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Unlocking melanoma Suppression: Insights from Plasma-Induced potent miRNAs through PI3K-AKT-ZEB1 axis

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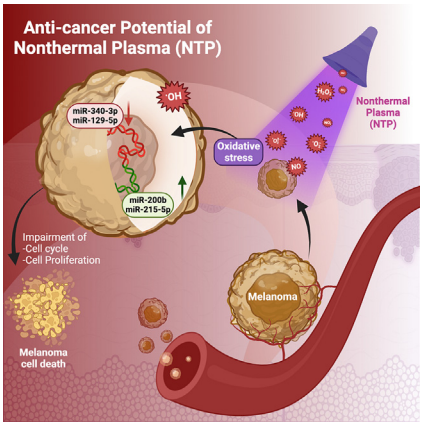
HIGHLIGHTS

- Non-thermal atmospheric pressure plasma (NTP) has anti-melanoma effects.
- miRNA sequencing identifies NTP-induced, differentially regulated miRNAs.
- NTP exposure upregulated several miRNAs, especially miR-200b-3p and miR-215-5p.
- miR-200b-3p and miR-215-5p upregulation decreased cell viability and migration.
- NTP dysregulated PI3K-AKT-ZEB1 axis-related miRNAs in melanoma cells *in vitro*.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Melanoma is a rare but highly malignant form of skin cancer. Although recent targeted and immune-based therapies have improved survival rates by 10–15%, effective melanoma treatment remains challenging. Therefore, novel, combinatorial therapy options such as non-thermal atmospheric pressure plasma (NTP) are being investigated to inhibit and prevent chemoresistance. Although several studies have reported the apoptotic and inhibitory effects of reactive oxygen species produced by NTP in the context of melanoma, the intricate molecular network that determines the role of microRNAs (miRNAs) in regulating NTP-mediated cell death remains unexplored.
Objectives: This study aimed to explore the molecular mechanisms and miRNA networks regulated by NTP-induced oxidative stress in melanoma cells.
Methods: Melanoma cells were exposed to NTP and then subjected to high-throughput miRNA sequencing to identify NTP-regulated miRNAs. Various biological processes and underlying molecular mechanisms were assessed using Alamar Blue, propidium iodide (PI) uptake, cell migration, and clonogenic assays followed by qRT-PCR and flow cytometry.
Results: NTP exposure for 3 min was sufficient to modulate the expression of several miRNAs, inhibiting cell growth. Persistent NTP exposure for 5 min increased differential miRNA regulation, PI uptake, and the expression of genes involved in cell cycle arrest and death. qPCR confirmed that miR-200b-3p and miR-215-5p upregulation contributed to decreased cell viability and migration. Mechanistically, inhibiting

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miR-200b-3p and miR-215-5p in SK-2 cells enhanced ZEB1, PI3K, and AKT expression, increasing cell proliferation and viability.
Conclusion: This study demonstrated that NTP exposure for 5 min results in the differential regulation of miRNAs related to the PI3K-AKT-ZEB1 axis and cell cycle dysregulation to facilitate melanoma suppression.

Introduction

The incidence of melanoma, the deadliest and most aggressive form of skin cancer, has been growing rapidly over the last three decades. It accounts for the majority of skin cancer-related deaths worldwide. Most melanomas detected from stages 0-III are surgically resected via lymph node management [1]. Chemotherapy, immunotherapy, and targeted therapy are the current treatments for unresectable stage III and IV and recurrent melanomas [2]. Despite reasonable treatment response rates in patients with the BRAF serine-threonine kinase V600E mutation, the mean disease-free survival of these patients remains less than six months [3]. Melanoma develops when melanocytes undergo malignant transformation due to exposure to intermittent ultraviolet (UV) light, creating an ideal microenvironment for oxidative stress [4]**.** Melanoma is caused by disrupting pathways involved in melanocyte growth and proliferation. Activating BRAF and neuroblastoma-RAS viral oncogene homolog (NRAS) mutations are observed in approximately 65 % and 20 % of melanomas, respectively [5]. The deletion of the CDKN2A locus, which regulates the activation of cyclin D/cyclin-dependent kinase 4 (CYCLIND/CDK4) and p53 to regulate cell cycle progression, is another genetic mutation prevalent in melanoma. Our comprehension of the epigenetic processes implicated in carcinogenesis, such as the control of microRNA (miRNA), is additionally enabling us to better understand the onset and spread of melanoma [6,7].

miRNAs are epigenetic factors that regulate various cellular processes, including cell proliferation, differentiation, senescence, survival, autophagy, migration, cellular metabolism, and genome integrity. Therefore, even minor changes in miRNA levels can lead to various diseases, including cancer [8–12]. miRNAs play a role in melanoma biology, and dysregulated miRNA expression has been linked to various diseases [13,14]. Specifically, miRNAs play a critical role in regulating the expression of microphthalmia-associated transcription factor (MITF), a crucial regulator of melanocyte differentiation, proliferation, survival, and melanoma formation [15]. Several miRNAs, including miR-137, miR-148, miR-182, miR-26a, miR-211, miR-542 3p, miR-340, miR-101, and miR-218, have also been identified.

Recent studies have revealed that miRNAs have great potential as biomarkers because they can distinguish between different malignancies, are chemically stable, and are resistant to RNase activity [16,17]. Strategies for delivering tumor-suppressive miRNAs or interfering with tumor-promoting miRNAs have been developed for therapeutic use [18,19]. Therefore, novel therapeutic methods are required to better understand the molecular mechanisms and signaling pathways associated with melanoma growth. Furthermore, to increase the success of metastatic melanoma treatment, therapeutic options using conventional chemotherapy alone or in conjunction with other medicines have been investigated [20,21].

