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Research Report

Profiles of oxidative stress-related microRNA and mRNA expression in auditory cells

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ARTICLE INFO

Article history:
Accepted 20 May 2010
Available online 26 May 2010

Keywords:
Auditory cell
Oxidative stress
Reactive oxygen species
MicroRNA
Expression profile
Microarray

ABSTRACT

Oxidative stress and high levels of reactive oxygen species (ROS) are risk factors of auditory cell injury and hearing impairment. MicroRNAs (miRNAs) are critical for the post-transcriptional regulation of gene expression and cell proliferation and survival. However, little is known about the impact of oxidative stress on the expression of miRNAs and their targeted mRNAs in auditory cells. We employed a cell model of oxidative stress by treatment of House Ear Institute-Organ of Corti 1 (HEI-OC1) cells with different concentrations of tert-butyl hydroperoxide (t-BHP) to examine the t-BHP-induced production of ROS and to determine the impact of t-BHP treatment on the relative levels of miRNA and mRNA transcripts in HEI-OC1 cells. We found that treatment with different concentrations of t-BHP promoted the production of ROS, but inhibited the proliferation of HEI-OC1 cells in a dose- and time-dependent manner. Furthermore, treatment with t-BHP induced HEI-OC1 cell apoptosis. Further microarray analyses revealed that treatment with t-BHP increased the transcription of 35 miRNAs, but decreased the expression of 40 miRNAs. In addition, treatment with t-BHP up-regulated the transcription of 2076 mRNAs, but down-regulated the levels of 580 mRNA transcripts. Notably, the up-regulated (or down-regulated) miRNAs were associated with the decreased (or increased) expression of predicted targeted mRNAs. Importantly, these differentially expressed mRNAs belonged to different functional categories, forming a network participating in the oxidative stress-related process in HEI-OC1 cells. Therefore, our findings may provide new insights into understanding the regulation of miRNAs on the oxidative stress-related gene expression and function in auditory cells.

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doi:10.1016/j.brainres.2010.05.059

1. Introduction

Oxidative stress and high levels of reactive oxygen species (ROS) are associated with the drug- and noise-induced, and age-related hearing injury and loss (Guthrie, 2008; Henderson et al., 2006; Riva et al., 2007). Cisplatin (Rybak et al., 2007; Im et al., 2010), aminoglycosides (Hirose et al., 1997; Guthrie, 2008) and continual noise (Ohlemiller et al., 1999; Henderson et al., 2006) can promote high levels of ROS production in hair cells. High levels of ROS can trigger hair cell apoptosis by breaking down DNA and damaging the cell membrane, oxidizing lipid and protein molecules, contributing to the hair cell lesion (Feghali et al., 2001; Clerici et al., 1995; Henderson et al., 2006). Ototoxic drugs can induce oxidative stress and destroy antioxidant homeostasis, leading to hair cell apoptosis (Vlajkovic et al., 2009; Cheng et al., 2005). Indeed, antioxidant reagents have been shown to support hair cell survival (Sha et al., 2001; Kawamoto et al., 2004; Darrat et al., 2007). In addition, steroids, neurotrophic factors, anti-apoptotic compounds, mitochondrial enhancers and pituitary adenylate cyclase activating polypeptide (PACAP) have also been reported to have anti-apoptotic activity and inhibit toxicant-triggered hair cell apoptosis (Seidman and Vivek, 2004; Racz et al., 2010). However, the pathogenic process of ROS-related hair cell death is poorly understood. Hence, discovery of the pathways involved in the pathogenesis of ROS-related hair cell cytotoxicity and illustration of molecular regulators of the pathogenic process will be of great significance.

Previous studies have revealed that high levels of ROS regulate the expression of epigenetic and transcriptional factors (Weigel et al., 2002; Vandenbroucke et al., 2008). Gentamicin and cisplatin can promote high levels of ROS production and modulate the transcription of a large number of genes in auditory hair cells (Nagy et al., 2004; Previati et al., 2004). Exposure of auditory cells to ROS modulates the activation and signal transduction of oxidation-sensitive signal pathways, including the mitogen-activated protein kinases (MAPK) and associated c-Jun N-terminal kinase (JNK) signaling pathway (Wang et al., 2003; Sugahara et al., 2006; Sha et al., 2009), the tumor suppressor p53 (Devarajan et al., 2002; Zhang et al., 2003), caspases (Van et al., 2004), and cytochrome c (Lee et al., 2004; Wang et al., 2004), and Bax pathways (Cheng et al., 2005) and others. Given that post-transcriptional regulation is crucial for gene expression and cell survival (Halbeisen et al., 2008), there are only a few studies of the ROS-related gene expression at the transcriptional level (Lin et al., 2009; Cheng et al., 2009), but no study on auditory cells.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that can negatively regulate gene expression by degradation and translational inhibition of their target mRNAs (Ambros, 2003; Pasquinelli et al., 2005). An individual miRNA can regulate the expression of its multiple target genes, and several miRNAs can also synergistically act on one target gene, regulating cell differentiation, proliferation/growth, mobility, and apoptosis (Farh et al., 2005; Bartel, 2004). MiRNAs also play an important role in the development and maturation of sensory epithelia in mouse inner ear (Soukup, 2009), and may be pivotal regulators of the process of hearing loss (Friedman et al., 2009a). Recent studies have shown that a mutation in microRNA-96 (miR-96) may be a causative factor for the development of progressive hearing loss in humans and mice (Mencía et al., 2009; Lewis et al., 2009). However, the effects of oxidative stress and high levels of ROS on the expression of miRNAs and their potential roles in the ROS-mediated gene regulation and biological functions in auditory cells have not been explored. Furthermore, little is known about how the expression profiles of miRNAs and mRNAs contribute to the regulatory networks in the ROS-related auditory cell injury and death.

This study aimed at determining the effects of treatment with tert-butyl hydroperoxide (t-BHP) on the production of ROS and the survival of House Ear Institute-Organ of Corti 1 (HEI-OC1) cells, and characterizing the ROS-related expression of miRNAs and mRNAs in HEI-OC1 cells in vitro. Furthermore, we analyzed the potential interaction of differentially expressed miRNAs with the targeted mRNAs in the process of ROS-induced auditory cells. Hence, our findings may provide new insights into understanding the regulation of miRNAs on the oxidative stress-related auditory cell injury and death.

