa-miR-223-5p	MIMAT0004570	CGTGTATTTGACAAGCTGAGTT
hsa-miR-223-3p	MIMAT0000280	TGTCAGTTTGTCAAATACCCCA

Table 6. Primers for mRNA Quantitative PCRs

Primer Name	Primer sequence (5' – 3')
ATM - Forward	CCGCGGTTGATACTACTTTGACC
ATM - Reverse	GCAGCA GGGTGACAATAAACAAGTAA
ERCC1 - Forward	GGGAATTTGGCGACGTAATTC
ERCC1 - Reverse	GCGGAGGCTGAGGAACAG
TAOK1 - Forward	TGCACGAGATGTGCGTACC
TAOK1 - Reverse	TGTGTTCACGTAAATAACAGCCT
TRIP13 - Forward	ACTGTTGCACTTCACATTTTCCA
TRIP13 - Reverse	TCGAGGAGATGGGATTTGACT
β-actin - Forward	CATGTACGTTGCTATCCAGGC
β-actin - Reverse	CTCCTTAATGTCACGCACGAT
WEE1- Forward	ATTTCTCTGCGTGGGCAGAAG
WEE1- Reverse	CAAAAGGAGATCCTTCAACTCTGC
Apaf-1 - Forward	GGGTTTCAGTTGGGAAACAA
Apaf-1 - Reverse	CACCCAAGAGTCCCAAACAT
RAD51 - Forward	CAGTGATGTCCTGGATAATGTAGC
RAD51 - Reverse	TTACCACTGCTACACCAAACTCAT

3. RESULTS

3.1. Prediction of miRNA-mRNA networks and critical miRNAs

miRNAs targeting specific biological processes influenced by nanoparticle exposure were predicted based on the genes in the "Summarized list" (including tissue-specific and biological response specific summarized list) and "Additional list". As a result, three tissue-specific miRNA-mRNA connecting networks and three biological response-specific miRNA-mRNA regulation networks were constructed. Based on selection criteria, six miRNAs (or miRNA families) with high likelihood to act as mediators in constructed networks were selected, and most of them were validated in mammals and/or zebrafish. These six miRNAs are: dre-miR-124, -144, -148, -155, -19a, -223. Among them, dre-miR-144 and -148 regulatory roles were predicted in 4 networks and both miRNAs were validated in mammals to target around 60 genes. dre-miR-124 and -155 were present only in one network but both of the miRNAs were validated in more than one mammalian study. Meanwhile, the expression of dre-miR-19a was also confirmed to change after zebrafish exposure to particulate matter and was top scoring in two of our networks. The dre-miR-223 was top predicted in all three networks so this miRNA was selected as the key regulator revealing the tissue damage induced by NPs in zebrafish. The summary of selection results is presented in table 4, which specifically stated the *in silico* prediction result of the six selected critical miRNAs in zebrafish following the selection criteria.

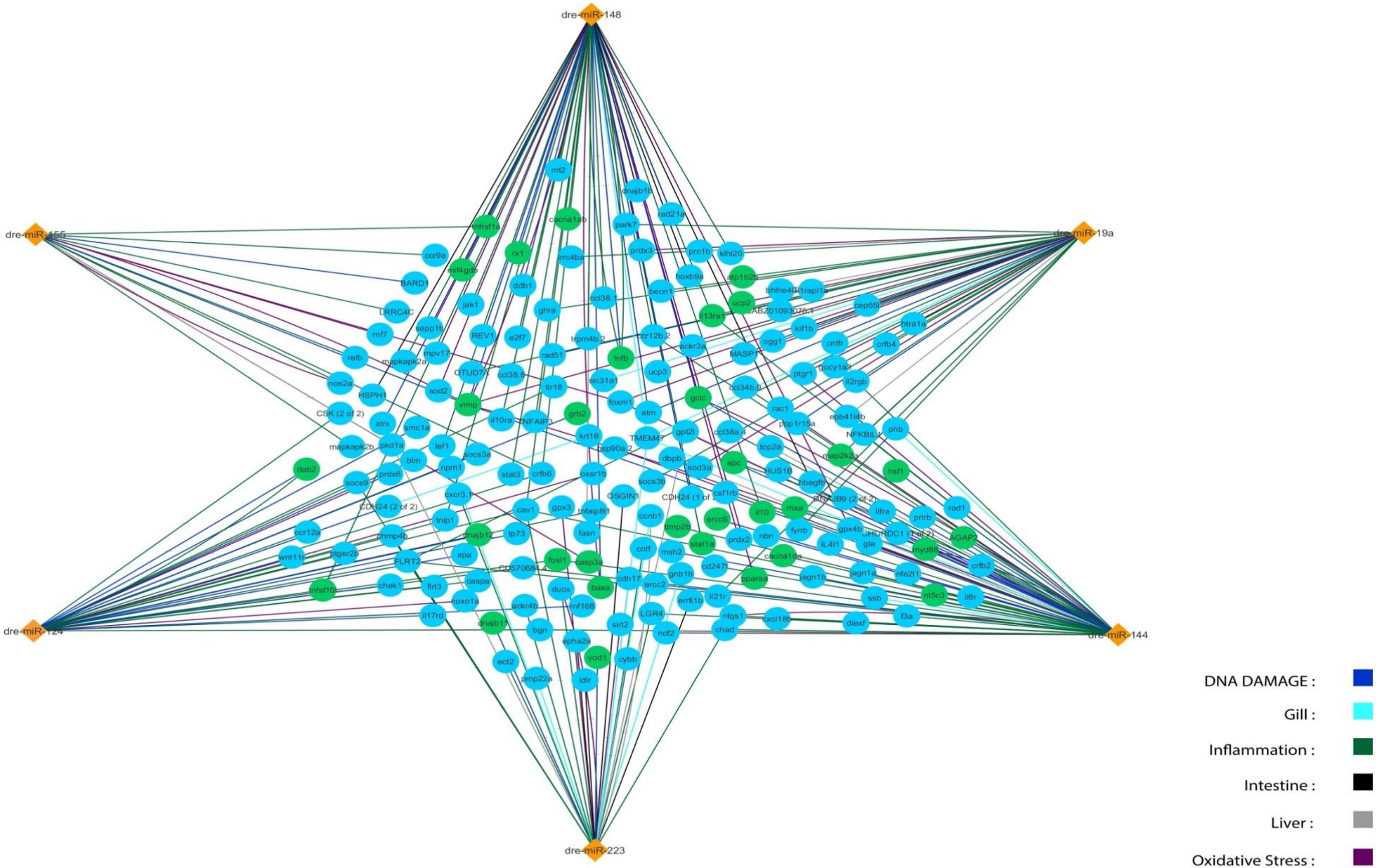
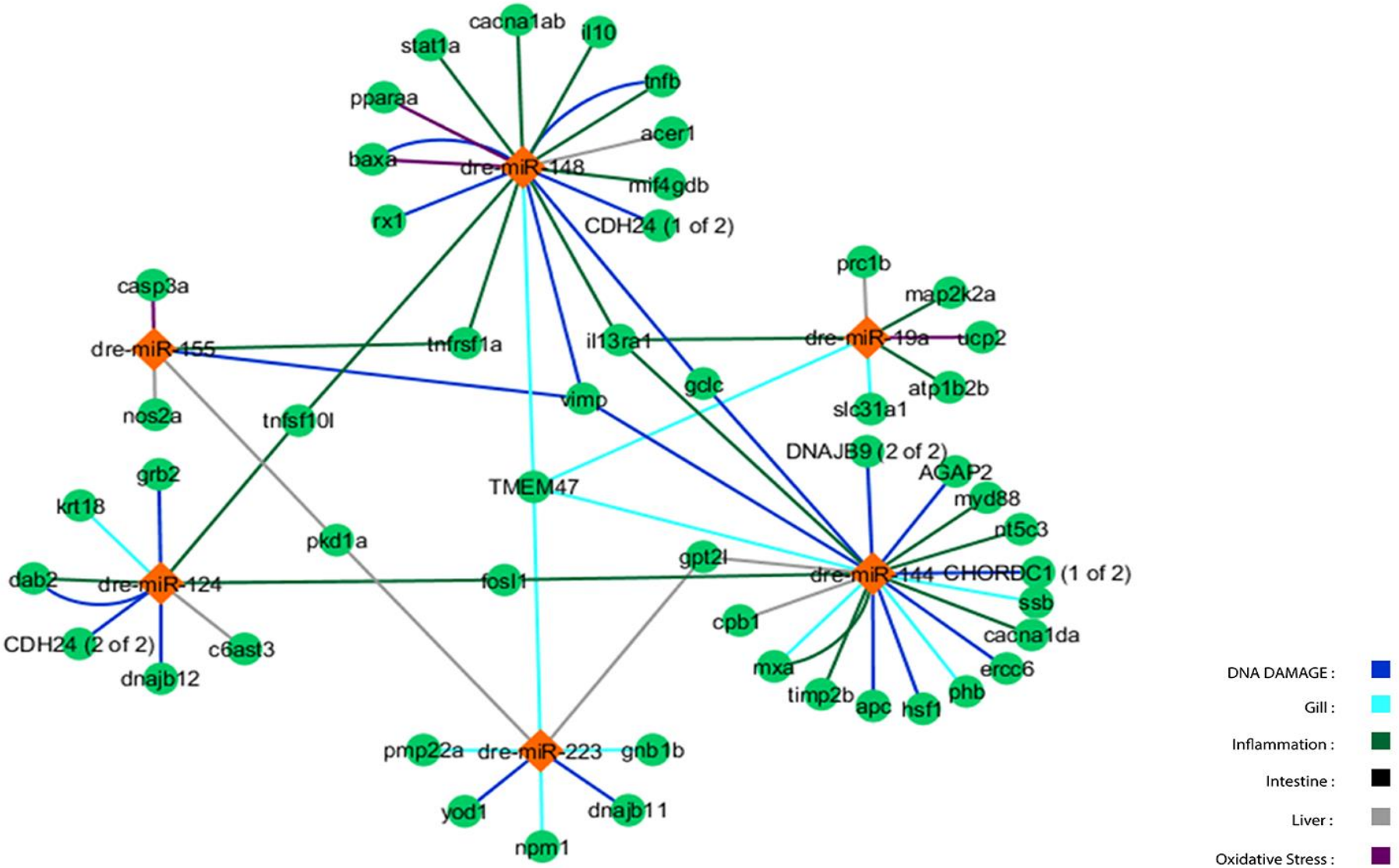