Advances in materials science, composites, nanotechnology, nanostructures, and plasma technologies have profoundly impacted the development of novel, environmentally friendly cancer detection and treatment methods in recent years [22–24]. As melanoma progression largely depends on oxidative stress, we speculated that non-thermal atmospheric pressure plasma (NTP) could be an effective tool for understanding its tumorigenesis and identifying key therapeutic targets. NTP is a partially ionized gas composed of molecules, free radicals, ions, electrons, and other physical elements such as photons, an electric field, and heat [25]. It has recently gained attention because of its potential medical applications, particularly in cancer treatment. This is because NTP specifically inhibits the proliferation of cancer cells compared to their healthy counterparts in various cancer types [26–28]. Cancer cells grown *in vitro*, including melanoma, breast, and lung cancer cells, exhibit growth retardation, an increased number of double-strand breaks, and increased apoptosis [27,29]. NTP also successfully treated xenograft cancer cells *in vivo* [30]. NTP produces different reactive oxygen and nitrogen species (RONS), which could increase the oxidative stress of cancer cells, ultimately killing them [31–33]. RONS levels can alter the cancer-related epigenetic pathways responsible for cancer pathophysiology and aggressiveness. miRNA biosynthesis involves RONS at each stage; therefore, the epigenetic state of miRNAs may be altered by RONS.

This study aimed to elucidate the transcriptomic regulation of melanoma growth mediated by miRNAs. Specifically, miRNAs differentially regulated by NTP-induced oxidative stress in melanoma cells were identified through computational and bioinformatic analyses of miRNA sequencing (miRNA-seq) data. We also identified pathways that are potentially beneficial for designing novel therapeutics against melanoma. Finally, we elucidated new target genes of differentially regulated miRNAs involved in melanoma progression and metastasis as therapeutic targets. It is the first time that NTP-mediated regulation of miRNA has been demonstrated to be effective in inhibiting melanoma growth. Following NTP exposure, several miRNAs previously reported to inhibit cancer cell growth were differentially regulated. Hence, NTP exposure can perform a dual function by upregulating miRNAs that target oncogenes and downregulating miRNAs that regulate the cell cycle and apoptosis. The findings of this study explain NTP-mediated cancer growth inhibition through miRNAs.

Materials and methods

Cell culture

The human melanoma cell lines SK-MEL-2 (SK-2), SK-MEL-31 (SK-31), and G-361 were cultured in minimum essential medium (MEM; Welgene, Gyeongsan, Korea) with 10 % fetal bovine serum (FBS), MEM (Welgene) with 15 % FBS, and RPMI-1640 (Welgene) with 10 % FBS, respectively, supplemented with 1 % penicillin/streptomycin. Cells were passaged every 2–3 days and maintained in a 37 °C incubator at 5 % CO_2.

Plasma treatment for melanoma cells

We used a soft-jet plasma with air as the input gas. The plasma device used in this study and its physical properties are similar to those previously described [34,35]. SK-2 cells were seeded at a density of 5×10^4 cells/well in a 24-well plate (SPL, Pocheon,

Korea) one day before the experiment to assess cell viability. After a 24-h incubation period, the cells were exposed to NTP, and viability was determined using Alamar Blue dye (Thermo Fisher, Seoul, Korea), as described previously [36,37].

Propidium iodide (PI) uptake analysis

The uptake of PI by the cells was measured to evaluate the inhibition of melanoma growth after NTP exposure. Briefly, 2×10^5 SK-2 cells were seeded in 35-mm dishes in triplicate. After 24 h, the cells were exposed to NTP and incubated for 48 h before harvesting for PI staining. PI-stained control and NTP-treated cells were analyzed using the BD FACSVerse suite (BD Biosciences, Seoul, Republic of Korea).

Clonogenic assay

Briefly, 2×10^5 SK-2 cells were seeded in 35-mm dishes in duplicate. After 24 h, cells were exposed to NTP, harvested using 0.25 % (w/v) trypsin, and seeded at a density of 150 cells/ml in 35-mm and 60-mm dishes in triplicate. The cells were incubated and monitored regularly for up to 12 d.

mRNA expression analysis

Total RNA was extracted from the plasma-treated SK-2 cells using RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer's instructions. Total RNA (2 μg) was used to synthesize template cDNA using a reverse transcription kit (Enzynomics, Daejeon, Korea) containing MMLV reverse transcriptase, RNase inhibitor, and MMLV RT buffer with dNTPs, according to the manufacturer's instructions and run on a thermocycler (Applied Biosystems). qPCR was performed on an iCycler IQ real-time system (Bio-Rad, Hercules, California, USA) using an iQ SYBR Green Supermix (Bio-Rad) for quantification. The primer sequences used in this study are listed in Table S1.