2. Results

2.1. *Effect of t-BHP on production of intracellular ROS in HEI-OC1 cells*

The t-BHP is a potent oxidant and can promote the generation of ROS. We first determined whether treatment with t-BHP could promote the formation of ROS in HEI-OC1 cells. HEI-OC1 cells were treated with different concentrations (0–400 μM) of t-BHP, and the generated ROS was measured using fluorescent dye DCFH-DA and observed by FACS analysis (Fig. 1A). Treatment with 50 μM of t-BHP induced significantly higher levels of ROS production and treatment with a higher concentration of t-BHP further elevated the levels of ROS in HEI-OC1 cells (Fig. 1B). Apparently, t-BHP promoted the formation of ROS in a dose-dependent manner.

2.2. *Cytotoxicity of t-BHP on HEI-OC1 cells*

The high levels of ROS formation usually inhibit the proliferation of many types of cells. To test the effect of t-BHP on auditory cell proliferation, HEI-OC1 cells were treated with different concentrations of t-BHP for 12 h, and their cell proliferation and viabilities were determined using CCK-8. While treatment with lower concentrations (25 and 50 μM) of t-BHP slightly reduced the proliferation of HEI-OC1 cells, treatment with higher concentrations (100, 200, and 400 μM) of it significantly reduced their viabilities (Fig. 2A). The inhibitory effects of different concentrations of t-BHP appeared to be dose-dependent. Furthermore, treatment of HEI-OC1 cells with 100 μM t-BHP for varying periods (0, 3, 6, 12, 24, and 48 h) revealed that the inhibitory effects of tBHP on the HEI-OC1 proliferation increased with extended time for exposure (Fig. 2B). Therefore, t-BHP inhibited HEI-OC1 cell proliferation in a dose- and time-dependent manner.

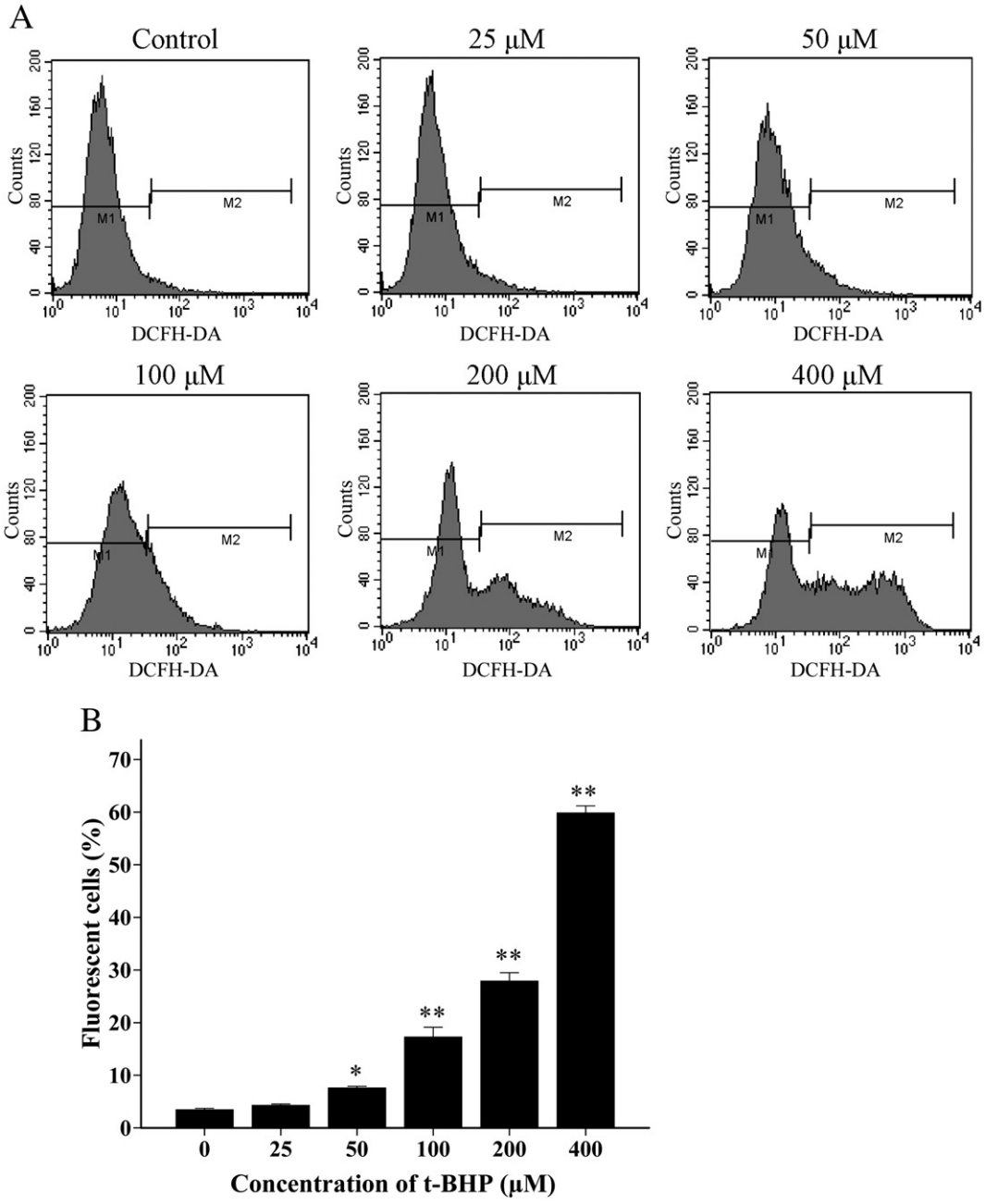

Fig. 1 – Treatment with t-BHP promotes the production of ROS in HEI-OC1 cells. (A) Flow cytometry analysis of the ROS production. HEI-OC1 cells were treated with 0, 25, 50, 100, 200, or 400 μM of t-BHP for 12 h and exposed to DCFH-DA, followed by flow cytometry analysis. Data shown are representative dot-plot graphs of different groups of cells from three independent experiments. M2 zone indicates the fluorescent intensity of ROS positive cells. (B) Quantitative analysis of the ROS production. Data are expressed as mean % ± SEM of fluorescent cells in different groups from three independent experiments. *P < 0.05, **P < 0.01 vs. control (0 μM).