dre-miR-148
dre-miR-455
dre-miR-19a
dre-miR-124
dre-miR-144
dre-miR-223
DNA DAMAGE :
Gill :
Inflammation :
Intestine :
Liver :
Oxidative Stress :

Fig. 2. miRNA-mRNA targeting network regulated by 6 miRNAs (Whole network)

Orange diamond indicating the miRNAs and the blue or green circles indicate the genes. The genes with blue circles indicating that these genes were belong to the additional lists which are the same as the definition in the Figure 1. Genes with green circles means genes were reported in previous articles. Lines in this figure showing the miRNA-mRNA targeting category and different color indicating the different network, color and their representing was specified in this figure.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stat1a
tnfb
pparaa
acer1
baxa
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rx1
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prc1b
map2k2a
casp3a
dre-miR-155
tnfrsf1a
il13ra1
dre-miR-19a
ucp2
atp1b2b
gclc
vimp
slc31a1
nos2a
tnfsf10l
DNAJB9 (2 of 2)
AGAP2
grb2
TMEM47
myd88
krt18
nt5c3
pkd1a
gpt2l
dab2
dre-miR-124
fosl1
dre-miR-144
CHORDC1 (1 of 2)
ssb
cpb1
cacna1da
CDH24 (2 of 2)
c6ast3
mxa
ercc6
timp2b
apc
hsf1
phb
dnajb12
pmp22a
dre-miR-223
gnb1b
yod1
dnajb11
npm1
DNA DAMAGE :
Gill :
Inflammation :
Intestine :
Liver :
Oxidative Stress :

Fig. 3. miRNA-mRNA targeting network regulated by 6 miRNAs (Network based on reported genes)

Interaction between 6 miRNAs and the genes were reported in previous articles. Orange diamond indicating the miRNAs while the green circles indicating genes. Lines in this figure are showing the miRNA-mRNA targeting category and different color indicates different connection type.

3.2. Results of genotoxicity induced by Polystyrene and Titanium dioxide Nanoparticles and potential MicroRNA regulations

3.2.1. Nanoparticle characterization

The PS NPs (stock solution, 25 mg ml^{-1}) with 50 nm in primary mean diameter holding a mean hydrodynamic diameter of 33.68 ± 4.47 nm with a single peak at 37.88 nm. The TiO_2-ARS NPs in a concentration of 5 mg ml^{-1} was characterized as a size distribution of 43.49 ± 19.26 nm. The zeta potential of PS NPs was -69.30 ± 2.44 mV while the zeta potential of TiO_2-ARS NPs was -3.23 ± 1.19 mV. The values of PS NPs indicate a low aggregation potential while the zeta potential values of TiO_2-ARS NPs indicated the higher aggregation preference for the TiO_2-ARS NPs than the PS NPs.

3.2.2. Time and dose dependent DNA damage profiling post nanoparticle exposure

Total of 3622 images were analyzed with the comet assay analytical software to establish *in vitro* DNA damage profile in THP-1 monocytic cell line after different time and dose exposures to PS NPs or TiO_2-ARS NPs. The percentage of comet "Tail" (% tail DNA) was referred to as the quantitative DNA damage parameter for comet assay in both alkaline and neutral conditions. The complete DNA damage profile is presented in Figures 4 and 5. Increased DNA damage was observed post 24 h incubation with 10 μg ml^{-1} PS NPs in alkaline comet assays. As for the cells treated with 100 μg ml^{-1} of PS NPs, the observable significant tail percentage increase ($p < 0.05$) was detected for the first time at 12 hours post exposure, with significance level observed at $p < 0.01$ at 24 hours of exposure in the alkaline comet assay. The only time point when a significant DNA damage occurred in the neutral

comet assay for 100 μg ml^{-1} PS NPs exposed cells was at 24 h post exposure. This result may indicate that PS NPs had higher ability to trigger the DNA single strand damages, rather than double strand breaks as the alkaline comet assay primarily detects SSBs (Single Strand Breaks) that are directly induced or formed as a result of BER (Base pair excision repair) and alkali labilization of AP sites while the comet assay in neutral conditions allows for the detection of DNA double-strand breaks (DSB) (McKelvey-Martin et al. 1993; Olive et al. 1991). Post PS NPs exposure at a concentration of 500 μg ml^{-1}, severe DNA damage ($p < 0.01$) was detected at all exposure time points (4 h, 8 h, 12 h and 24 h) in the alkaline comet assay comparing to the blank control while the significant tail content increase was detected at 24 hours of exposure in the neutral comet assay. This phenomenon implies that PS NPs are able to induce a severe DNA damage in both manners of SSB and DSB at a high dose towards the THP-1 cell. On the other hand, at the dose of 500 μg ml^{-1}, TiO_2-ARS NPs were able to induce the significant comet tail percentage increase at every exposure time point in both alkaline and neutral comet assays. Concentration of 100 μg ml^{-1} of TiO_2-ARS NPs induced a severe SSB at both 12 and 24 hours post exposure while significant DSB was observed only at 8 hours post exposure. For TiO_2-ARS NPs exposure at a mild concentration (10 μg ml^{-1}), a significant tail content increase occurred at 24 hours post treatment in the alkaline comet assay and 8 hours post-treatment in the neutral comet assay.