Melanoma cell migration assay

A wound healing scratch test was used to examine cell migration in untreated control cells and cells exposed to NTP. The cells were serum-starved for 12 h after seeding until the end of the experiment. A scratch wound was then created in the 95 % confluent SK-2 cell monolayer using a Sartorius wound maker, followed by NTP exposure. The medium was treated with air/soft-jet plasma for 3 min before being used for treatment. After plasma treatment, the plates were incubated in Sartorius Incucytes (Korea). Image scanning was stopped after two days for data analysis using Incucyte software. In another experiment, cells were manually scratched and were manually photographed using a bright-field microscope and analyzed using ImageJ software at desired time points.

miRNA sequencing of melanoma cells

Total RNA from NTP-treated or untreated SK-2 cells was extracted according to the manufacturer's protocol using the RNAiso Plus RNA extraction method (Takara, Shiga, Japan) and delivered to Ebiogen Pvt. Ltd. for high-throughput RNA sequencing on an Illumina NextSeq500 platform (Ebiogen, Seoul, Korea).

miRNA inhibition

Specific inhibitors for miR-200b-3p (5′-UAAUACUGCCUG GUAAUGAUGA-3′), miR-215-5p (5′-AUGACCUAUGAAUUGACA GAC-3′), and negative control miRNA synthesized by Genolution (Korea) were transfected into cells using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. After 48 h, the cells were exposed to NTP and processed for further experiments at the indicated time points.

Flow cytometry analysis

After 24 h of culture, SK-2 cells from each group (miRNA inhibitor + NTP, NTP, and control) were seeded in 35-mm dishes and exposed to NTP for 5 min. The cells were then harvested after 48 h using trypsin and incubated with primary antibodies against AKT (Santa Cruz, Texas, USA), PI3K (Cell Signaling, USA), and ZEB1 (Abcam, Cambridge, UK), and their corresponding APC-conjugated secondary antibodies (Santa Cruz, Texas, USA) for 40 min in the dark, according to their respective manufacturer's instructions. The acquired cell samples were analyzed using a FACSVerse system (BD Biosciences, Seoul, Republic of Korea) as described previously [38].

Bioinformatics analysis

The control and plasma-treated groups were subjected to high-throughput small-RNA sequencing, and t-tests were used to identify differentially expressed genes based on a fold-change (FC) of 1.5. FC differences between the plasma-treated and control groups were examined. Differentially expressed miRNAs were identified using p-values < 0.01 and FC > 1.5 (upregulated) or FC < 1.5 (downregulated). Heatmaps and scatter plots were created for differentially expressed miRNAs using ExDEGA software. Functional clustering, gene ontology annotations, pathways, and network analyses were carried out using the miRNA enrichment turned network (Mienturnet) web tool (http://userver.bio.uniroma1.it/apps/mienturnet/) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). Further expression and survival data analyses were performed using Gene Expression Profiling Interactive Analysis 2 (GEPIA 2) (http://gepia2.cancer-pku.cn/#index) and OncoLnc (http://www.oncolnc.org/).

Statistics

Experimental data values were expressed as the mean ± standard deviation evaluated from at least three independent experiments. Statistical differences between groups were examined using PRISM10 software, and the statistical tests performed are specified in the respective figure legends, wherever applicable. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were used to denote statistically significant differences.

Results

Effect of NTP on melanoma cells

We first determined the dose of NTP required to inhibit melanoma cell growth. Briefly, we exposed melanoma cells (SK-2) to air/soft-jet plasma for different durations (0.5, 1, 1.5, 2, or 4 min) and measured cell viability after 48 h of incubation. We observed no significant reduction in cell viability in the 0.5–1.5 min NTP exposure groups, whereas there was a significant reduction in the 2- and 4-min NTP exposure groups compared to the unexposed control (Fig. 1A). We replicated this test in multiple melanoma cell lines: SK-2, SK-31, and G-361 cells, exposing them to NTP for 0, 3, or 5 min. The results indicated a dose-dependent reduction in the viability of all melanoma cell lines after NTP exposure (Fig. 1B). SK-2 cells showed a greater reduction in viability than the SK-31 and G-361 cells following NTP exposure; hence, SK-2 cells were used for subsequent experiments.

