Mir-573 regulates cell proliferation and apoptosis by targeting Bax in human degenerative disc cells following hyperbaric oxygen treatment

Song-Shu Lin
Chang Gung Memorial Hospital Linkou Main Branch: Chang Gung Memorial Hospital

Chi-Chien Niu
Chang Gung Memorial Hospital Linkou Main Branch: Chang Gung Memorial Hospital

Li-Jen Yuan
E-Da Hospital/E-Da University

Tsung-Ting Tsai
Chang Gung Memorial Hospital Linkou Main Branch: Chang Gung Memorial Hospital

Po-Liang Lai
Chang Gung Memorial Hospital Linkou Main Branch: Chang Gung Memorial Hospital

Kowit-Yu Chong
Chang Gung University College of Medicine

Kuo-Chen Wei
Department of Neurosurgery, Chang Gung Memorial Hospital, Chang Gung University

Chiung-Yin Huang
Department of Neurosurgery, Chang Gung Memorial Hospital, Chang Gung University

Meng-Ling Lu
Chang Gung Memorial Hospital Kaohsiung Branch

Chuen-Yung Yang
Chang Gung Memorial Hospital Linkou Main Branch: Chang Gung Memorial Hospital

Steve W. N. Ueng (mailto: wenneng@adm.cgmh.org.tw)
Chang Gung Memorial Hospital Linkou Branch

Research article

Keywords: microRNA-573, Bax, caspase, HBO, NPC

Posted Date: October 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-88633/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Version of Record: A version of this preprint was published on January 7th, 2021. See the published version at https://doi.org/10.1186/s13018-020-02114-6.
Abstract

Background: MicroRNA (miRNA) plays a vital role in the intervertebral disc (IVD) degeneration. The expression level of miR-573 was downregulated whereas Bax was upregulated notably in human degenerative nucleus pulposus cells (NPCs). In this study, we aimed to investigate the role of miR-573 in human degenerative NPCs following hyperbaric oxygen (HBO) treatment.

Methods: NPCs were separated from human degenerated IVD tissues. The control cells were maintained in 5% CO$_2$/95% air and the hyperoxic cells were exposed to 100% O$_2$ at 2.5 atmospheres absolute. MiRNA expression profiling was performed via microarray and confirmed by real-time PCR, and miRNA target genes were identified using bioinformatics and luciferase reporter assays. The mRNA and protein levels of Bax were measured. The proliferation of NPCs were detected using MTT assay. The protein expression levels of Bax, cleaved caspase 9, cleaved caspase 3, pro-caspase 9 and pro-caspase 3 were examined.

Results: Bioinformatics analysis indicated that the 3’ untranslated region (UTR) of the Bax mRNA contained the “seed-matched-sequence” for hsa-miR-573, which was validated via reporter assays. MiR-573 was induced by HBO and simultaneous suppression of Bax was observed in NPCs. Knockdown of miR-573 resulted in upregulation of Bax expression in HBO-treated cells. In addition, overexpression of miR-573 by HBO increased cell proliferation and coupled with inhibition of cell apoptosis. The cleavage of pro-caspase 9 and pro-caspase 3 was suppressed while the levels of cleaved caspase 9 and caspase 3 were decreased in HBO-treated cells. Transfection with anti-miR-573 partly suppressed the effects of HBO.

Conclusion: Mir-573 regulates cell proliferation and apoptosis by targeting Bax in human degenerative NPCs following HBO treatment.

Background

Intervertebral disc degeneration (IDD) is considered to be the pathological basis of degenerative spinal diseases, leading to intervertebral disc herniation, spinal canal stenosis and lower back pain [1]. Apoptosis is a key component responsible for the decrease in the cell number of nucleus pulposus cells (NPCs) during degeneration [2, 3]. There were two apoptotic factors Bax (pro-apoptotic protein) and Bcl-2 (anti-apoptotic protein); the former binds to the mitochondrial membrane and induces the release of cytochrome c [4] and the latter prevents the formation of Bax homodimers [5], which inhibits the cytosolic accumulation of cytochrome c and activation of caspase 3 [6]. Bcl-2 prevents or delays apoptotic induction by a large variety of stimuli in many cell types [7]. Overexpression of bcl-2 in intervertebral disc (IVD) cells reduced the mRNA expression level of caspase 3 and prevented in vitro apoptotic cell death [8].