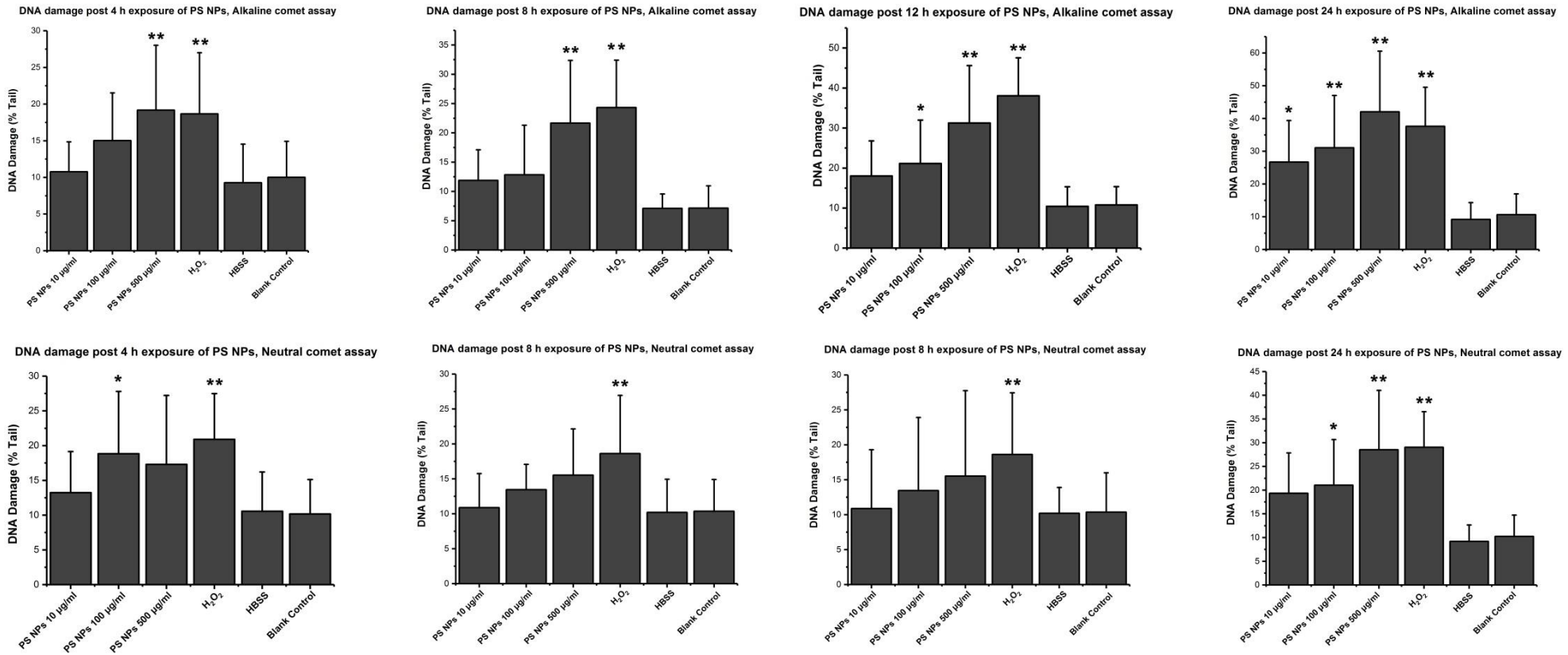

Fig. 4. Time and dose dependent DNA damage profile induced by Polystyrene Nanoparticles.

DNA damage was evaluated by comet assay in alkaline condition and neutral condition. Asterisk (*) indicates the significant fluctuation ($p < 0.05$), double asterisks indicate the very significant change ($p < 0.01$). All data compared with blank control

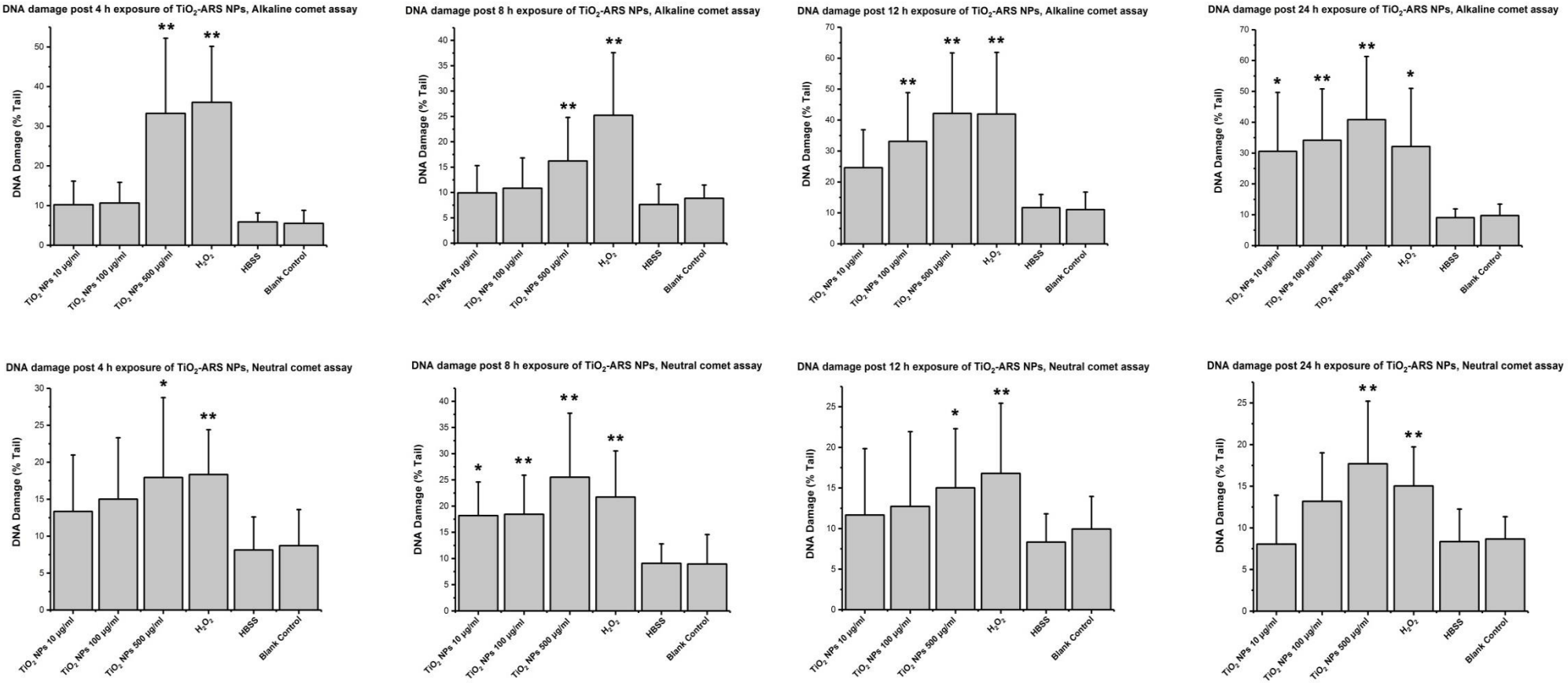

Fig. 5. Time and dose dependent DNA damage profile induced by ARS labeled Titanium Dioxide Nanoparticles.

DNA damage evaluated by comet assay in alkaline condition and neutral condition. Asterisk (*) indicates the significant fluctuation ($p < 0.05$), double asterisks indicate the very significant change ($p < 0.01$). All data compared with blank control.

3.2.3. MicroRNA expression after nanoparticles exposure

In our first objective, six miRNA-mRNA regulation networks were constructed using *in silico* analysis approaches and data summary. Among them, six zebrafish miRNAs that were predominately present in our networks were selected as most promising candidates for further studies: dre-miR-124, -144, -148, -155, -19a, -223. So in our second objective, the expression profile of these six miRNA categories was analyzed post PS NPs or TiO_2-ARS NPs exposure in human THP-1 monocytic cell line. The relative expression level of these miRNAs compared with control is presented in Figure 6. Post 8 hour exposure of PS NPs at a concentration of 10 μg ml^{-1}, five strands of miRNA showed significantly down-regulated expression compared to control. Significant decrease in expression was detected for hsa-miR-124-3p, -148a-3p, -155-3p, -155-5p and -223-3p ($p < 0.05$). Among them, miR-124-3p and miR-155-5p were the most significantly down-regulated ones ($p < 0.01$). Expression of miR-144-3p and miR-19a-5p was elevated, but not significantly up-regulated post PS NPs exposure.