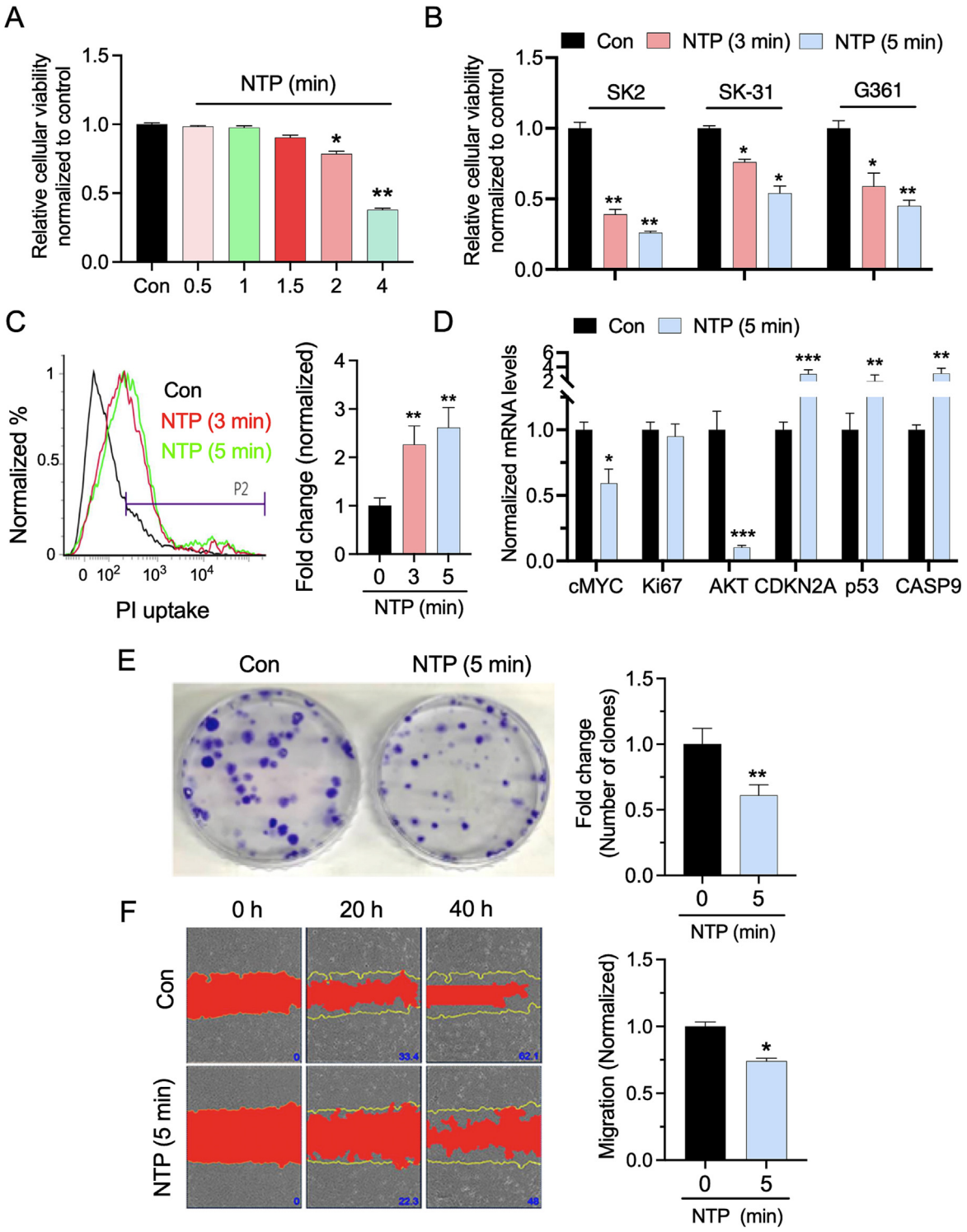

Fig. 1. Melanoma growth inhibition by NTP. (A) Graph depicting viability of SK-2 cells after increasing exposure (0.5 min, 1 min, 1.5 min, 2 min, and 4 min) to NTP, (B) Graph depicting viability of SK-2, SK-31 and G-361 cells after NTP exposure (0, 3 min, and 5 min), (C) Histograms and a graph showing results of cell death (PI uptake) analysis using flow cytometry in SK-2 cells following NTP exposure, (D) Graphs showing gene expression of markers involved in cellular proliferation (c-Myc, Ki-67), survival (AKT), cell cycle (CDKN2A and p53) and cell death (Casp9) –by qPCR analysis, (E) Representative image and corresponding graph indicating results of clonogenic assay for SK-2 cells post 12 days of NTP exposure. (F) Representative images and a graph indicating SK-2 cell migration analysis using Incucyte scratch wound assay. NTP, nonthermal atmospheric pressure plasma; PI, propidium iodide; CDKN2A, cyclin-dependent kinase inhibitor 2A; CASP9, Caspase-9; Statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) was determined using unpaired t-tests or Dunnett's/Tukey's multiple comparison tests.

The growth inhibition of SK-2 cells under the given NTP exposure conditions was evaluated using PI uptake and an mRNA expression analysis of key markers involved in cellular growth, including cellular myelocytomatosis (*c-MYC*), nuclear protein Ki67 (*Ki67*), AKT serine-threonine kinase (*AKT*), cell cycle (*CDKN2A* and *p53*), and cell death (*CASP9*). Aside from decreased cell viability, we also observed enhanced PI uptake (Fig. 1C) and reduced *c-MYC* and *AKT* expression, whereas *Ki-67* expression remained unaffected. A significant increase in cysteine aspartase 9 (*CASP9*), *CDKN2A*, and *p53* mRNA expression was also observed,

indicating cell cycle arrest and subsequent cell death (Fig. 1D). We also evaluated the cellular potential of the developing clones following NTP exposure. As shown in Fig. 1E, the number of colonies in the NTP-exposed groups was significantly lower than in their control counterparts. Lastly, to assess the metastatic potential of melanoma cells after NTP exposure, we evaluated and observed reduced cell migration in NTP-exposed groups compared to control groups (Fig. 1F). Overall, these findings indicated that plasma exposure inhibited melanoma growth and reduced cellular migration.

RNA-Seq analysis of NTP-exposed melanoma cells for differentially expressed miRNAs