MicroRNAs (miRNAs) are endogenous non-coding small RNAs consisting of 20–25 nucleotides that serve to mediate gene regulatory events by pairing with the 3’ untranslated region (3’ UTR) of their target messenger RNAs (mRNAs) and, thus, modulating their expression [9]. Various miRNAs are dysregulated in IDD and functionally implicated in its pathogenesis. MiR-21 promotes human NPCs proliferation by affecting PTEN/AKT signaling [10]. MiR-494 induced cell apoptosis via directly combining with SOX9 in human degenerative NPCs [11]. Therefore, miRNAs might play an important role in the development and progression of IDD through regulating NPCs proliferation and apoptosis.

Human IVD is the largest avascular tissue in the body [12]. During degeneration, structural changes with vascular depletion, endplate calcification and disc size increase make oxygen diffusion harder and the oxygen concentration in the IVD becomes even lower [13]. Hyperbaric oxygen (HBO) treatment can serve to improve hypoxic conditions by increasing tissue and/or microvascular O$_2$ levels [14]. The expression level of miR-573 was downregulated whereas Bax was upregulated notably in degenerative NPCs [15]. However, the role of miR-573 in degenerative NPCs following HBO treatment has not been fully elucidated.

In this study, we demonstrated that HBO treatment increased miR-573 expression in degenerated NPCs, as assessed via microarray analysis and confirmed by real-time PCR. We used bioinformatics to identify putative target sequences for miR-573 in the human Bax mRNA and confirmed these by way of luciferase reporter assays. Subsequently, we examined the cell
proliferation, Bax protein, cleaved-caspase 9 protein, pro-caspase 9 protein, cleaved-caspase 3 protein, and pro-caspase 3 protein expression after HBO treatment. Our findings provide a new therapeutic target for the HBO treatment of IDD.

Materials And Methods

The experimental protocol was approved by the Human Subjects Institutional Review Board at Chang Gung Memorial Hospital, Taiwan.

Nucleus pulposus cell isolation and culture. Fresh abnormal disk tissue was harvested from the degenerated lumbar IVD of 28 patients who receive total discectomy and posterior lumbar interbody fusion (n = 28; male = 10, female = 18, age = 63.1 ± 12.7). NPCs were separated from the nucleus tissues by performing sequential enzymatic digestion, first with 0.4% pronase (Sigma) for 1 h and subsequently with 0.025% collagenase P (Boehringer) and 0.004% DNase II (Sigma) at 37°C overnight. After digestion, the cells were washed with DMEM/F-12 and seeded in three fresh flasks at a density of 5,000 cells/cm² and incubated in a humidified atmosphere of 5% CO₂ and 95% air until the cells attained confluence. Cells were used at passage 2 for each experiment.

Cells exposure to intermittent HBO. Approximately 2 × 10⁵ cells were plated on a 100-mm cell culture dish containing 10 ml DMEM/F-12 supplemented with 10% FBS. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were either maintained in 5% CO₂/95% air throughout the experiment as a control or in HBO-treated protocol containing three times of 100% O₂ at 1.5 atm (atmosphere) for 25 min each, two times of air break (5% CO₂/95% air) at 1.5 atm for 5 min each in a hyperbaric chamber (Sigma II; Perry Baromedical, USA). The total duration for HBO-treated protocol is about 120 min. HBO treatment administered a total of 120 min every 48 h.

MicroRNA Profiling. Total RNA was extracted from cells using mirVana miRNA isolation kit (Ambion, Austin). MiRNA expression profiling was accomplished using TaqMan Human MicroRNA Array B Cards containing 384 mature human microRNAs (Applied Biosystems, USA) and an ABI 7900 real-time PCR System according to the manufacturer’s protocol. MiRNA expression profiling was performed on eight samples from four patients (with or without HBO treatment). Briefly, 3 µL of total RNA from each sample was reverse-transcribed using the Taq-Man miRNA Reverse Transcription Kit (Applied Biosystems, USA) and the stem-loop Megaplex Primer Pool Sets. A total of 7.5 µL of reaction mixture was immediately incubated under the following conditions: 40 cycles at 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s, and 85 °C for 5 min. Then, 2.5 µL of the resultant Megaplex RT products were mixed with 2.5 µL of Megaplex PreAmp Primers and 12.5 µL of TaqMan PreAmp Master Mix. A total of 25 µL of the reaction mixture was incubated using the following program: 95 °C for 10 min, 55 °C for 2 min, and 72 °C for 2 min followed by 12 cycles at 95 °C for 15 s, 60 °C for 4 min, and 99.9 °C for 10 min. The pre-amplified cDNA was diluted with 0.1 × TE (10 mM Tris, pH 8.0, 1 mM EDTA) to 100 µL and used for PCR. The relative miRNA expression levels were calculated by the 2−ΔΔCt method as follows: ΔCt (test) = Ct (miRNA of interest, test) − Ct (internal reference, test), ΔCt (calibrator) = Ct (miRNA of interest, calibrator) − Ct (internal reference, calibrator), ΔΔCt = ΔCt (test) − ΔCt (calibrator). The inter-individual variability of the efficiency of our procedures was controlled by spiking of U6 snRNA. The hierarchical cluster analysis of differentially expressed miRNAs was performed using CLUSTER 3.0 [16], and the hierarchical clustering heat map was visualized by Tree View [17].