After exposure to TiO_2-ARS NPs, miRNA expression patterns suggested that half of the investigated miRNAs were not significantly different from the controls (miR-144-3p, -144-5p, -148a-3p, 148a-5p, -19a-3p and -19a-5p) and a non-significant elevation was observed in miR-155-3p. The exposure of TiO_2-ARS NPs only significantly down-regulate the expression of miR-155-5p.

It has been reported that the expression of miRNA could be altered either by engaging in the endocytosis or by the pro-inflammatory responses that induced following the phagocytosis of PAMPs (Pathogen Associated Molecular Patterns) in monocytes and their differentiated macrophages (Navarro et al. 2008). In attempt to exclude potential interference of pro-inflammatory reactions following PAMP related phagocytosis, an additional

experiment was performed by exposing the THP-1 cells with a type of nanoparticles that could trigger endocytosis but without induction of pro-inflammatory responses or toxicity responses (PLGA nanoparticle) (Hirota and Terada 2012). Similarly, to exclude possible PAMP derived miRNA induction, a bacterial derived bio-particle (*E. coli* BioParticles) was used. The miRNA expression profiles after the exposure to all four particle types (PS/TiO_2-ARS NPs, PLGA, BioParticles) was presented as a heat-map in Figure 7. In this figure, miR-155-5p was not significantly up-regulated after the exposure of PLGA nanoparticles and *E. coli* BioParticles, but was down-regulated after the inducement of both PS NPs and TiO_2-ARS NPs. Based on all collected data, the hsa-miR-155-5p shows the most promise to be used as possible biomarker of regulatory mechanisms activated by PS NPs and TiO_2-ARS NPs.

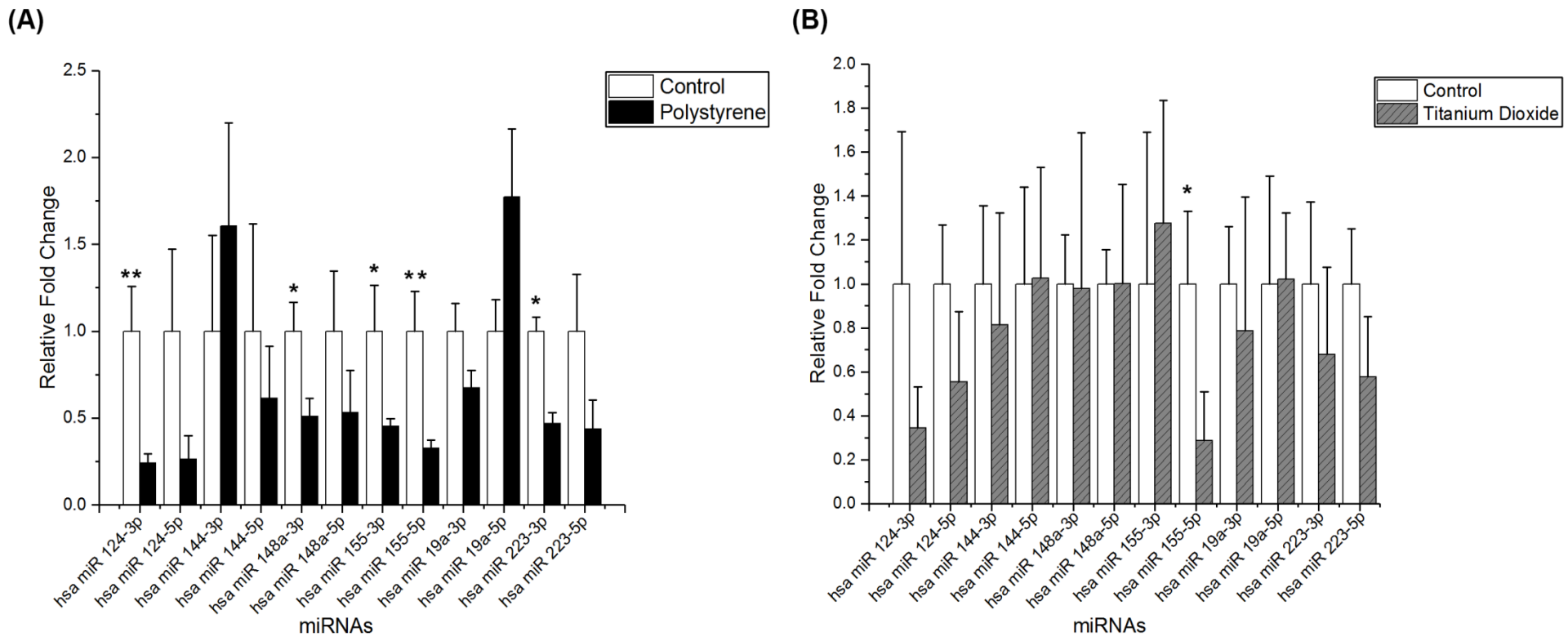

Fig. 6. Relative expression level of selected miRNAs in THP-1 Human monocytic cell line after the exposure of Polystyrene nanoparticles and ARS labeled Titanium Dioxide Nanoparticles.

Relative expression level of all groups compared with the control. Single asterisk (*) represents the significant alteration ($p<0.05$), double asterisks (**) indicate the very significant change ($p<0.01$).