To further understand the transcriptomic regulation of plasma-mediated melanoma cell growth inhibition, we conducted a comprehensive RNA-seq of the control and plasma-exposed groups. RNA-Seq provides a more thorough understanding of transcriptome complexity and has the ability to identify a dynamic range of expression levels, novel transcripts, short RNAs, single-nucleotide polymorphisms, alternative splicing products, sense and antisense transcripts, fusion transcripts, and transcription initiation sites than other technologies, such as hybridization-based microarrays and Sanger sequencing-based approaches [39–42]. The steps involved in the bioinformatics analysis of the raw data obtained from RNA-seq are illustrated in Fig. 2A. The principal component analysis shown in Fig. 2B reveals the variations between samples. A panel of 2588 miRNA probes was used to screen for differentially expressed miRNAs through RNA sequencing. The expression of all the miRNAs in the panel is shown as a heatmap (Fig. 2C). For differential expression analysis, the FC cut-off criteria was set to 1.5, with a p-value < 0.01 and a normalization standard of $\log_2$ 3 to denote significant upregulation or downregulation. Bioinformatics analysis revealed 33 upregulated and 31 downregulated miRNAs in the 3-min NTP exposure groups, while the 5-min NTP exposure groups revealed 82 upregulated and 66 downregulated miRNAs. Furthermore, scatter plots were constructed, which showed a significant deviation in the top differentially regulated miRNAs compared with the control in both cases (Fig. 2D–E). Next, we investigated the number of miRNAs that overlapped and were upregulated or downregulated in both NTP-exposed (3 min and 5 min) groups. The Venn diagram shown in Fig. 2F reveals that 23 miRNAs were upregulated and 16 were downregulated in both NTP-exposed conditions, whereas one miRNA was downregulated. These miRNAs are sensitive to oxidative stress and are sustained in cells subjected to prolonged oxidative stress. In contrast, 10 upregulated and 14 downregulated miRNAs were exclusive to the 3 min NTP-exposed groups. These miRNAs may be highly sensitive to oxidative stress or act as first-line enforcers of NTP-mediated action. The 5 min NTP-exposed groups showed 58 upregulated and 50 downregulated differentially expressed miRNAs owing to persistently high oxidative stress following NTP exposure. These may be referred to as the late or final enforcement of NTP-mediated actions.

Differentially regulated miRNAs and their gene categories

Most of the genes identified through bioinformatics analysis can be categorized into various cellular processes, such as aging, angiogenesis, apoptosis, autophagy, cell cycle, cell differentiation, cell migration, cell proliferation, DNA repair, immune response, inflammatory response, neurogenesis, and secretion. The miRNAs can also be categorized into these processes. Using ExDEGA software, we investigated the extent of the contribution of the upregulated and downregulated miRNAs to key cellular processes such as aging, apoptosis, autophagy, cell cycle, cell proliferation, and DNA repair, which are involved in cellular proliferation and are often dysregulated during growth inhibition. These processes are central to melanoma or cancer cell growth, and their targeting can facilitate effective intervention. The graph shown in Fig. 3A indicates the number of upregulated and downregulated miRNAs after 3 min of NTP exposure, while Fig. 3B provides the complete list of miRNAs differentially regulated after 5 min of NTP exposure. Exposure to NTP for 3 min led to an extensive upregulation of several miRNAs compared to the downregulated miRNAs. In contrast, 5 min of NTP exposure resulted in miRNA downregulation in melanoma cells. Several miRNAs, such as hsa-miR-215-5p, hsa-miR-34c-5p, and hsa-miR-708-5p, are considered the most sensitive to oxidative stress and are easily upregulated by NTP exposure. In contrast, hsa-miR-148a-3p expression was downregulated following NTP exposure. The expression of these miRNAs was sustained at high or low levels with an increase in NTP-induced oxidative stress. As oxidative stress continues to increase, other miRNAs become upregulated or downregulated. The miRNA profile consists of several miRNAs common to apoptosis, the cell cycle, cellular proliferation, and DNA repair, indicating that their critical functions are triggered by NTP exposure.

Functional enrichment of differentially regulated miRNAs in NTP-exposed melanoma cells

We used the mierturnet tool and selected the top dysregulated miRNAs for pathway enrichment and annotation using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Fig. 4A shows the top KEGG annotated pathways enriched in the upregulated miRNAs, and Fig. 4B shows those enriched through upregulated miRNAs in the 3-min NTP-exposed groups. Only pathways with significant levels ($p < 0.05$), represented in dark red, were considered. As expected, pathways such as apoptosis, p53 signaling, and transcriptional regulation were enriched, and most miRNAs were shown to play a role. miR-769-5p is enriched in several cancer types, but not in melanoma. Certain upregulated miRNAs, such as miR-17-3p, miR-184, and miR-34, were enriched in melanoma conditions and miRNAs in cancer pathways, while downregulated miRNAs indicating melanoma and miRNA pathway enrichment included miR-128–1-5p, miR-152-5p, and miR-424-5p in the 3 min NTP-exposed groups. In addition, the upregulated miRNAs in the 5 min NTP-exposed groups include miR-135a-5p, miR-335-5p, and mir-1-3p; the downregulated miRNAs are enriched in most processes that are often dysregulated in cancers. The miRNAs involved in critical pathways, such as apoptosis, miRNAs in cancer, the p53 pathway, and the PI3K-AKT pathway, include miR-128, miR-139, miR-200b-3p, miR-215-5p, miR-365, and miR-340 (Figure S1A and S1B). Overall, KEGG functional enrichment indicated cell death. Following basic pathway enrichment, we investigated the data for gene ontology (GO) annotation and function.

GO analysis of differentially regulated miRNAs in NTP-exposed melanoma cells

Next, GO analysis was performed for miRNAs using the microRNA enrichment turned network webtool (http://userver.bio.uniroma1.it/apps/mienturnet/), while DAVID (https://david.ncifcrf.gov/) tools were used to analyze the enriched genes. Data were selected and analyzed based on their FDR (<0.05) and *p*-value (<0.05). GO analysis showed significantly ($p < 0.05$) dysregulated GO categories, including "positive regulation of gene expression," "protein phosphorylation," "protein kinase activity," "cellular response to insulin stimulus," "negative regulation of the apoptotic process," and "positive regulation of mitochondrial membrane potential" from the upregulated miRNAs of the 3 min NTP-exposed samples compared with the control group (Fig. 5A).