2.3. *Apoptosis of HEI-OC1 cells induced by t-BHP*

To assess whether t-BHP could induce HEI-OC1 cell apoptosis, HEI-OC1 cells were treated with 0–400 μM of t-BHP for 12 h. The cells were stained with FITC-Annexin V and propidium iodide (PI), and the t-BHP-induced HEI-OC1 cell apoptosis was characterized by flow cytometric analysis (Fig. 3A). Obviously, treatment with 0, 25, 50, 100, 200, and 400 μM of t-BHP induced 9.6 ± 1.69%, 11.39 ± 0.28%, 15.41 ± 1.31%, 16.8 ± 1.35%, 18.29 ± 1.85% and 20.46 ± 1.54% of HEI-OC1 cell apoptosis, respectively. However, the percentage of the PI positive and Annexin V negative cells was not statistically significantly different between the control and the t-BHP treated groups ($P > 0.05$, data not shown). Apparently, treatment with higher concentrations (≥50 μM) of t-BHP significantly promoted HEI-OC1 cell apoptosis (Fig. 3B).

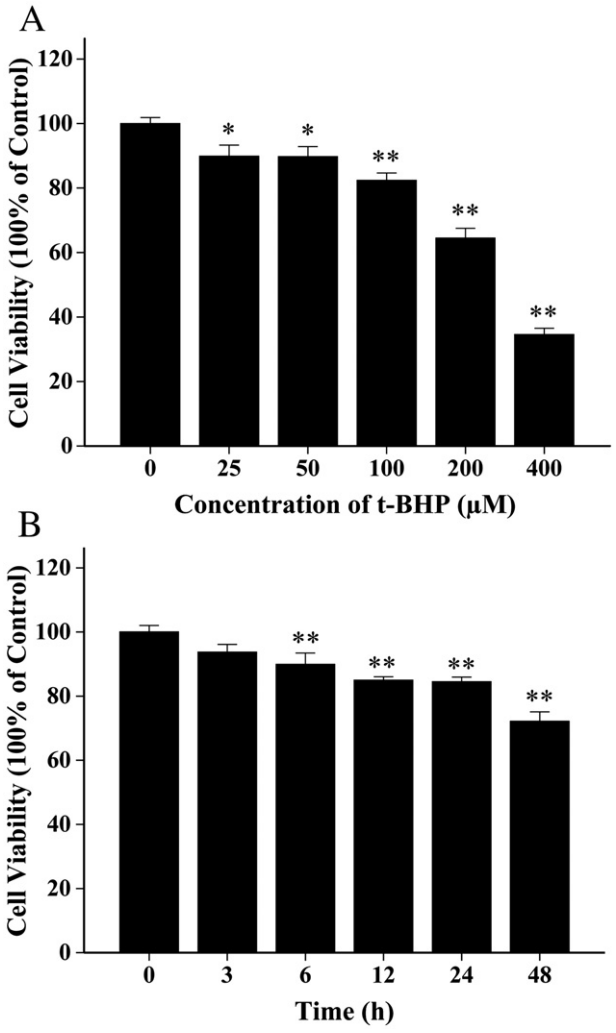

Fig. 2 – Treatment with t-BHP inhibits the proliferation of HEI-OC1 cells. (A) Dose effect of t-BHP. HEI-OC1 cells were treated in triplicate with the indicated doses of t-BHP for 12 h and the proliferation of cells was determined by CCK-8 assays. (B) Time effect of t-BHP. HEI-OC1 cells were treated in triplicate with 100 μM of t-BHP for the indicated periods and the cell viabilities were characterized by CCK-8 assays. The percentage of survived unmanipulated cells was designated as 100%. Data are expressed as mean ± SEM of the relative percentage of survived cells in each group from eight separate experiments. *P < 0.05, **P < 0.01 vs. control (0 μM).

2.4. *Treatment with t-BHP modulates the relative levels of miRNA expression in HEI-OC1 cells*

MiRNAs are crucial regulators of the translation of mRNA, regulating cell proliferation and apoptosis, including oxidative stress-induced cell apoptosis. To understand the molecular mechanisms by which t-BHP induced HEI-OC1 cell apoptosis, HEI-OC1 cells were treated with t-BHP (0, 50, 100, and 200 μM) for 12 h, and their total RNA was extracted. Subsequently, the relative levels of miRNA transcripts were determined using the highly sensitive and specific LNA-modified capture probes (Castoldi et al., 2006; Braasch and Corey, 2001; Tolstrup et al., 2003). In comparison with that in unmanipulated control cells, treatment with t-BHP (50, 100, and 200 μM) significantly increased the transcription levels of 35 miRNAs, but decreased the expression of 40 miRNAs. Treatment with 50, 100, or 200 μM of t-BHP up-regulated the relative levels of 21, 19, and 21 miRNAs, but down-regulated the transcription levels of 30, 17, and 33 miRNAs, respectively (Table 1). Furthermore, these differentially expressed miRNAs belonged to different groups, determined by clustering analysis using the CLUSTER 3.0/TreeView software (Supplementary Fig. 1). These data suggest that t-BHP modulated the expression of many miRNAs, regulating the proliferation and apoptosis of HEI-OC1 cells in vitro.

2.5. *Treatment with t-BHP modulates the relative levels of mRNA expression in HEI-OC1 cells in vitro*

Next, we examined the impact of t-BHP treatment on the relative levels of mRNA transcripts in HEI-OC1 cells. Following treatment with t-BHP, total RNA was extracted from HEI-OC1 cells and hybridized to microarray chips containing a total of 41,000 mouse gene transcripts. In comparison with that of untreated control HEI-OC1 cells, treatment with 50, 100, and 200 μM of t-BHP modulated the transcription of 2656 genes by up-regulating 2076 and down-regulating 580 gene transcriptions (the lists of differentially expressed transcripts in Additional File 2). Notably, treatment with 50, 100, and 200 μM of t-BHP significantly up-regulated the transcription of 62, 1803, and 533 genes, but down-regulated the expression of 26, 298, and 367 genes in HEI-OC1 cells, respectively (Fig. 4).

2.6. *Validation of miRNA and mRNA expression by real-time qRT-PCR*

To further characterize the effect of t-BHP treatment, HEI-OC1 cells were treated with 0, 50, 100, and 200 μM of t-BHP for 12 h. Subsequently, total RNA was extracted and reversely transcribed into cDNA, and the relative levels of miRNA and mRNA transcripts were determined by quantitative RT-PCR. Obviously, treatment with 100 and 200 μM of t-BHP significantly reduced the transcription of mmu-miR-203, while treatment with 200 μM of t-BHP increased the expression of mmu-miR-29a (Fig. 5A). Analysis of the relative levels of mRNA transcripts revealed that treatment with 50, 100, or 200 μM of t-BHP significantly reduced the relative levels of CCND2 transcripts. Furthermore, treatment with 100 or 200 μM of t-BHP also up-regulated the expression of ATF7IP (Fig. 5B). Collectively, these data indicated that the expression profiles of these miRNAs and mRNAs were consistent with that observed in the microarray assays.