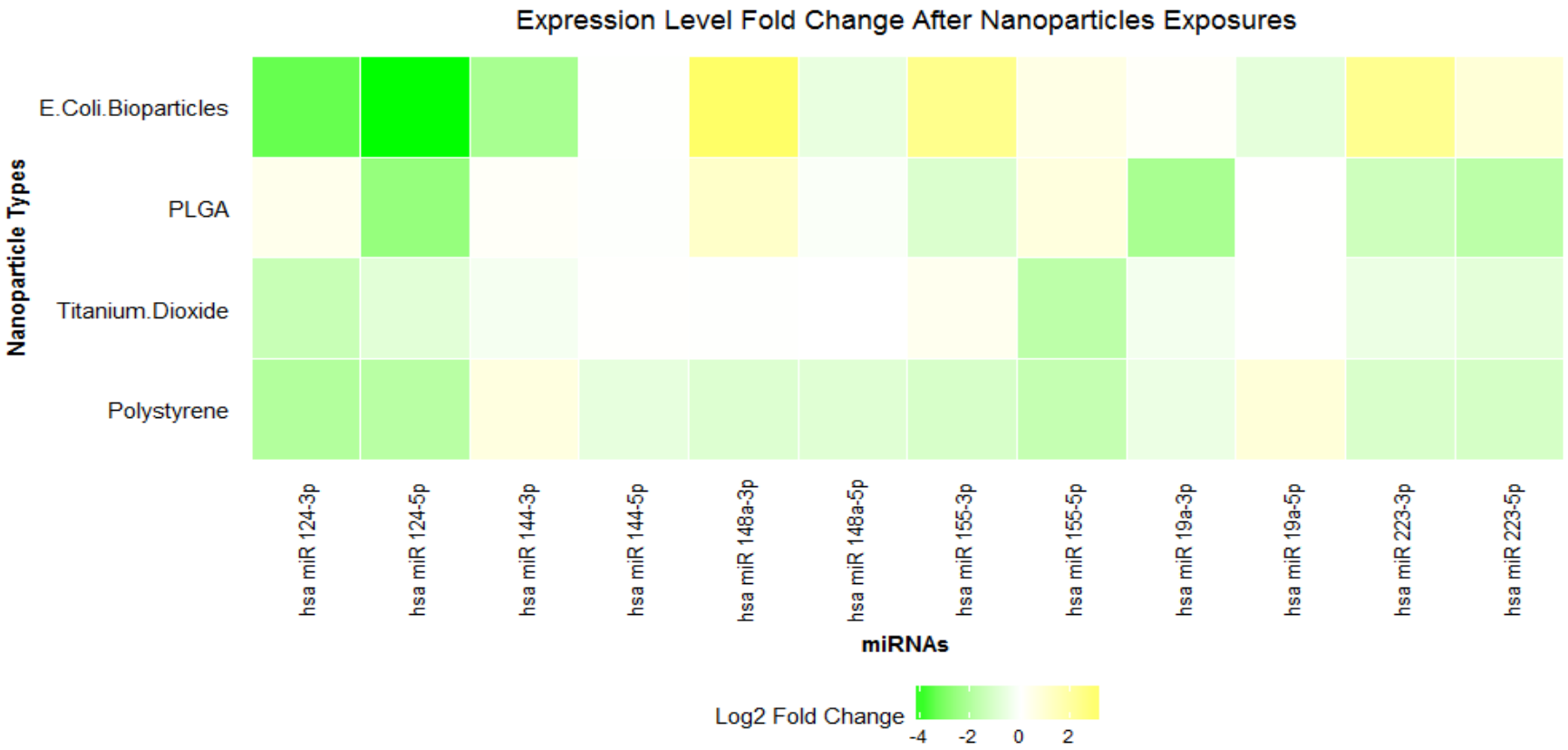

Fig. 7. Expression alteration of selected miRNAs after exposure of Polystyrene nanoparticles, ARS labeled Titanium dioxide nanoparticles, PLGA nanoparticles and *E.Coli* bioparticles in THP-1 cell line.

Relative expression level presented as the Log_2 Fold Change comparing with control in our heat-map.

3.2.4. DNA biomarker expression and potential miR-155-5p targeting genes expression

Based on our previous result that hsa-miR-155-5p is a potential biomarker for PS NPs and TiO_2-ARS NPs, we performed the miRNA functional study by transfecting miR-155-5p mimic to simulate of miRNA overexpression. The result from our preliminary experiment indicated that the miR-155-5p was significantly up-regulated after transfection with 10 nM mimic, compared to the negative control mimic (Table 7). Then, we checked the expression of three categories of genes: 1) DNA damage biomarker genes (*ATM, ERCC1*), 2) genes *in silico* predicted to be targeted by miR-155-5p and responsible for DNA damage repairing (*TAOK1, TRIP13*) and 3) experimentally validated miR-155-5p target genes engaged in a variety of DNA damage repairing processes (*WEE1, APAF-1* and *RAD51*). The expression of selected genes was measured in the following conditions: 1) THP-1 cells exposed with PS NPs or TiO_2-ARS NPs; 2) THP-1 cells transfected with miR-155-5p mimic; and 3) Mimic transfected cells with the exposure of PS or TiO_2-ARS NPs. All data were presented as relative expression fold change comparing to their control. Post TiO_2-ARS NPs stimulation, five out of seven genes were significantly affected in expression levels while only *TAOK1* was significantly up-regulated post PS NPs exposure (Fig. 8). This result may indicate that DNA damage repairing (DDR) processes are initiated by TiO_2-ARS NPs, but not by PS NPs.

TAOK1, TRIP13, WEE1, APAF-1 and *RAD51* are either experimentally proven, or predicted with *in silico* analysis to be targeted by miR-155-5p. In our experiment, a majority of these genes are significantly ($p<0.01$) suppressed in their expression level after the transfection of miR-155-5p mimic (*TAOK1, WEE1, ATM, TRIP13 and ERCC1*) and the rest of the genes show overall down-regulation trend ($p<0.05$) except *RAD51* (Fig. 9). Moreover, significant gene expression suppressions were observed in *ATM* and *ERCC1* post miR-155-5p overexpression. This finding is interesting, because the relations between miR-155-5p and these two genes were not reported previously. Our results suggest that miR-155-5p, may be

directly targeting *ATM* and *ERCC1*, and could also be a part of the network to regulate the function of these genes. On the other hand, strong activation of *ATM*, *TAOK1*, *TRIP13*, *APAF-1* and significant attenuation of *ERCC1* was detected in miR-155-5p mimic transfected cells post PS NPs exposure while significant up-regulation of *ATM*, *APAF-1* and *RAD51* was found in transfected cells after TiO_2-ARS NPs (Fig. 10). The different gene expression profiles between mimic transfected and mimic un-transfected cells post NPs exposure indicate significant engagement of miR-155-5p in the NPs induced adverse outcomes such as DNA damage.

Table 7. miR-155-5p expression level changes after the transfection of miR-155-5p mimic in different concentrations

	miR-155-5p Mimic Concentrations			
	10 nM	5 nM	1 nM	Negative Control
Mean of $2^{-\Delta\Delta Ct}$ values	10.50	4.58	2.80	1.37
Standard Deviation of $2^{-\Delta\Delta Ct}$ values	2.35	2.97	0.90	0.32
p-Value (Comparing to Control)	0.002612	0.068275	0.060584	N/A

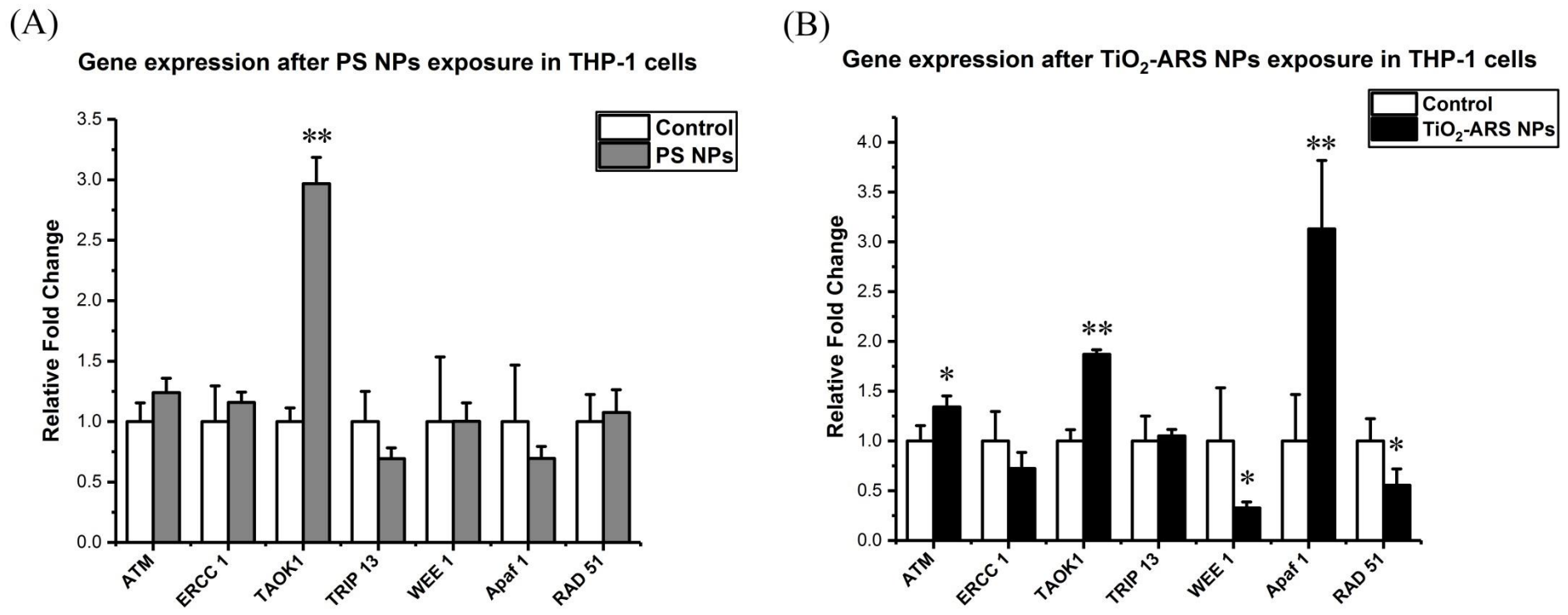

Fig. 8. DNA damage repairing responsible gene expression alteration post the exposure of PS NPs or TiO_2-ARS NPs in THP-1 cells

These genes are DNA damage biomarkers (*ATM, ERCC1*), DNA damage repairing genes that are *in silico* predicted targeted (*TAOK1, TRIP13*) or experimental proved targeted (*WEE1, APAF-1* and *RAD51*) by hsa-miR-155-5p. Single asterisk (*) represents the significant alteration ($p<0.05$), double asterisks (**) indicate the very significant change ($p<0.01$).