Real-time PCR. TaqMan miRNA assays (ABI PRISM; Applied Biosystems, USA) were used to detect the expression levels of the mature miR-573. For the reverse transcription (RT) reactions, 10 ng of total RNA was mixed with the RT primer. RT reactions were performed at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then maintained at 4 °C. Following the RT reactions, 1.5 µl of cDNA was used for a polymerase chain reaction (PCR) using 2 µl of TaqMan primers. The PCR was conducted at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in an ABI 7900 real-time PCR system (Applied Biosystems, USA). The fold change in the miRNA expression in each sample relative to the average expression in the control was calculated based on the threshold cycle (CT) value using the 2−ΔΔCt method.

MiRNA target prediction and dual-luciferase reporter assays. Target Scan 7.2 (http://www.targetscan.org) online software were used to analyze the putative target genes of miR-573. The 3’ UTR of Bax containing the miR-573 binding site was cloned into
pmirGLO dual-luciferase miRNA reporter vectors (Promega, USA). A mutated 3' UTR of Bax was introduced into the potential miR-573 binding site. The reporter vectors containing the wild-type or mutant Bax 3' UTR were transfected into NPCs using Lipofectamine 3000 (Invitrogen, USA). After incubation with or without HBO, transfected cells were lysed. Firefly and Renilla luciferase activities were detected using the dual-luciferase assay system (Promega, USA) in accordance with the manufacturer's instructions.

**Transfection of NPCs with anti-miRNAs and analysis after HBO treatment.** NPCs were seeded into 24-well plates at a density of 2 x 10^4 cells/cm^2^ in culture medium without antibiotics and divided into control, HBO, and HBO + Anti-miR-573 groups. The next day (day 1), cells were transfected with anti-miR-573 (100 nM; Ambion, USA) using RNAiMAX (Invitrogen, USA) and cultured in an incubator at 37 °C with 5% CO₂. After 8 h of transfection, the culture medium was changed to DMEM/F-12 with 5% FBS, and the cells were exposed to HBO treatment. Cell proliferation was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. Forty-eight hours after HBO treatments, cells were washed once in serum-free DMEM/F-12 medium, then 900 µL/well medium and 100 µL/well MTT (5 mg/mL in PBS; Sigma) were added for 4 h at 37 °C. The dye solution was removed and cells washed once with PBS before adding 1,000 µL/well dimethyl sulfoxide (DMSO) and mixing in an orbital shaker for 30 to 60 min at room temperature in the dark. Finally, 150 µL samples were removed from each well and put into 96-well plates, and absorbance was read at 570 nm against a blank of DMSO using a microplate reader (Dy nex MRX; Dynex Technologies Ltd., UK). Data expressed the (absorbance value) ratio of HBO and HBO + Anti-miR-573 to the control group.

NPCs were seeded into six-well plates at a density of 2 x 10^5 cells/well in culture medium without antibiotics. The next day (day 1), cells were transfected with anti-miR-573 (100 nM) using RNAiMAX (Invitrogen) and cultured in an incubator at 37 °C with 5% CO₂. After 8 h of transfection, the culture medium was changed to DMEM/F-12 with 5% FBS, and the cells were exposed to HBO treatment. On days 3 and 5, the cells were re-transfected once and exposed to HBO. At 12 h after the third HBO treatment, cellular RNA was isolated using an RNeasy mini kit (Qiagen, USA) and reverse-transcribed into cDNA with the ImProm-II reverse transcription system (Promega, USA) for real-time PCR detection of Bax transcripts. At 24 h after the third HBO treatment, cells were washed with PBS and extracted using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, USA) for immunoblotting experiments. The proteins were separated via SDS-PAGE and transferred onto nitrocellulose membranes using a protein transfer unit (Bio-Rad, USA). After blocking with 10% nonfat milk, the membranes were incubated overnight at 4 °C with a 1000-fold dilution of mouse antibodies against Bax (Abcam, UK) or β-actin (Abcam, UK) or a 1000-fold dilution of rabbit antibodies against procaspase-9, cleavedcaspase9, procaspase-3, cleavedcaspase3 (Cell Signaling Technology, MA). After washing, the membranes were further incubated for 2 h with a 10,000-fold dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (CalBiochem, USA) or goat anti-rabbit IgG conjugated to horseradish peroxidase (CalBiochem, USA). The membranes were then washed and rinsed with ECL detection reagents (Amersham Pharmacia Biotech, UK). The band images were photographed using Hyperfilm (Amersham Pharmacia Biotech, UK). The intensity of the staining for Bax, procaspase-9, cleavedcaspase9, procaspase-3, cleavedcaspase3, and β-actin was quantified using an image analysis system (Image-Pro plus 5.0; Media Cybernetics, USA).