2.7. *Integrated analysis of the miRNA and mRNA expression profiles in the t-BHP-treated HEI-OC1 cells*

Although the precise mechanism(s) by which miRNAs induce mRNA degradation and regulate protein expression by post-transcriptional silencing have not been fully elucidated (Filipowicz et al., 2008; Pillai, 2005), perfect miRNA seed/target complementarity is predominantly associated with the destabilization of target genes (Filipowicz et al., 2008). Accordingly, the levels of miRNA should be inversely correlated to the

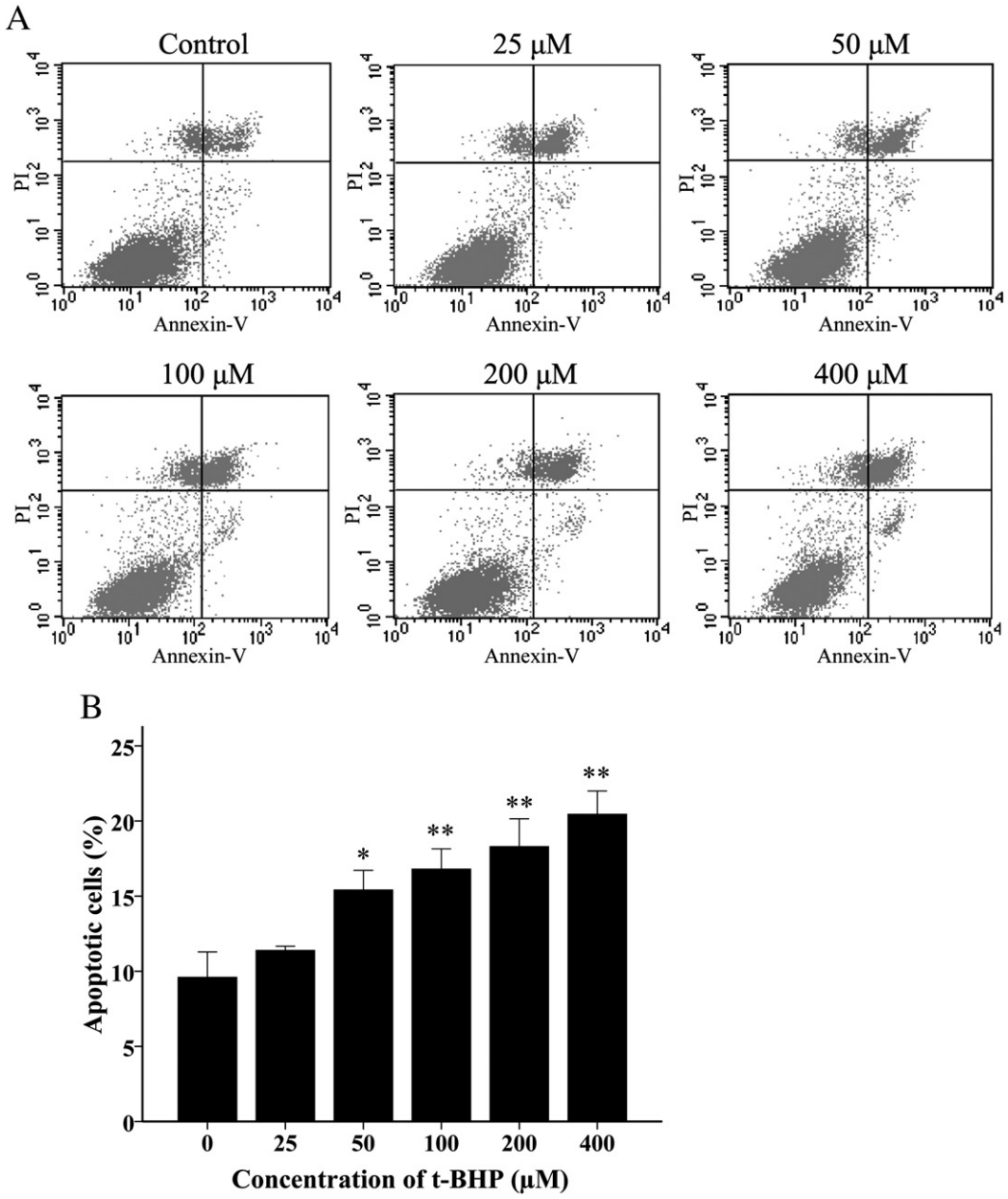

Fig. 3 – Treatment with t-BHP induces the apoptosis of HEI-OC1 cells. HEI-OC1 cells were treated with the indicated concentrations of t-BHP for 12 h and the cells were stained by FITC-Avenix V and PI and analyzed by flow cytometry. (A) FACS analysis of the t-BHP-induced HEI-OC1 apoptosis. Data shown are representative dot-plot graphs of different groups from three independent experiments. (B) The frequency of apoptotic cells was determined by FACS analysis. Data are expressed as mean %± SEM of apoptotic cells in each group of cells from three independent experiments. *P<0.05, **P<0.01 vs. control (0 μM).

levels of its target gene transcripts (Nielsen et al., 2009). To understand the mechanisms underlying the action of t-BHP in the oxidative stress of HEI-OC1 cells, we analyzed the potential association of differentially expressed mRNAs with miRNAs.

First, the potential target genes for each of the differentially expressed miRNAs were predicted by screening mRNA database using TargetScan 5.1, and then compared with the identified mRNAs that were differentially expressed in the t-BHP-treated HEI-OC1 cells. Furthermore, the 3′ un-translated region (3′ UTR) of the differentially expressed mRNAs was characterized for potential miRNA binding sites. Interestingly, 9, 141, or 128 differentially expressed mRNAs containing the binding sites of miRNAs in 50, 100, or 200 μM of t-BHP-treated cells, respectively. In addition, the relative levels of 5, 20, and 63 potential miRNA-targeted mRNA transcripts were down-regulated, while 4, 121, and 65 potential miRNA-targeted mRNA transcripts were up-regulated in the 50, 100, or 200 μM of t-BHP-treated cells, respectively (Additional File 3).