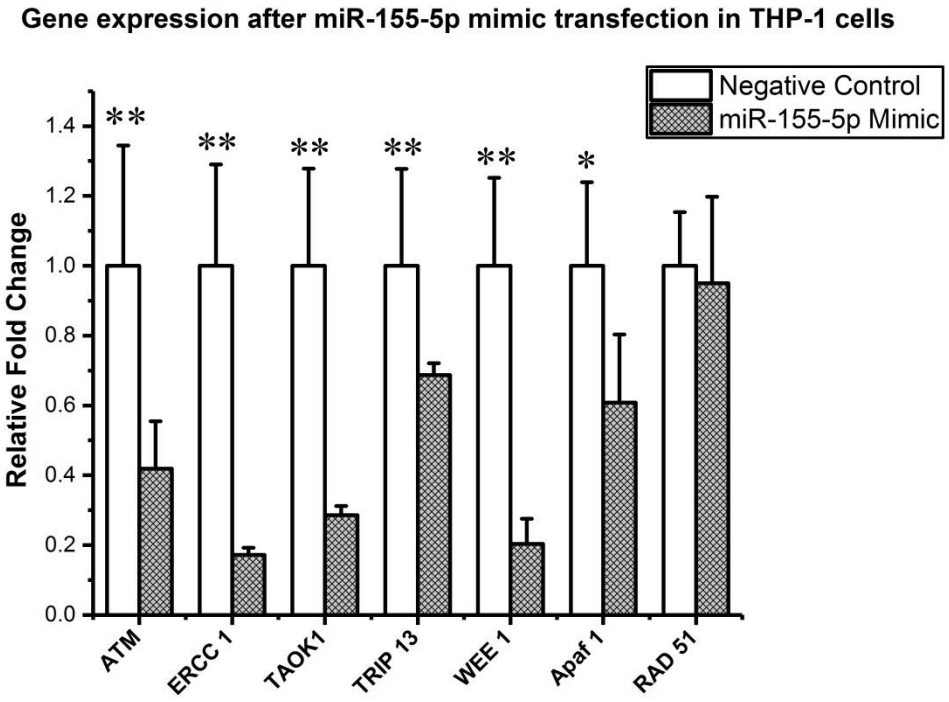

Fig. 9. The expression alteration of DNA damage repairing genes after miR-155-5p transfection in THP-1 cells

Negative control indicates the negative control mimic. All expressions of genes were comparing with the negative control transfected group. Single asterisk (*) represents the significant alteration ($p<0.05$), double asterisks (**) indicate the very significant change ($p<0.01$).

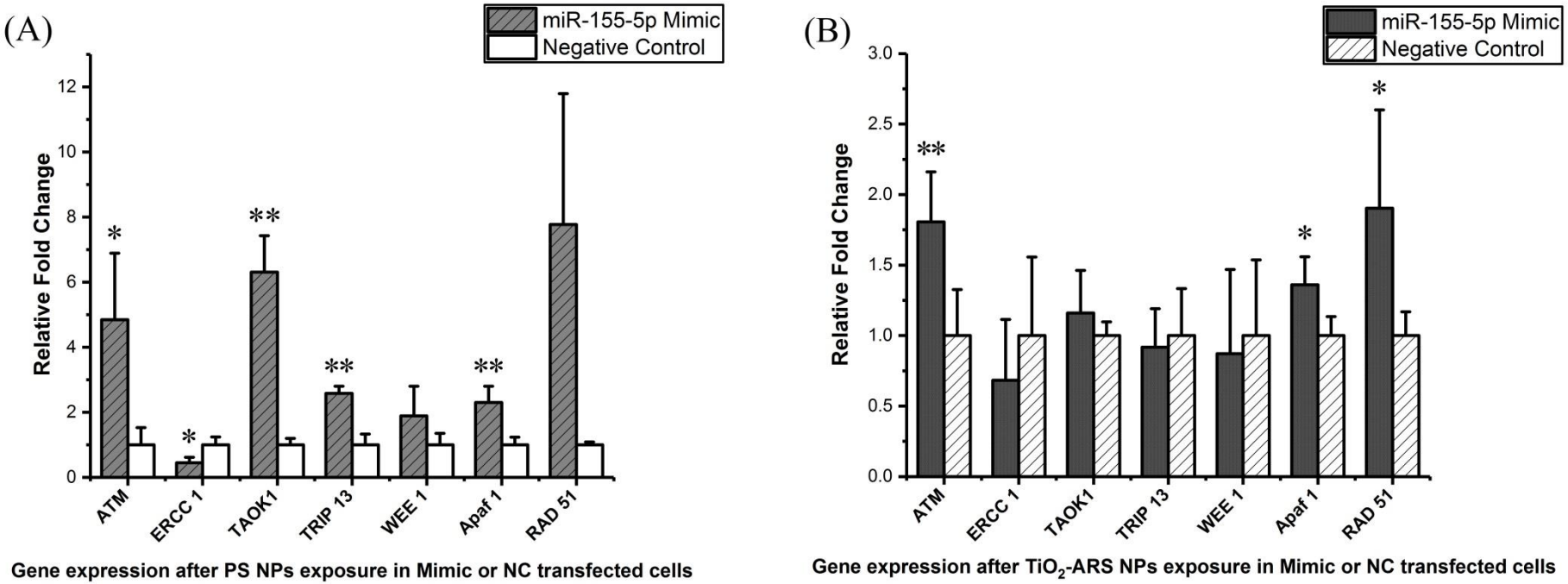

Fig. 10. The expression alteration of DNA damage repairing genes after miR-155-5p transfection in miR-155-5p mimic transfected cells or Negative control transfected cells

Mimic stands for the miR-155-5p mimic and NC stands for the negative control. Single asterisk (*) represents the significant alteration ($p<0.05$), double asterisks (**) indicate the very significant change ($p<0.01$).

4. DISCUSSION

4.1. Critical miRNAs and their potential regulatory role in nano-toxicology

During *in silico* analysis and delineation of nanoparticle induced mRNA-miRNA regulation networks (Fig. 2), total of six zebrafish miRNAs, or miRNA families, were identified as potential biomarkers of nanoparticle toxicity. These are dre-miR-124, -144, -148, -155, -19a, -223.

4.1.1. Dre-miR-124

In mammals, miR-124 is engaged in regulation of neuron development, cellular proliferation, and cytokine production by targeting corresponding mRNAs, and was implied in mediation of the development of Parkinson's disease and cancer (Sun et al. 2016; Wang et al. 2014; Xie and Chen 2016). In Parkinson's disease, the miR-124 suppression significantly increased cell apoptosis rate and mTOR pathway-related protein expression, indicating that miR-124 could be involved in cell apoptosis (Gong et al. 2016). Apoptosis can be triggered by NPs induced oxidative stress, also in zebrafish, where dre-miR-124 has been described as an orthologue of mammalian miR-124. In our predictions, dre-miR-124 targeted *wnt11r* and *grb2b*, two genes which serve as essential components of mTOR pathway (Fig. 2 and Fig. 3). Expression of *wnt11r* can be down-regulated during the NPs treatment (Park and Yeo 2013b), therefore, it is possible that miR-124 can function as a regulator of NPs toxicity induction by controlling effectors of mTOR pathway and apoptosis. Moreover, NPs can activate Wnt signaling cascade in mammalian models (Section 1.2.3 of this dissertation), and overexpression of miR-124 leads to a powerful upregulation of Wnt/β-catenin reporter

activity detected by the duel-luciferase reporter assay in human 293T cell line (Liu et al. 2011b). In the present prediction, dre-miR-124 was targeting *wnt11r* and *fosl1* which are engaged in the Wnt pathway indicating that this miRNA may also function in the Wnt signaling cascade upon the treatment of NPs.