**Statistical analysis.** Data are expressed as mean ± standard deviation (SD). The p value for the Student's t test was calculated, and a p value of < 0.05 was considered statistically significant.

**Results**

**Heat maps of miRNA expression in degenerated NPCs after HBO treatment.** To identify the miRNAs involved in the molecular regulation of NPCs after HBO treatment, the miRNA expression profile of NPCs was performed using a TaqMan Human MiRNA Array B Card. As shown in Figs. 1a, there were 185 miRNAs upregulated and 37 downregulated by at least 1.5-fold following HBO treatment. Among these, miR-573 was chosen for further investigation (Figs. 1b, Table 1, n = 4) as previous studies revealed that miR-573 expression is lower in degenerative NPCs [15].
Table 1. Differentially expressed of miRNA-573 in NPCs following HBO treatment

| Detector Mean_DeltaCt_C Expression_C Mean_DeltaCt_H Expression_H Delta_Mean_DeltaCt Fold_Change Regulation |
|--------------------------------------------------------------------------------------------------|----------------------------------------------------------|
| hsa-miR-573-001615                                        | 23.67                                                   | 7.48E-08                                               | 22.07                                                   | 2.28E-07                                               | -1.61                                                   | 3.04 | Up |

C: control group; H: HBO group

**HBO treatment increased miR-573 expression in degenerated NPCs.** HBO treatment increased miR-573 expression in NPCs (4.05 ± 1.78 fold, *p < 0.05, n = 4; Figs. 1c). These results indicated that miR-573 might play an important role in inhibiting the progression of IDD in NPCs after HBO treatment.

**Seed sequence of miR-573 in the 3' UTR of the Bax mRNA.** To investigate the potential molecular targets of miR-573, we screened for putative target genes of miR-573 using Target Scan 7.2 (http://www.targetscan.org) online software. We found that Bax, an important regulator of apoptosis, was likely a direct target of miR-573, as the 3' UTR of Bax contained a potential binding element for miR-573 with a 7-nt match to the miR-573 seed region (Figs. 2a). Additionally, cross-species conservation of the miR-573 seed sequence in the 3' UTR of the Bax mRNA was confirmed by the Target Scan algorithm (Figs. 2b). These findings suggested that hsa-miR-573 might target the Bax mRNA by directly recognizing its seed-matched sequence present in the 3' UTR.

**Bax is a direct target of miR-573.** To validate the direct targeting of Bax by miR-573, the wild type (WT) or a mutant variant (Mut) of the Bax 3' UTR containing the target sequence was cloned into a dual-luciferase reporter vector (Figs. 3a). Overexpression of miR-573 after HBO treatment significantly inhibited luciferase activity of the WT Bax 3' UTR (Figs. 3b, 0.53 ± 0.06 fold, **p < 0.01, n = 4), whereas mutation of the miR-573 binding sites abolished this inhibitory effect of miR-573 in the degenerated human NPCs (Figs. 3b, 1.05 ± 0.05 fold, *p > 0.05, n = 4). These observations support the conclusion that Bax is a target gene of miR-573 following HBO treatment.

We next examined the expression of Bax in NPCs transfected with an anti-miR-573 construct. As shown in Figs. 4a, HBO treatment decreased the mRNA expression of Bax (HBO/Control: 0.56 ± 0.09 fold, **p < 0.01, n = 4), whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (HBO + Anti-miR573/Control: 0.80 ± 0.08 fold, p*** < 0.05, n = 4). Western blot analysis was performed to examine the protein level of Bax (Figs. 4b), and the results indicated that HBO treatment led to a significant decrease in the protein level of Bax (HBO/Control: 0.55 ± 0.08 fold, **p < 0.01, n = 4), whereas knockdown of miR-573 partly suppressed the effects of HBO treatment (HBO + Anti-miR573/Control: 0.84 ± 0.06 fold, p*** < 0.05, n = 4). These data indicated that Bax was negatively mediated by miR-573 at the post-transcriptional level in NPCs after HBO treatment, as overexpression of miR-573 after HBO treatment significantly inhibited the mRNA (Figs. 4a) and protein (Figs. 4b) expression of Bax in these cells.