Analysis of the identified miRNAs revealed that 11 out of 35 up-regulated miRNAs (31.4%) had 81 down-regulated mRNA targets, while 15 out of 40 down-regulated miRNAs (37.5%) had 180 up-regulated target mRNAs in the t-BHP-treated HEI-OC1 cells (50, 100, and 200 μM) (Additional File 4). The potential

Table 1 – Treatment with t-BHP modulates the expression profiles of miRNA in HEI-OC1 cells.

t-BHP (μM)	Regulation	Number of miRNA	Differentially expressed miRNAs
50	Down	30	miR-122, miR-27b*, miR-28*, miR-335-5p, miR-377, miR-383, miR-675-3p, miR-743b-5p, miR-871, miR-874, miR-208a, miR-337-3p, miR-452, miR-181d, miR-18a*, miR-206, miR-291a-5p, miR-291b-5p, miR-323-3p, miR-326, miR-381, miR-425*, miR-500, miR-540-3p, miR-701, miR-763, miR-92a*, miR-17*, miR-671-5p, miR-686
	Up	21	miR-133a*, miR-17, miR-191*, miR-214, miR-467b*, miR-690, miR-691, miR-2142, miR-669e, miR-709, miR-720, miR-1192, miR-126-3p, miR-141, miR-200c, miR-466e-5p, miR-125b-5p, miR-136*, miR-181a, miR-320, miR-466c-5p
100	Down	17	miR-122, miR-203, miR-27b*, miR-28*, miR-335-5p, miR-377, miR-383, miR-675-3p, miR-743b-5p, miR-871, miR-874, miR-208a, miR-337-3p, miR-452, miR-299*, miR-743a, miR-330*
	Up	19	miR-133a*, miR-17, miR-191*, miR-214, miR-467b*, miR-690, miR-691, miR-2142, miR-669e, miR-709, miR-720, miR-199a-5p, miR-466f-3p, miR-466i, miR-142-5p, miR-16, miR-700, miR-706, miR-744
200	Down	33	miR-122, miR-203, miR-27b*, miR-28*, miR-335-5p, miR-377, miR-383, miR-675-3p, miR-743b-5p, miR-871, miR-874, miR-181d, miR-18a*, miR-206, miR-291a-5p, miR-291b-5p, miR-323-3p, miR-326, miR-381, miR-425*, miR-500, miR-540-3p, miR-701, miR-763, miR-92a*, miR-299*, miR-743a let-7a*, miR-107, miR-186*, miR-212, miR-346, miR-688
	Up	21	miR-133a*, miR-17, miR-191*, miR-214, miR-467b*, miR-690, miR-691, miR-1192, miR-126-3p, miR-141, miR-200c, miR-466e-5p, miR-199a-5p, miR-466f-3p, miR-466i, miR-101b, miR-144, miR-29a, miR-451, miR-881, miR-881*

Differentially expressed miRNAs included were expressed at 2-fold difference levels and significant at Welch t-test ($P<0.05$), as compared with controls. *Some miRNA hairpin precursors give rise to two excised miRNAs, one from each arm. An asterisk has been used to denote the less predominant form.

interaction between those miRNAs and mRNAs was analyzed in t-BHP-treated HEI-OC1 cells (Fig. 6), suggesting that these miRNAs and mRNAs may form a network that regulates the t-BHP-induced oxidative stress in HEI-OC1 cells.

GO (see Additional File 5) and pathway analyses (see Additional File 6) of differentially expressed mRNAs induced by treatment with t-BHP revealed that the cellular process was the top GO category of up-regulated mRNAs, associated with down-regulated miRNAs; while the regulation of biological process was the top GO category of down-regulated mRNAs, related to the up-regulated miRNAs.

3. Discussion

Low levels of ROS are physiological regulators of cell differentiation, proliferation, and migration, while high levels of ROS can cause cell apoptosis and death by modulating the expression of many genes (Nagy et al., 2004; Previati et al., 2004; Van et al., 2004). High levels of ROS are associated with hearing loss and hair cell death (Guthrie, 2008; Henderson et al., 2006; Sugahara et al., 2006). In this study, we employed an in vitro cellular model of oxidative stress in auditory HEI-OC1 cells. The experiments were done by culturing HEI-OC1 cells under the permissive (proliferative) conditions. Although the cellular mechanisms from the in vitro study on undifferentiated hair cells may not completely represent the mature organ of Corti in vivo, the findings may partially reflect the mature organ in vivo because both differentiated and permissive auditory cells share with some molecular characteristics (Kalinec et al., 2003). Ototoxic drugs are important factors for the development of prenatal deafness and induce oxidative stress, leading to hearing injury and loss (Guthrie, 2008; Rybak et al., 2007). In addition, HEI-OC1 cells cultured under the permissive conditions have been used for the study of ototoxic mechanisms and screening of otoprotective agents (Kalinec

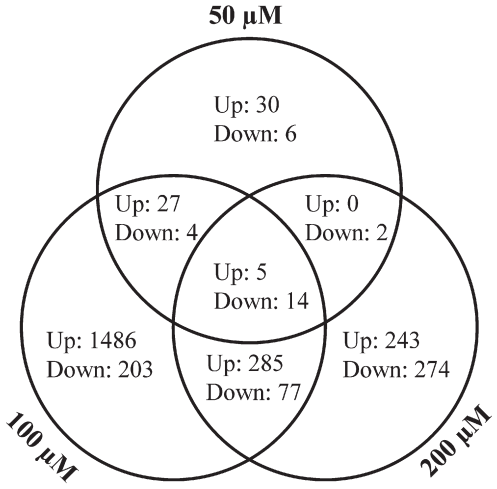

Fig. 4 – Analysis of mRNA expression in the t-BHP-treated HEI-OC1 cells. HEI-OC1 cells were treated with 0, 50, 100, or 200 μM of t-BHP for 12 h and their total RNAs were extracted for microarray analysis. The relative levels of individual mRNA transcripts with 2-fold difference and statistical significance by Welch t-test ($P<0.05$), as compared with that of untreated control cells, were identified differentially expressed mRNAs. Data shown are real number of up-regulated (Up) and down-regulated (Down) mRNAs. The numbers in the overlapped areas represent the number of differentially transcribed mRNAs shared by two or three groups.