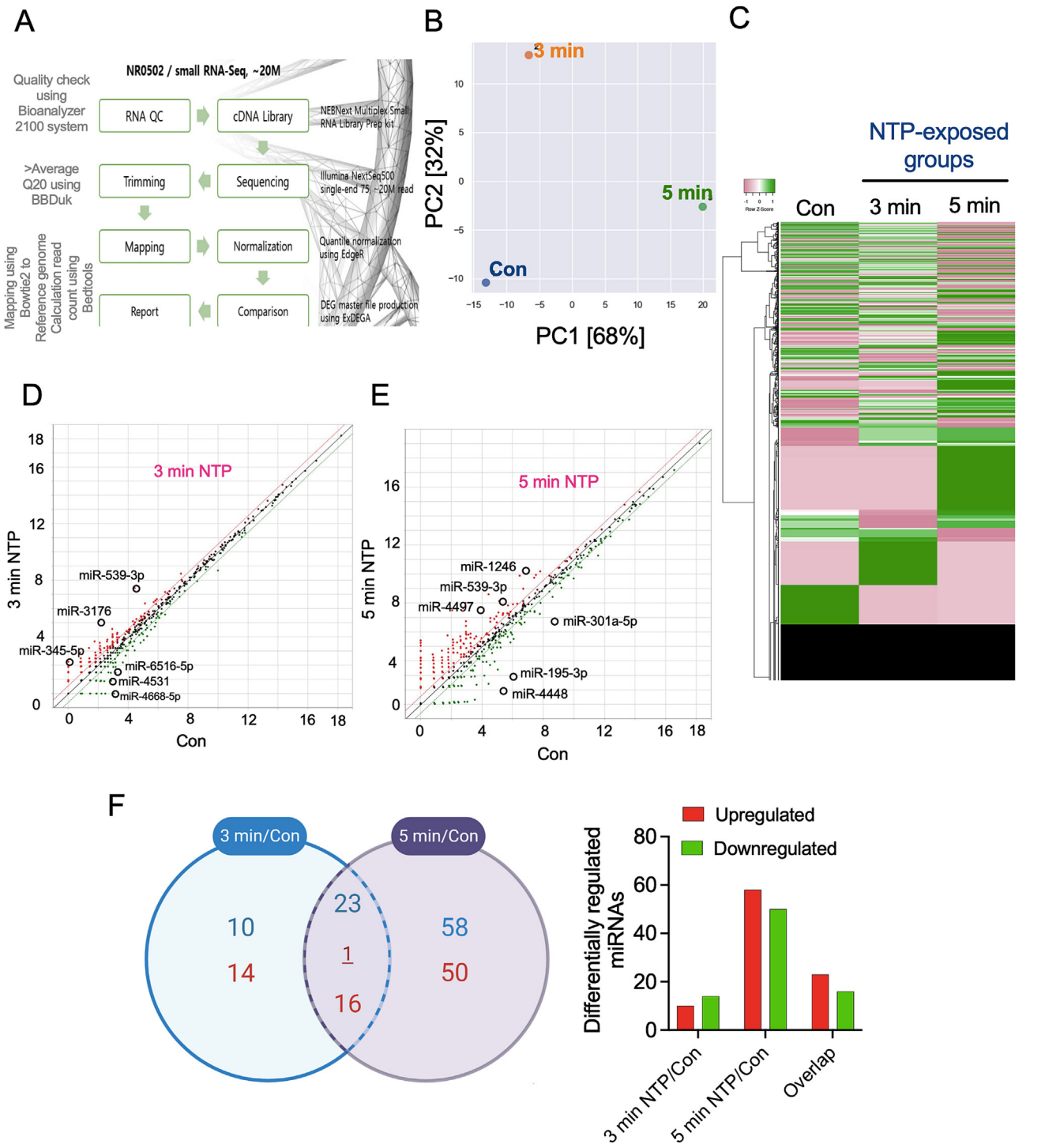

Fig. 2. Global transcriptome analysis. (A) Schematic presentation of High throughput RNA sequencing for miRNA (small RNA seq) (B) Plot showing principal component analysis (PCA) of the control (con) and NTP-treated samples (C) Heat map showing differentially expressed miRNAs after NTP exposure (D, E) Scatter plots showing the spread of upregulated and downregulated miRNAs in 3 min NTP and 5 min NTP exposed samples respectively, and (F) Venn diagram indicating numbers of upregulated and downregulated miRNAs differentially regulated and overlapping in both NTP groups (left panel). Similar data has been shown using a bar graph (right panel). NTP, non-thermal plasma.

GO analysis of downregulated miRNAs in the 3 min NTP-exposed samples revealed "cell cycle," "cyclin-dependent protein kinase complex," "cell division," "positive regulation of G2/M transition of the mitotic cell cycle," and "regulation of cell cycle," among others. In samples exposed to NTP for 5 min, GO categories enriched by upregulated miRNAs mainly include basic transcription machinery and associated regulation. For instance, GO categories related to transcription, transcriptional regulation, regulation of transcription by RNA polymerase, and DNA binding were prevalent. Although these GO categories were also enriched in the enrichment of downregulated miRNAs, several different GO categories, such as suppressors of mothers against decapentaplegic (SMAD) binding, were also enriched (Fig. 5B). The GO pathway categories, along with the KEGG and Reactome pathways, were then chosen under the default conditions and biological process criteria in DAVID.