**Overexpression of miR-573 by HBO treatment increases cell proliferation in degenerative NPCs.** To investigate the roles of miR-573 in cell proliferation of degenerative NP cells after HBO treatment, the MTT assay was employed. As shown in Figs. 5, overexpression of miR-573 by HBO treatment markedly increased NPC cell proliferation (HBO/Control: 1.63 ± 0.18 fold, **p < 0.01, n = 4), whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (HBO + Anti-miR573/Control: 1.33 ± 0.12 fold, p** < 0.05, n = 4).

**Overexpression of miR-573 by HBO treatment blocks the mitochondrial apoptotic pathway.** To investigate whether miR573 may regulate the mitochondrial apoptotic pathway by suppressing apoptotic associated protein expression in NPCs following HBO treatment, western blotting was used to detect the cleaved caspase 9, procaspase 9, cleaved caspase 3 and procaspase 3 expression levels in NPCs. It was observed that, HBO treatment significantly down-regulated the cleaved caspase 9 and cleaved caspase 3 expression, whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (Figs. 6a, 6b for cleaved caspase 9: HBO/Control: 0.32 ± 0.04 fold, **p < 0.01, n = 4; HBO + Anti-miR-573/Control: 0.59 ± 0.10 fold, **p < 0.01, n = 4; Figs. 6a, 6c for cleaved caspase 3: HBO/Control: 0.37 ± 0.06 fold, **p < 0.01, n = 4; HBO + Anti-miR-573/Control: 0.68 ± 0.02 fold, **p < 0.01, n = 4).
for the regulation of apoptosis in NPCs following HBO treatment, transfection with anti-miR-573 only partly suppressed the mitochondrial apoptotic pathway. However, there are some limitations in this study. Because other miRNAs may also be relevant, our data indicated that MiR-573 is one of the identified miRNAs upregulated in human degenerated NPCs following HBO treatment by microarray (Figs. 1a, 1b) and confirmed this expression via real-time PCR (Figs. 1c). HBO treatment increases the O2 levels to improve hypoxia conditions thus may regulate the expression of miRNAs.

Bioinformatics analysis showed that Bax [15], apolipoprotein M (apoM) [21], and E2F transcription factor 3 (E2F3) [22] was a potential target of hsa-miR-573 in diverse cells. Bax was the first identified pro-apoptotic member in the protein family of the B cell lymphoma-2 (Bcl-2) [23], and Bcl-2 is also a key anti-apoptotic protein [24]. Epigenetic knockdown of miR-143 regulated cell apoptosis in IDD by targeting Bcl-2 [25]. Low expression of miR-125a was found in IDD by targeting pro-apoptotic Bcl-2 antagonist killer 1 [26]. Furthermore, the expression of miR-573 was decreased whereas that of Bax was increased oppositely in human IDD tissues [15]. HBO treatment upregulates the ratio of Bcl-2 to Bax expression and reduced the apoptosis of ischemic tissue [27, 28] or degenerated NPCs [29]. In this study, we further found the epigenetic regulations of Bax mRNA expression. The 3’ UTR of Bax containing the miR-573 binding site (Figs. 2). HBO treatment decreased luciferase activity in the WT 3’UTR of Bax compared with that of mutant forms (Figs. 3). Taken together, our present study suggested that Bax was a direct target of miR-573. Moreover, overexpression of miR-573 decreased the mRNA and protein levels of Bax (Figs. 4), which indicated a potential regulatory relationship between miR-573 and Bax following HBO treatment. HBO exerted its biological functions on NPCs via modulation of miR-573/Bax axis.

Increasing numbers of studies have demonstrated that miRNAs play vital roles in diverse pathological and biological processes by impacting on cell proliferation and apoptosis [23]. MiR-98 increased cell proliferation in NPCs [25]. Overexpression of miR-573 by mimic transfection increased cell viability in degenerated NPCs [15]. However, miR-573 upregulation inhibited melanoma cell proliferation also has been reported [30]. HBO treatment may promote [31–33] or inhibit [34] cell proliferation in diverse cell types. In consistent with previous studies, overexpression of miR-573 by HBO treatment markedly increased NPC cell proliferation, whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment in the present study (Figs. 5). MiR-573 overexpression increased cell proliferation and suppressed cell apoptosis in degenerative NPC cells following HBO treatment.