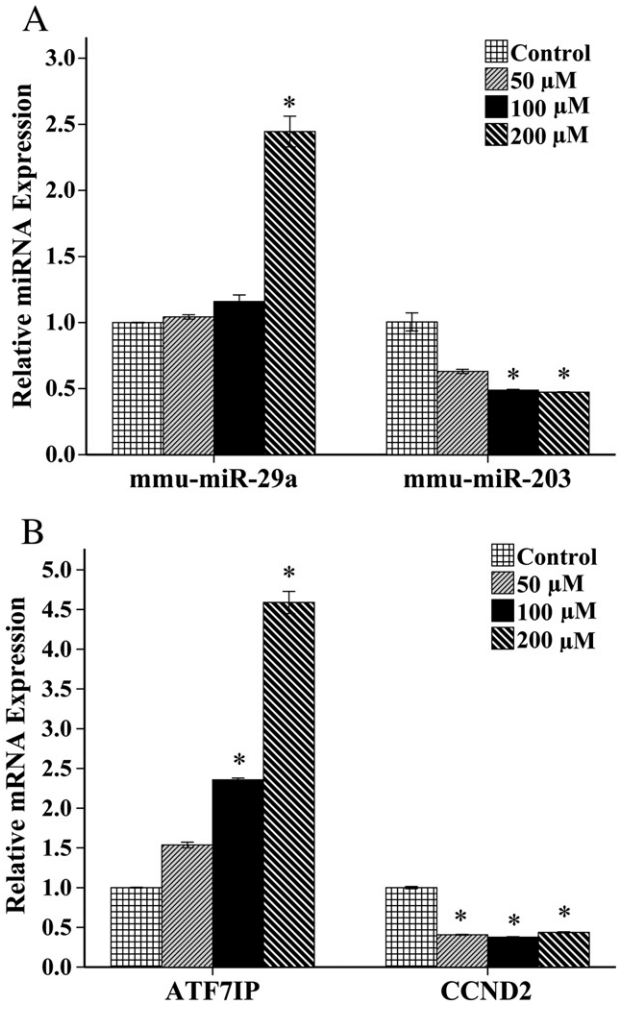

Fig. 5 – Analysis of miRNAs and mRNAs by quantitative RT-PCR. HEI-OC1 cells were treated with t-BHP (0, 50, 100, and 200 μM) for 12 h and the relative levels of miRNA and mRNA transcripts to the internal control were determined by quantitative RT-PCR. (A) Analysis of miRNA expression. (B) Analysis of mRNA expression. The relative levels of the indicated miRNA and mRNA transcripts to the internal control in unmanipulated control cells were designated as 1, and data are expressed as mean ± SEM of the relative levels of miRNA and mRNA transcripts in each group of the cells from three experiments. *Fold change >2 and $P<0.05$ vs. control (0 μM).

et al., 2005; Kim et al., 2006). Moreover, previous studies have shown that treatment with t-BHP induces oxidative injury and cell death of permissive HEI-OC1 cells (Choi et al., 2007; Choi and Kim, 2008).

Our study found that treatment with t-BHP promoted the production of ROS in a dose-dependent manner. Furthermore, treatment with t-BHP inhibited HEI-OC1 cell proliferation, which was associated with inducing HEI-OC1 cell apoptosis. In addition, we found that treatment with t-BHP up-regulated the transcription of 35 miRNA, but down-regulated the expression of 40 miRNAs. Further analysis of the potential targeted mRNAs revealed that treatment with t-BHP increased the transcription levels of 180 mRNAs, but decreased the expression of 81 mRNAs. Conceivably, these miRNAs and their targeted genes regulate the t-BHP-related oxidative stress-mediated auditory cell death.

Importantly, the t-BHP-up-regulated miRNAs were associated with decreases in the transcription levels of predicted target mRNAs, while the t-BHP-down-regulated miRNAs were related to the increased transcription levels of potential target mRNAs. For example, the transcription levels of t-BHP up-regulated miR-29a, miR-214, miR-17, miR-199a-5p, miR-200c, miR-141, miR-451, miR-144, miR-142-5p, miR-16, and miR-181a were negatively correlated with the expression levels of their predicted target genes (Fig. 6A). Similarly, the levels of t-BHP-down-regulated miR-206, miR-381, miR-326, miR-181d, miR-122, miR-203, miR-323-3p, miR-377, miR-212, miR-107, miR-874, miR-208a, miR-337-3p, miR-383, and miR-452 were also inversely associated with the increased transcription of their predicted target genes (Fig. 6B). The inverse relationships suggest that the oxidative stress-related miRNAs negatively regulate the expression of their targeted genes by inducing degradation of mRNAs and/or inhibiting the translation of mRNAs in HEI-OC1 cells (Shyu et al., 2008; Yekta et al., 2004). However, the precise relationship between those miRNAs and the expression of their potential target mRNAs remains to be further validated.

The inverse correlation between the expression of miRNAs and their predicted target mRNAs identified in this study supports the hypothesis that miRNAs may modulate the expression of genes required in the oxidative stress-related death of auditory cells. Further GO analysis revealed that the differentially regulated mRNAs could be classified as predicted targets of miRNAs. Moreover, the targets of these miRNAs were chosen to perform a pathway analysis on DAVID Bioinformatics Resources 6.7 (Additional File 6).

Insulin-like growth factor 1 (IGF-1) is a member of the insulin receptor family that regulates tissue development and homeostasis (Piper et al., 2008; Russo et al., 2005), and provides pivotal cell survival and differentiation signals during the development of inner ear (Sanchez-Calderon et al., 2007). A recent study has found the spatiotemporal expression of the IGF signaling elements during the development of inner ear and reveals that IGF-1, through novel regulatory mechanisms, promotes sensory and neural cell survival and differentiation (Sanchez-Calderon et al., 2010). In age-related hearing loss (ARHL) of CD/1 mice, Schwann cells of the spiral ganglion seemed to be more vulnerable to free radical damage than the neurons, and degenerated more rapidly. Since oxygen radicals are required for the post-translational stabilization of HIF-1alpha during hypoxia, ROS induced multiple reactions within the cochlea, such as inhibition of neuronal protection mechanisms with repression of IGF-1 (Riva et al., 2007). Notably, our data indicated that the expression of IGF-1 signaling events (include IGF-1, PIK3R1, and PTPN11) were down-regulated, which was associated with increased transcription of miR-29a, miR-17, and miR-200c. Interestingly, IGF-1 is a potential target of miR-29a, PIK3R1 is a potential target of miR-29a and miR-17, and PTPN11 is a potential target of miR-200c. Apparently, t-BHP induced oxidative stress inhibited the IGF-1 mediated signaling by up-regulating the transcription of related miRNAs in HEI-OC1 cells.