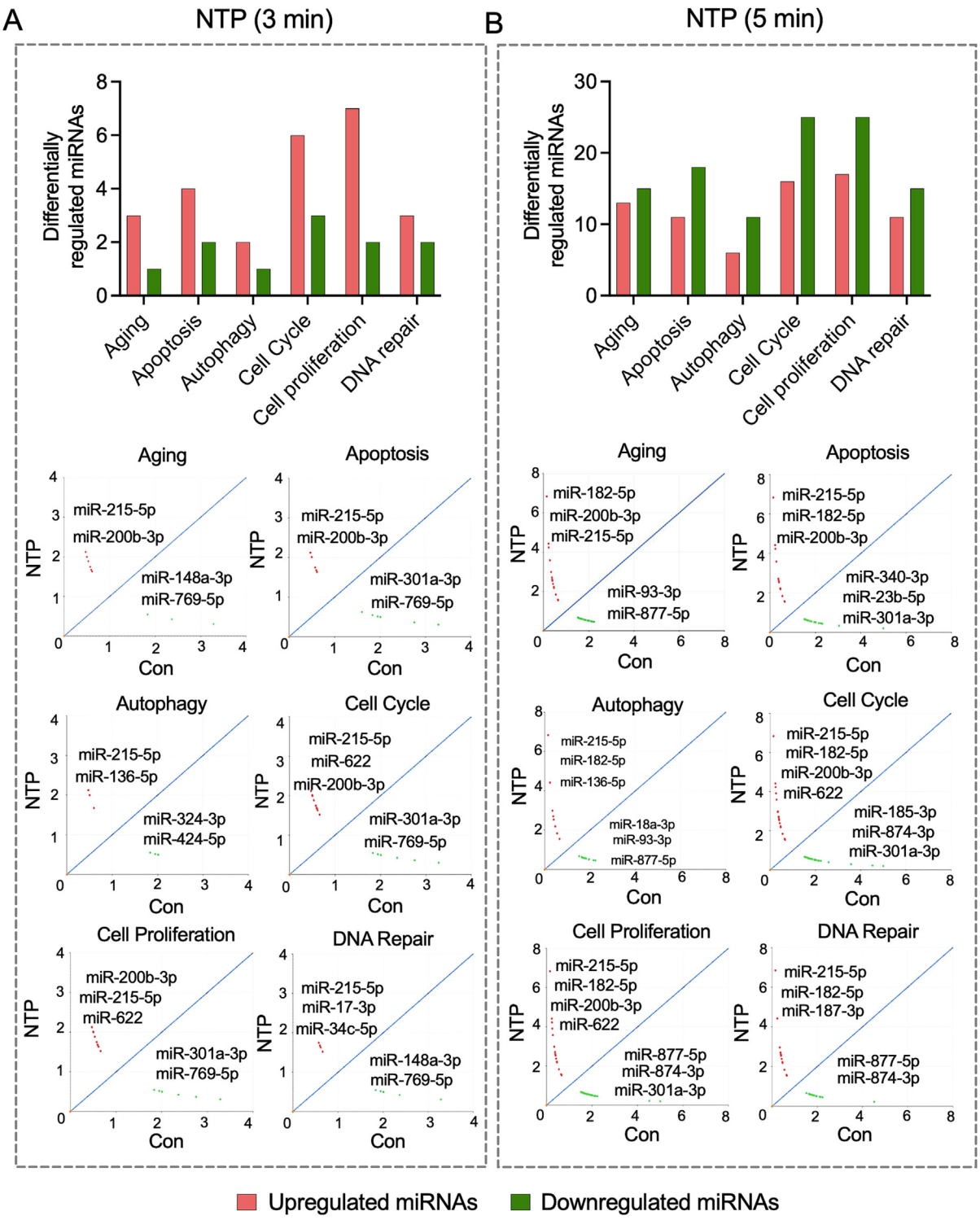

Fig. 3. Cellular process-based categorization of differentially regulated miRNAs. Graph and representative scatter plots showing differentially regulated miRNAs involved in biological processes in (A) 3 min NTP and (B) 5 min NTP groups. NTP, non-thermal plasma.

Analyzing the role of differentially expressed transcripts in the inhibition of melanoma growth

We have previously shown that plasma exposure modulates signal transduction pathways and induces cancer cell death [27,43,44]; the current miRNA-seq data agree with these results. NTP-mediated apoptotic modulation has been intensively studied as the primary effect of NTP exposure; therefore, we focused our analysis on other possible molecular interactions leading to cancer cell inhibition, especially miRNAs. Fig. 6A shows the top differentially regulated miRNAs after 5 min of plasma exposure as determined via RNA-Seq. These differentially expressed miRNAs were further validated using qPCR in the SK-2 and G-361 cell lines (Fig. 6B and S2A). The top differentially regulated miRNAs were selected as inputs to derive an enriched network of interactions using Mienturnet. As shown in Fig. 6C, the resulting network