Mitochondria serve an important role in the apoptotic process by releasing apoptogenic molecules, including cytochrome c [35, 36]. Bax has been identified to have a proapoptotic effect, which may open the permeability transition pore on the mitochondrial membrane to trigger the release of cytochrome c from mitochondria into the cytoplasm [37]. Cytochrome c normally functions as a part of the respiratory chain, but when released into the cytosol it becomes a critical component of the apoptosis execution machinery, where it activates caspases and causes apoptotic cell death. Cytochrome c may trigger the caspase9-mediated cascade amplification reaction in the mitochondrial apoptotic pathway, which in turn processes procaspase 3 to generate active caspase 3 [38]. The present results suggested that upregulation of miR573 decreased the Bax (Figs. 4), cleavedcaspase 9 and cleavedcaspase 3 expression levels, and reduced the activation (cleavage) of procaspase 9 and procaspase 3 expression levels, whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (Figs. 6). Collectively, these results suggested that miR573 may exert its therapeutic effects on degenerated NPCs following HBO treatment by blocking the mitochondrial apoptotic pathway. However, there are some limitations in this study. Because other miRNAs may also be relevant for the regulation of apoptosis in NPCs following HBO treatment, transfection with anti-miR-573 only partly suppressed the
effects of HBO treatment. In the future, further systematic and indepth studies investigating the apoptosis in NPCs following HBO treatment will be conducted.

In conclusion, the results of this study indicate that HBO treatment of degenerated NPCs exerts a protective effect by mitigating apoptosis and its activation. Mir-573 regulates cell proliferation and apoptosis by targeting Bax in human degenerative NPCs following HBO treatment.

**Abbreviations**

IDD: Intervertebral disc degeneration  
NPCs: nucleus pulposus cells  
miRNAs: microRNAs  
3'UTR: 3' untranslated regions;  
mRNAs: messenger RNAs  
HBO: hyperbaric oxygen  
MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide  
WT: wild type  
Mut: mutant variant  
apoM: apolipoprotein M  
E2F3: E2F transcription factor 3

**Declarations**

**Ethics approval and consent to participate**

The experimental protocol was reviewed and approved by the Human Subjects Institutional Review Board at Chang Gung Memorial Hospital, Taiwan.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

Not applicable.

**Authors’ contributions**

LSS, NCC, CKY, WKC, and USWN did the conception and design. LSS, NCC, YLJ, YCY, and LMM are responsible for the acquisition, analysis, and interpretation of the data. LSS, NCC, TTT, LPL, and HCY drafted the manuscript. All authors did the final approval of the manuscript.

**Acknowledgments**
This research was supported in part by grants from the National Science Council and Chang Gung Memorial Hospital, Taiwan (CRP3K0061).

References

1. Sudo H, Yamada K, Iwasaki K, et al. Global identification of genes related to nutrient deficiency in intervertebral disc cells in an experimental nutrient deprivation model. PLoS One. 2013; 8:e58806.

2. Chen B, Fellenberg J, Wang H, et al. Occurrence and regional distribution of apoptosis in scoliotic discs. Spine 2005; 30: 519–524.

3. Kim KW, Kim YS, Ha KY, et al. An autocrine or paracrine Fas-mediated counterattack: a potential mechanism for apoptosis of notochordal cells in intact rat nucleus pulposus. Spine 2005; 30:1247–1251.

4. Gogvadze V, Robertson JD, Zhivotovsky B, et al. Cytochrome c release occurs via Ca2t-dependent and Ca2t-independent mechanisms that are regulated by Bax. J Biol Chem 2001; 276:19066–19071.

5. Reed JC, Zha H, Aime-Sempe C, et al. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 1996; 406:96–112.

6. Zhao H, Yenari MA, Cheng D, et al. Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. J Neurochem 2003; 85:1026–1036.

7. Burlacu A. 2003. Regulation of apoptosis by Bcl-2 family proteins. J Cell Mol Med 2003; 7: 249–257.

8. Sudo H, Minami A. Regulation of apoptosis in nucleus pulposus cells by optimized exogenous Bcl-2 overexpression. J Orthop Res 2010; 28:1608–1613.