Hearing loss can be caused by a variety of insults, including acoustic trauma and exposure to ototoxins, which principally

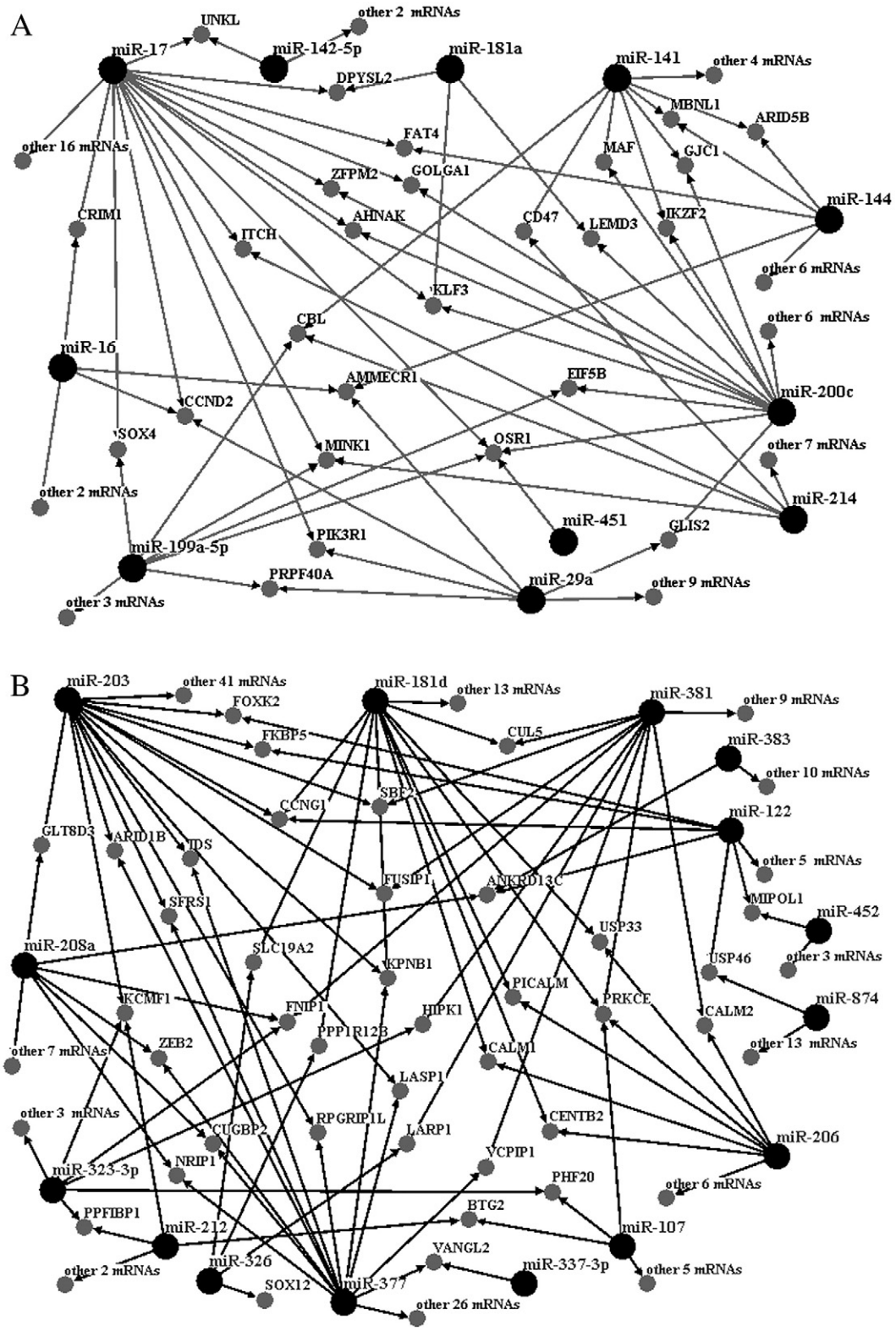

Fig. 6 – Network analysis of the relationship of miRNAs and mRNAs. (A) The up-regulated miRNA-associated network. The larger circles represent the up-regulated miRNAs, while the smaller circles indicate the down-regulated target genes in the t-BHP-treated HEI-OC1. (B) The down-regulated miRNA-associated network. The larger circles represent the down-regulated miRNAs while the smaller circles indicate the up-regulated target genes in the t-BHP-treated HEI-OC1 cells. The miRNA binding site was predicted in the 3′ UTR of the mRNA using TargetScan 5.1 (connected lines). The "other number mRNAs" means that these mRNAs may be regulated by one miRNA and shown in the Additional File 4.

affect the viability of sensory hair cells by regulating the MAPK and JNK signaling pathway (Wang et al., 2003). Furthermore, increased levels of p38 MAPK and JNK phosphorylation are observed in the cochleae of aging CBA/J mice. These suggest that multiple cell death pathways, which are related to oxidative stress, are activated in the hair cells of the auditory organ in aging mice (Sha et al., 2009). Indeed, corticosteroids have long been used for the intervention of several types of acute sensorineural hearing loss because of their anti-inflammatory and anti-apoptotic effects. Other anti-apoptotic drugs that target the MAPK/c-JNK signal cascade are promising both in vitro and in laboratory animal studies (Dinh et al., 2009). Notably, our data revealed that the MAPK signaling events of FOS, SOS2, PPP3R1, PPM1B, FLNC, and PPP5C were up-regulated by oxidative stress and their expressions were likely regulated by miR-383, miR-208a, miR-377, miR-181d, miR-377, and miR-874, respectively.

Interestingly, we detected a positive correlation between the expression of a few miRNAs and their potential target mRNAs (either down-regulated or up-regulated transcription). These data suggest a positive regulatory role of miRNAs for their target genes by some alternative indirect mechanisms, particularly for those genes involved in cell cycle regulation (Vasudevan et al., 2007). We are interested in further investigating how oxidative stress modulates the transcription of those miRNAs and their predicted target genes in auditory cells.

In summary, we employed an in vitro model of oxidative stress in auditory cells and displayed the profiles of t-BHP-induced differentially expressed miRNAs and mRNAs in HEI-OC1 cells. Although our study is very preliminary, we believe that our findings provide a basis for further investigation of the ROS-related pathogenic process and its associated signal transduction pathways in auditory cells. We are interested in further validating our findings in primarily cultured auditory cells and the mature organ of Corti in vivo. Apparently, modulation of those related miRNA transcription may aid in the design of new therapies for the prevention and treatment of hearing loss at clinic.