4.1.2. Dre-miR-144

A study with pulmonary tuberculosis patients revealed significant overexpression of miR-144 mainly in T cells and transfection of miR-144 precursor inhibited cytokine production, indicating that miR-144 is involved in regulating human immune responses through down-stream modification of cytokine synthesis and secretion (Liu et al. 2011d). Such biological activity may suggest there is direct interaction between miR-144 and TLR2 and TLR pathway activation (Li et al. 2015). However, in our prediction, dre-miR-144 was not found to target *TLR2* (Fig.2). This controversial phenomenon may suggest a different mechanism in cytokine synthesis induced by NPs in zebrafish. Moreover, miR-144 was experimentally proven to directly regulate key mediators of oxidative stress response such as *Caspase3*, *Nfe2l2* and *Rac1* (Ovcharenko et al. 2007; Sangokoya et al. 2010; Wang et al. 2012). Among them, *nfe2l2* and *rac1* were also predicted as targets in our networks revealing that dre-miR-144 may possibly mediate the protective signaling cascade toward oxidative stress induced by NPs through regulation of *nfe2l2* and *rac1* in zebrafish.

4.1.3. Dre-miR-155

In mammalian organisms, miR-155 is a mediating component of different types of inflammatory processes signaling. For instance, it has been shown that miR-155 expression is

induced by bacterial lipopolysaccharide (LPS), and also by cytokines including IFN-β and TNF-α in human monocytic cell line (Taganov et al. 2006). Furthermore, inhibition of the c-Jun N-terminal kinase (JNK) blocks expression of miR-155, suggesting that miR-155 could induce signaling cascade of the JNK pathway (O'Connell et al. 2007). The inflammatory response can be triggered by exposure to NPs through activation and stimulation of innate immune effectors (Stevenson et al. 2011). The overexpression of miR-155 was shown to block manganese (Mn) NPs-induced increase of TNF-α and IL-6 expression, suggesting possible role of miR-155 in mediation of NPs induced inflammatory responses in mammalian cell lines (Grogg et al. 2016). Moreover, the expression of miR-155 was significantly up-regulated during exposure of human lung fibroblasts to gold NPs while suppressing the expression of *PROS1* gene (Ng et al. 2011).

Our predictions suggested that dre-miR-155 is likely involved in the inflammatory responses through interplay between tumor necrosis factor receptor superfamily (TNFRs) and engagement in the MAPK signaling and/or cytokine production in zebrafish (Fig.2 and Fig.3). On the other hand, miR-155 was found to be one of the most potent miRNAs to suppress apoptosis, which could attribute to the underlying mechanism that miR-155 can directly or indirectly block the caspase-3 activity (Ovcharenko et al. 2007). In fact, it was described earlier that miR-155 can directly target caspase-3 and repress apoptosis in human *nucleus pulposus* (Wang et al. 2011), which is supporting to our prediction that dre-miR-155 can bind with the 3'-UTR of caspase-3. These phenomena may imply the existence of conserved regulation between miR-155 and caspase-3 mediating apoptosis and oxidative stress responses in toxicity induction and disease development by NPs.

4.1.4. Dre-miR-19a

A recent study has reported that exposure to particulate matter 2.5 micrometer ($PM_{2.5}$) could down-regulate dre-miR-19a in zebrafish embryos, which is the first evidence connecting miR-19a with particle induced toxicity (Duan et al. 2017). miR-19a was identified to be one of crucial oncogenes, as it could not only regulate several of cancer development landmarks, but has also been reported to engage in the regulation of inflammatory responses (Olive et al. 2009; Wang and Chen 2015). Functional studies revealed that miR-19a could decrease the expression in several suppressors of cytokine signaling proteins (SOCS, e.g. SOCS3) by direct binding, and this was also confirmed by our network prediction (Fig.2) indicating that in zebrafish, dre-miR-19a may bind with *socs3* and regulate inflammatory responses induced by nano-toxicity (Collins et al. 2013). Furthermore, miR-19a can interact with cytokines, as it was shown that it can regulate IFN-α and interleukin-6 through the mediation of SOCS3 involved in the JAK-STAT signal transduction pathway (Collins et al. 2013). In addition, the experiments to target validation of relationship between miR-19a and STAT3 (or TNF-beta) in mammalian studies are not currently available. As this miRNA-mRNA relationship was also indicated during our prediction analysis and found in our zebrafish network (Fig.2), we speculate that validation information about this predicted relationship could confirm the importance of this miRNA in the basic molecular defensive mechanism protecting various species against NPs induced toxicity (Li et al. 2016; Liu et al. 2011a).

4.1.5. Dre-miR-148

In our prediction, dre-miR-148 could potentially regulate a majority of inflammation-related genes such as *il-10* and *tnf-beta*, indicating that this miRNA may be involved in the

immune responses to NPs (Fig.2 and Fig.3) in zebrafish. According to a study by Huang et al. (Huang et al. 2015), miR-148 was upregulated in human dermal fibroblasts upon the Gold NPs treatment and engaged into the MAPK pathway controlling upstream activation of innate immunity effectors. However, miRNA targeting and functional validation experiments were rarely focused on the miR-148 across species from mammals to zebrafish, and the only overlapping miR-148 targeting between the existing validations in mammals and our prediction in zebrafish is towards the mRNA *bax*, a gene responsible for pro-apoptosis (Grimson et al. 2007).

4.1.6. Dre-miR-223

miR-223 is cross-species conserved microRNA involved in immune cells differentiation and tumor suppression (Fukao et al. 2007; Xu et al. 2011). Our prediction indicated that dre-miR-223 was predominantly regulated in all three tissue-specific networks revealing that this microRNA possesses potential to influence tissue damage induced by NPs in zebrafish. In mammals, miR-223 would negatively regulate the differentiation processes of myeloid cells including neutrophils and their precursors and their further activation (Johnnidis et al. 2008). Main regulatory mechanism is direct targeting between miR-223 and *IGF1R* (insulin-like growth factor 1 receptor) (Lu et al. 2013). In our predicted miRNA-mRNA liver specific network in zebrafish, dre-miR-223 also targeted *igf1r* (Fig.2). This result provides a theoretical evidence that the binding between of miR-223 and *igf1r* is a conserved mechanism and would further be associated with the differentiation and hyper-activation of neutrophils in the organismal defenses, which may also include the exposure to NPs (Lu et al. 2013).

4.2. Genotoxicity and the potential regulation of hsa-miR-155-5p in PS NPs and TiO_2-ARS NPs induced toxicity

The potential of nanoparticles to possibly induce DNA damage has been recently raising attention since they are increasingly used in cosmetics and pharmaceuticals. One of the major categories of NPs that are of high concern is the titanium dioxide (TiO_2) NPs due to their observed potential for triggering carcinogenicity (Jugan et al. 2012; Lu et al. 1998; Proquin et al. 2016). However, the genotoxicity of TiO_2 NPs remains controversial as EFSA panel concluded that TiO_2 NPs are unlikely to raise genotoxic concern *in vivo* as a food additive even though direct aggregation of TiO_2 NPs around the cell nuclei was observed in the vicinity of the endoplasmic reticulum *in vitro* (Additives and Food 2016; Zucker and Daniel 2012). Moreover, another type of nanoparticle that is being used extensively is the nano-sized plastic beads, with majority of them with polystyrene core (PS NPs). The genotoxicity induced by PS NPs is rarely reported as the toxicity of PS NPs is not fully understood (Liu et al. 2011c; Paget et al. 2015). In present study, we used comet assay to determine if different exposure times and doses of both TiO_2 and PS NPs could cause significant DNA damage in attempt to close fulfill the knowledge gap regarding the PS NPs genotoxicity and also further investigate mutagenesis potency of the TiO_2 NPs with and without fluorescent dye (ARS) labeling.