9. Croce CM, Calin GA. MiRNAs, cancer, and stem cell division. Cell 2005; 122:6–

10. Liu H, Huang X, Liu X, et al. miR-21 promotes human nucleus pulposus cell proliferation through PTEN/AKT signaling. Int. J Mol Sci. 2014; 15: 4007–

11. Kang L, Yang C, Song Y, et al. MicroRNA-494 promotes apoptosis and extracellular matrix degradation in degenerative human nucleus pulposus cells. Oncotarget. 2017; 8: 27868–

12. Ciapetti G, Granchi D, Devescovi V, et al. Ex vivo observation of human intervertebral disc tissue and cells isolated from degenerated intervertebral discs. Eur Spine J 2012; 21: S10–S19.

13. Galbusera F, Mietisch A, Schmidt H, et al. Effect of intervertebral disc degeneration on disc cell viability: a numerical investigation. Comput Methods Biomech Biomed Engin 2013; 16: 328–337.

14. Korhonen K, Kuttila K, Niinikoski J: Subcutaneous tissue oxygen and carbon dioxide tensions during hyperbaric oxygenation: an experimental study in rats. Eur J Surg 1999;165: 885–90.

15. Wang R, Wen B, Sun D. MiR-573 regulates cell proliferation and apoptosis by targeting Bax in nucleus pulposus cells. Cellular & Molecular Biology Letters 2019; 24:2.

16. Zhai Y, Li Y, Zhang J et al., “Identification of the gene cluster for bistropolone-humulene meroterpenoid biosynthesis in Phoma sp.,” Fungal Genetics and Biology, vol. 129, pp. 7–15, 2019.

17. Page RD, TreeView, Glasgow University, Glasgow, UK, 2001.

18. Camps C, Saini HK, Mole DR, et al. Integrated analysis of microRNA and mRNA expression and association with HIF binding reveals the complexity of microRNA expression regulation under hypoxia. Mol Cancer 2014; 13: 28.

19. Rupaimoole R, Wu SY, Pradeep S, et al. Hypoxia-mediated downregulation of miRNA biogenesis promotes tumour progression. Nat Commun 2014; 5: 5202.

20. Niu CC, Lin SS, Yuan LJ, et al. Upregulation of miR-107 expression following hyperbaric oxygen treatment suppresses HMGB1/RAGE signaling in degenerated human nucleus pulposus cells. Arthritis Research & Therapy 2019; 21:42.

21. Hu YW, Chen ZP, Hu XM, et al. The miR-573/apoM/Bcl2A1-dependent signal transduction pathway is essential for hepatocyte apoptosis and hepatocarcinogenesis. Apoptosis 2015; 20:1321-37.

22. Chen P, Wang R, Yue Q, et al. Long non-coding RNA TTN-AS1 promotes cell growth and metastasis in cervical cancer via miR-573/E2F3. Biochem Biophys Res Commun. 2018; 503: 2956-2962.
23. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993; 74: 609–619.

24. Cleary ML, Smith SD, Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 1986; 47:19–28.

25. Zhao K, Zhang Y, Kang L, et al. Epigenetic silencing of miRNA-143 regulates apoptosis by targeting BCL2 in human intervertebral disc degeneration. Gene 2017; 628:259–

26. Liu P, Chang F, Zhang T, et al. Downregulation of microRNA-125a is involved in intervertebral disc degeneration by targeting pro-apoptotic Bcl-2 antagonist killer 1. Iran J Basic Med Sci. 2017; 20:1260–

27. Godman CA, Chheda KP, Hightower LE, et al. Hyperbaric oxygen induces a cytoprotective and angiogenic response in human microvascular endothelial cells. Cell Stress Chaperones. 2010;15: 431-42.

28. Qi Y, Ruan J, Wang M, et al. Effects of hyperbaric oxygen treatment on gastric cancer cell line SGC7901. Biomed Rep. 2017; 6: 475-479.

29. Lu XY, Cao K, Li QY, et al. The synergistic therapeutic effect of temozolomide and hyperbaric oxygen on glioma U251 cell lines is accompanied by alterations in vascular endothelial growth factor and multidrug resistance-associated protein-1 levels. J Int Med Res. 2012; 40: 995-1004.

30. Yuan LJ, Niu CC, Lin SS, et al. Additive effects of hyperbaric oxygen and platelet-derived growth factor-BB in chondrocyte transplantation via up-regulation expression of platelet-derived growth factor-beta receptor. J Orthop Res. 2009; 27:1439-46.