4. Experimental procedures

4.1. *Cell culture*

Conditionally immortalized mouse auditory cells, HEI-OC1, were kindly provided by Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA, USA). The cells can be cultured under a permissive condition (33 °C) for proliferation while under a nonpermissive condition (39 °C) for differentiation. In our study, the cells were cultured under permissive conditions (at 33 °C, 10% CO_2) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO) (Kalinec et al., 2003). The cells at 70–80% of confluence were used for the following experiments.

4.2. *Detection of intracellular ROS*

The contents of intracellular ROS were determined using 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), according to the manufacturer's instructions (Beyotime, China). Briefly, HEI-OC1 cells at 1×10^5 cells/well were cultured in 6-well plates and treated with different concentrations (0–400 μM) of t-BHP (Wako, Japan) for 12 h. Subsequently, the cells were exposed to 2 μM of DCFH-DA for 15 min and washed with PBS three times. DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). DCF fluorescence was detected by FACS analysis on a Becton Dickinson FACScan flow cytometer.

4.3. *Cell viability assay*

The viability of HEI-OC1 cells responding to t-BHP treatment was measured using Cell Counting Kit-8 (CCK-8), according to the manufacturer's instruction (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells at 5×10^3 cells/well were cultured in 96-well microplates. The cells were treated with different concentrations (0, 25, 50, 100, 200, or 400 μM) of t-BHP for 12 h, and exposed to CCK-8 (10 μl) for 1 h, followed by measuring at 450 nm on a microplate reader (Anthos 2010, China). Additionally, the cells were treated with 100 μM of t-BHP for varying periods (0, 3, 6, 12, 24 and 48 h). The OD450 value was proportional to the number of viable cells. Cell viability was calculated as: Absorbance of treatment group/Absorbance of untreated control group × 100%.

4.4. *Measurements of apoptosis*

The t-BHP-induced HEI-OC1 cell apoptosis was analyzed by FACS analysis using the FITC-Annexin V Apoptosis Detection Kit I, according to the instructions from the manufacturer (BD Pharmingen, USA). Briefly, HEI-OC1 cells (about 1×10^6 cells) were cultured in 10% FBS DMEM and treated with t-BHP (0, 25, 50, 100, 200, or 400 μM) for 12 h. Subsequently, the cells were washed with cold PBS twice, and stained with FITC-Annexin V and PI. The apoptotic cells were examined by FACS analysis on a Becton Dickinson FACScan flow cytometer. A total of 10,000 events were characterized for each sample and analyzed with the FACScan Cell Quest software (Apple, USA).

4.5. *RNA isolation and quantification*

HEI-OC1 cells were treated with t-BHP (0, 50, 100 and 200 μM) for 12 h and harvested. Their total RNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. The quality and quantity of extracted RNA were assessed by spectrophotometry at A260/A280 on a Nanodrop ND-1000 Spectrophotometer, USA and the denaturing agrose gel electrophoresis. The RNA samples were used for miRNA microarray, mRNA microarray, and qRT-PCR experiments.

4.6. *MicroRNA microarray*

Individual RNA samples were labeled using the miRCURY Hy3 labeling kit (Cat. 208032, Exiqon) and concentrated with the RNeasy Mini Kit (Cat. 74104, Qiagen), following the manufacturer's instruction. After evaluating the labeling efficiency, the labeled RNA samples were hybridized on the miRCURY LNA™ (locked nucleic acid, LNA) Array (v. 11.0, Exiqon). Three independent hybridizations for each sample were performed

on chips with each miRNA spotted in quadruplicate. The resulting signals were scanned using the GenePix 4000B scanner (635 nm, Axon Instruments) and the values of signal intensity were normalized to per-chip median values, and then used to obtain geometric means and standard deviations for each miRNA using the GenePix Pro V6.0 software (Axon Instruments). A miRNA with 2-fold difference in mean normalized values and statistical significance in Welch *t*-test ($P<0.05$) between control and t-BHP-treated HEI-OC1 cells was considered as a differentially expressed miRNA.

4.7. *mRNA microarray*

Individual RNA samples were amplified and labeled using the Quick Amp labeling kit (p/n 5190-0442, Agilent) and hybridized in triplicate with the Agilent Whole Mouse Genome 4×44 k Oligo Microarray Kit format (p/n G4122F), according to the manufacturer's instructions. After hybridization and washing, the processed slides were scanned with the Agilent microarray scanner (p/n G2505B) using settings recommended by Agilent Technologies. The resulting text files extracted from Agilent Feature Extraction Software (v10.5.1.1) were analyzed using the Agilent GeneSpring GX software (version 11.0). The microarray data were normalized using the Agilent FE one-color scenario (mainly median normalization). Individual genes with 2-fold difference in normalized expression and statistical significance in Welch *t*-test ($P<0.05$) between control and t-BHP-treated HEI-OC1 cells were identified as differentially expressed genes.

4.8. *Quantitative RT-PCR*

The levels of individual gene transcripts were determined by qRT-PCR using the qRT-PCR mRNA detection kit, according to the manufacturer's instruction (Promega). Briefly, total RNA was reversely transcribed into cDNA and used as templates for qRT-PCR analysis of the mmu-miR-29a, mmu-miR-203, ATF7IP, and CCND2 expression, respectively, on a Roche Lightcycler 480 Detection System. The U6 miRNA and GAPDH mRNA expression were used as internal controls, respectively. The relative levels of individual gene transcripts to control U6 miRNA or GAPDH were determined using the threshold cycle (Ct) and expressed as $2^{-(\Delta Ctsample-\Delta Ctcontrol)}$ (Lin et al., 2009; Cheng et al., 2009).

4.9. *Integrated analysis of miRNA and mRNA*

The relationships of differentially expressed miRNAs and mRNAs were further analyzed. The potential mRNA targets of individual differentially expressed miRNAs were predicted using the TargetScan version 5.1 (Friedman et al., 2009b). Their potential ontology, pathway and networks were analyzed using the DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003; Huang et al., 2009) and the Osprey 1.2.0, respectively (Breitkreutz et al., 2003).

4.10. *Statistical analysis*

All data were expressed as mean±standard error of the mean (SEM) from at least three independent experiments. The difference among groups was analyzed by one-way ANOVA using SPSS 16.0. A value of $P<0.05$ was considered as statistically significant.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (no. 30971193), the Key Program of Medical Scientific Research Foundation of Guangzhou Municipality (no. 2009-ZDi-07; no. 2006-ZDi-06), and the Medical Scientific Research Foundation of Guangzhou Municipality (no. 2008-YB-114) and Guangdong Province (no. B2009218), China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.05.059.

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