TiO_2-ARS NPs could generally induce a dose and exposure time dependent DNA strand damage as shown with comet assay and in concert with majority of studies (Jomini et al. 2012; Landsiedel et al. 2010). Starting at 4 hours post exposure (hpe), 500 μg ml^{-1} TiO_2-ARS NPs (ARS labeled) induced significant increase in both single strand DNA (ssDNA) and double strand DNA (dsDNA) damage, and severe ssDNA damage was also observed post 100 μg ml^{-1} TiO_2-ARS NPs exposure after 12 hours (Fig. 5). This result is similar to a

previous study that the stimulation of TiO_2 NPs results a significant ssDNA fragmentation at a concentration of 25 μg ml^{-1} post 3 hours of exposure (Ghosh et al. 2013). For dsDNA breaks, our pH neutral comet assay detected that the most severe DNA damage in cells after exposure to 10 and 100 μg ml^{-1} TiO_2-ARS NPs was observed at 8 hours post exposure. This finding is supported by significant dsDNA damage after 3 hours of 20 μg ml^{-1} TiO_2 NPs exposure reported by Saquib et al (Saquib et al. 2012). These results indicate high potential for TiO_2-ARS NPs to induce genotoxicity similar to unlabeled TiO_2 NPs *in vitro*.

PS NPs were shown to induce a significant ssDNA damage starting from 4 hours post exposure (hpe) and a significant dsDNA damage at 24 hpe in the highest concentration tested (500 μg ml^{-1}) (Fig. 4). This concentration of PS NPs is not environmentally relevant but high enough to induce the cellular injury including the ssDNA damage and the double strand DNA damage (Arora et al. 2009) (Fig. 4). The dose of PS NPs of 100 μg ml^{-1} induced ssDNA damage from 12 hpe and dsDNA damage from 24 hpe. This result is different from the previous study of Paget et al. (2015) where non-functionalized PS NPs in concentration of 8.1 μg cm^{-2} could not induce a significant raise of γ-H2Ax-foci counts in THP-1 cells except at 1 hpe (Paget et al. 2015). One of the possible reasons for this discrepancy is that NPs genotoxicity is generally dose dependent, and the concentration of 8.1 $\mu g/cm^2$ is considerably lower than our 100 μg ml^{-1} tested dose (Park et al. 2010; Tiwari et al. 2011).

Interestingly, low concentration exposure with PS NPs (10 μg ml^{-1}) did not induce a significant increase of the DNA damage during pH neutral comet assay, but did induce a significant increase of DNA damage level in the alkaline comet assay environment. This may suggest DNA strain break could be directly triggered by the PS NPs, but also some other considerations needed to be taken as this result differs from the well-known low-toxicity that this kind of nanoparticles could potentially induce (Ekkapongpisit et al. 2012; Liu et al. 2011c). During incubations prior to the comet assay, “Naked DNA” is exposed due to the

degradation of nuclear membranes, while the cell internalized NPs remain and persist in the lysosome (Oh and Park 2014). The interaction between nanoparticles and the bare DNA, outside of the cell environment results in the additional DNA strand breaks (Karlsson et al. 2015).

It should be noted that most studies investigating potential genotoxicity induced by polystyrene nanoparticles or micro-particles have been based on using γ-H2Ax-foci assay or transcriptomic analysis, but not comet assay (Avio et al. 2015; Paget et al. 2015), even though comet assay is directly quantifying the amount of breaks occurring in the DNA (Tice et al. 2000). The only study that was using comet assay to illustrate the genotoxicity triggered by PS NPs is showing results similar to our study, and reports that non functionalized PS NP concentration of 50 μg ml^{-1} applied for 48 hours exposure time causes significant DNA damage increase in HeLa cells (Ferraro et al. 2016). As the authors were also questioning the use and reliability of comet assay in the mutagenesis evaluation of nanoparticles, they suggested that the outcome of comet assay could be interfered by nanoparticles itself since strong interaction between NPs and DNA was indicated (Ferraro et al. 2016).

To further investigate this potential issue with comet assay use, we analyzed the expression of DNA damage biomarker genes to compare the results of comet assay and the DNA damage post exposure of TiO_2-ARS NPs and PS NPs. In the follow-up gene expression experiments, DNA Damage repairing related genes were not significantly activated after the stimulation with PS NPs. This appears supportive to concerns of other authors about use of comet assay in assessment of nanoparticles induced genotoxicity. However, our study also revealed that *TAOK1* (thousand and one kinase 1) was strongly activated after the stimulation of PS NPs, and since TAOK1 is involved in the activation of JNK pathway in response to DNA damage (Raman et al. 2007), we can't completely exclude the possibility

that PS NPs induced the actual DNA damage, and that results of the comet assay can at least partially be attributed to the DNA damage caused by PS NPs.

Based on the results from Objective 1, the expression profile of top six predicted miRNA after exposure to TiO_2-ARS NPs and PS NPs was determined. Only miR-155-5p was significantly down-regulated after exposure to both types of NPs. We also identified the miRNA expression patterns after stimulation of cells with PLGA nanoparticles, and *E. coli* BioParticles, in order to exclude miRNAs that functionally engaged in endocytosis (PLGA) or pro-inflammatory (BioParticles) responses. The results supported that miR-155-5p is the best candidate for functional validation (Figure 7.).

MicroRNAs normally regulate mRNA function through direct binding with mRNAs and formation of the RNA-induced silencing complex, effectively suppressing the transcription process (Bartel 2004). Transfection of miRNA mimics into the cell is used to simulate an overexpression of mature miRNA strands (Wang 2011). In our experiment, the transfection of miR-155-5p mimic lead to significant down-regulation of the genes that are either experimentally proven, or predicted *in silico*, to be targeted by this miRNA. Our results strongly suggested that miR-155-5p has regulatory function in expression of these genes.

Furthermore, a significant induction of *ATM*, *TAOK1*, *TRIP13* and *APAF-1* was detected in miR-155-5p over-expressed cells post PS NPs exposure while strong up-regulation of *ATM, APAF-1* and *RAD51* was found in miR-155-5p mimic transfected cells after TiO_2-ARS NPs. This phenomenon not only implies that miR-155-5p regulated these genes after exposure with TiO_2-ARS NPs or PS NPs but also indicates that existence of miR-155-5p is necessary for regulation of multiple DNA damage repairing processes that were induced by TiO_2-ARS NPs, or potentially triggered by PS NPs. One example is the involvement of APAF-1 (apoptotic protease activating factor 1) in regulation of DNA damage-induced apoptosis by acting as a p53 downstream factor (Moroni et al. 2001). In

addition, miR-155-5p was identified to target *APAF-1* supporting its engagement in DNA damage regulation and apoptosis via mitochondrial apoptotic pathway (Zang et al. 2012). In our experiment, the expression of *APAF-1* was elevated in miR-155-5p over-expressed cells after TiO_2-ARS NPs and PS NPs exposure, suggesting an activation of APAF-1 apoptotic pathway could be induced by nanoparticles via interaction between miR-155 and *APAF-1*.

5. CONCLUSION

Six miRNA-mRNA regulation networks were constructed using data summary and *in silico* predicting approaches and six miRNAs that were predominately present in our networks were selected as most promising candidates for further studies: dre-miR-124, -144, -148, -155, -19a, -223. Subsequent validation experiments indicated that miR-155-5p is the most promising biomarker for TiO_2-ARS NPs and PS NPs.

The role of TiO_2-ARS nanoparticles in DNA damage was supported with results of time and dose dependent DNA damage assessment taken together with DNA damage repairing related genes expression profile. However, the role of polystyrene nanoparticles as possible causative agent of DNA damages remained unclear. Over-expression of miR-155-5p in PS NPs treated cells increased expression of *ATM, TAOK1, TRIP13,* and *APAF-1*, and attenuated the expression of *ERCC1* while *ATM, APAF-1* and *RAD51* were strongly activated post TiO_2-ARS NPs exposure in miR-155-5p mimic-transfected cells.

We conclude that there is significant potential of miR-155-5p to be used as a biomarker of nanoparticle induced toxicity. Possible future applications include research and regulatory toxicology in different levels of biological complexity, becoming a valuable tool in human, and also animal and environmental monitoring of potential nanoparticle effects.

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