31. Niu CC, Lin SS, Yuan LJ, et al. Hyperbaric oxygen treatment suppresses MAPK signaling and mitochondrial apoptotic pathway in degenerated human intervertebral disc cells. J Orthop Res. 2013; 31: 204–209.

32. Li Z, Yu X, Shen J, et al. MicroRNA in intervertebral disc degeneration. Cell Prolif 2015; 48: 278–

33. Ji ML, Lu J, Shi PL, et al. Dysregulated miR-98 contributes to extracellular matrix degradation by targeting IL-6/STAT3 signaling pathway in human intervertebral disc degeneration. J Bone Miner Res. 2016; 31: 900-9.

34. Wang HF, Chen H, Ma MW, et al. miR-573 regulates melanoma progression by targeting the melanoma cell adhesion molecule. Oncol Rep. 2013; 30: 520-6.

35. Malhotra R, Lin Z, Vincenz C and Brosius FC III: Hypoxia induces apoptosis via two independent pathways in Jurkat cells: Differential regulation by glucose. Am J Physiol Cell Physiol 2001; 281: C1596-C1603.

36. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397: 441-446.

37. Lu Z, Chen H, Zheng XM and Chen ML: Experimental study on the apoptosis of cervical cancer Hela cells induced by juglone through c-Jun N-terminal kinase/c-Jun pathway. Asian Pac J Trop Med 2017; 10: 572-575.

38. Zou H, Li Y, Liu X and Wang X: An APAF-1-cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 1999; 274: 11549-11556.

Figures
Hierarchical cluster of miRNAs. HBO modulates the expression of miRNAs in degenerated NPCs. (a) A heat map of the miRNAs with significantly changed expression levels in the HBO-treated group compared with the control group. Average expression value is shown for each miRNA in each class. The red and green colors indicate that the ΔCt value is below (relatively high expression) and above (relatively low expression levels) the median of all ΔCt values. (b) MiR-573 was chosen for further investigation. (c) HBO treatment increased miR-573 expression in NPCs (*p < 0.05).
Seed sequence of miR-573 in the 3'UTR of the Bax mRNA. Target Scan predicted the duplex of miR-573 with the seed sequence in the 3' UTR of the human Bax mRNA. The sequences in white are the locations of the potential seed-matched sequences for the miRNAs assessed. Cross-species conservation of the miR-573 seed sequence in the 3' UTR of the human Bax mRNA as identified via the Target Scan algorithm (sequences in red).
Bax is a direct target of miR-573. Diagram of the binding site between miR-573 and the Bax 3' UTR. The reporter vectors contain the wild-type (WT) or mutant (Mut) Bax 3' UTR. Dual luciferase reporter assay of the Bax 3' UTR. The reporter vectors containing the WT or Mut Bax 3' UTR were transfected into NPCs. Luciferase activity was shown to be significantly down-regulated after HBO treatment (**p < 0.01; n = 4) in the constructs harboring the WT but not in the Mut 3' UTR (p > 0.05, n = 4).
Figure 4

Real-time PCR and Western blot analysis of Bax expression in degenerated NPCs transfected with miR-573 inhibitors following HBO treatment. (a) Bax mRNA expression was down-regulated after HBO treatment (**p < 0.01; n = 4). Anti-miR-573 partly reversed the suppressive effects of HBO (※p < 0.05; n = 4). (b) Bax protein expression was significantly down-regulated after HBO treatment (**p < 0.01; n = 4). MiR-573 inhibitors partly reversed the suppressive effects of HBO (※p < 0.05; n = 4). Values were normalized against β-actin. WT: wild type, Mut: mutant.
**Figure 5**

Overexpression of miR-573 by HBO treatment increases cell proliferation in degenerative NPCs. HBO treatment markedly increased NPC cell proliferation (**p < 0.01, n = 4), whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (×p < 0.05, n = 4).

**Figure 6**

Overexpression of miR-573 by HBO treatment blocks the mitochondrial apoptotic pathway. HBO treatment significantly down-regulated the cleaved caspase 9 (a, b, ***p < 0.01, n = 4) and cleaved caspase 3 (a, c, **p < 0.01, n = 4) expression, whereas transfection with anti-miR-573 partly suppressed the effects of HBO treatment (b, c, ××p < 0.01, n = 4). At the same time, HBO treatment significantly reduced the activation (cleavage) of pro caspase 9 (a, b, **p < 0.01, n = 4) and pro caspase 3 proteins (a, c, **p < 0.01, n = 4), whereas transfection with anti-miR-573 partly suppressed the effects of HBO (b, **p < 0.01; c, ×p < 0.05, n = 4) in NPCs.