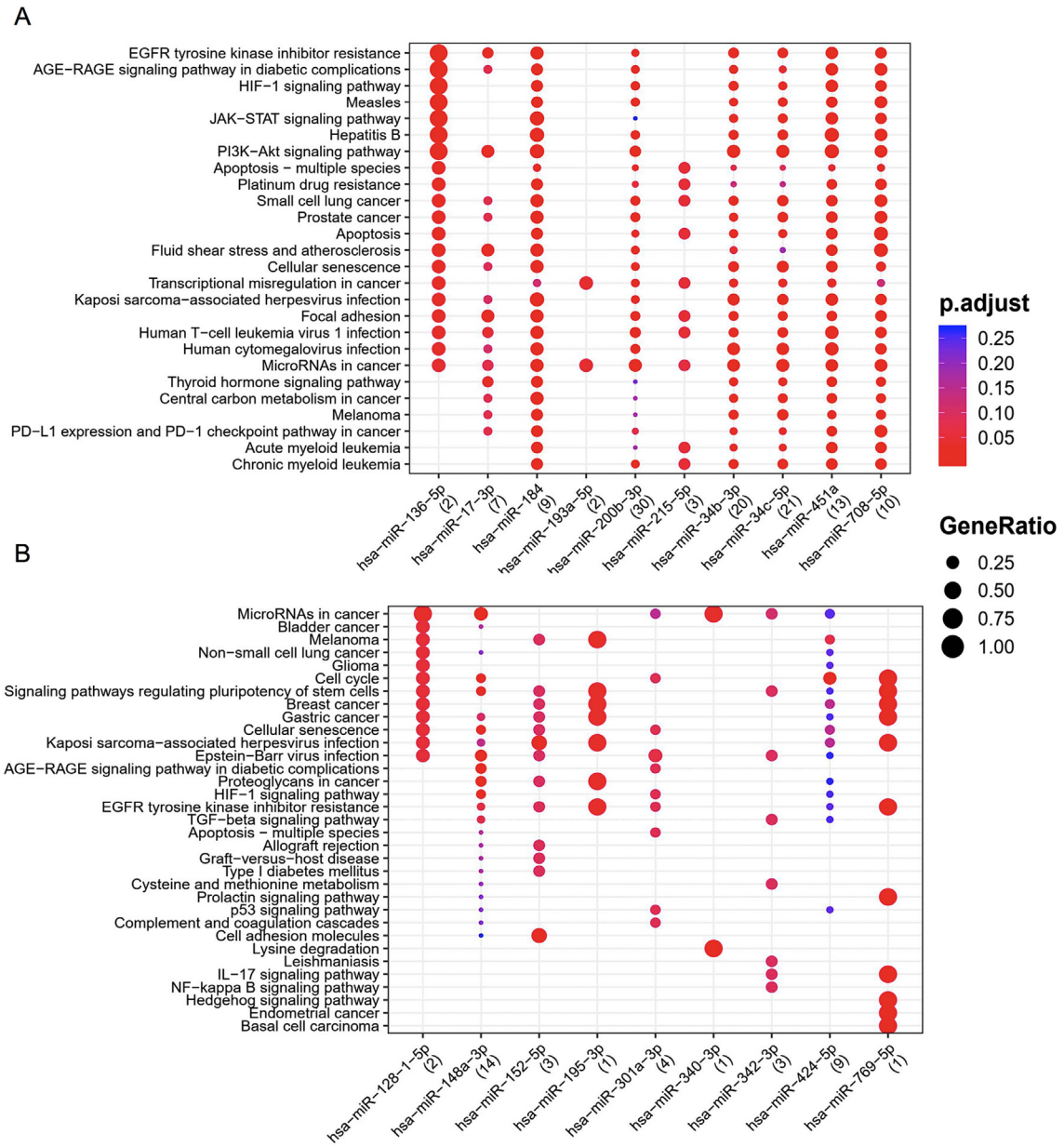

Fig. 4. Pathway enrichment analysis using the Mierturnet tool. Functional enrichment of differentially regulated miRNAs in 3 min NTP exposed melanoma cells. (A) KEGG enrichment for upregulated miRNAs, and (B) KEGG enrichment for downregulated miRNAs. KEGG, Kyoto encyclopedia of genes and genomes; NTP, non-thermal plasma.

contained two clusters of target gene networks: hsa-miR-200b-3p and hsa-miR-215-5p. To further elucidate the roles of these miRNAs in melanoma growth and migration, the effects of miR-200b-3p and miR-215-5p on NTP-treated SK-2 cells were explored. To achieve this, we treated SK-2 melanoma cells with miRNA inhibitors before NTP exposure and evaluated their effects after 48 h of incubation. Significant downregulation of various miRNAs was observed after treatment with their respective miRNA inhibitors in SK-2 melanoma cells (Fig. 6D). Following the confirmation of inhibition efficiency, we determined the viability of SK-2 cells after plasma treatment in the presence or absence of these miR inhibitors. Interestingly, no significant reduction in cell viability was observed in the NTP-exposed groups in the presence of miRNA inhibitors. However, there was a decrease in viability in the 5 min NTP-exposed groups, including the negative control miRNA, compared to the unexposed control group (Fig. 6E). These results suggest that the downregulation of miR-200b-3p and miR-215-5p increases the viability of melanoma cell lines after NTP exposure. Cell death was then evaluated via PI uptake to further confirm the growth inhibition of SK-2 cells under the given NTP exposure conditions. Aside from decreased cell viability, we observed remarkably higher PI uptake in the NTP-exposed groups, suggesting enhanced cell death or membrane permeability (Figure S2B). We also assessed the migratory ability and cloning efficiency of SK-2 cells after NTP treatment in the presence or absence of miRNA inhibitors. Notably, treatment with miRNA inhibitors (miR-200b-3p and miR-215-5p) abolished the effect of NTP treatment on SK-2 cells, as evidenced by the increased number of clones and migration rate (Fig. 6F, 6G, and S2C).

NTP-mediated melanoma growth inhibition via the miR-200b-3p/ZEB axis

As shown in Fig. 6C, the common node between hsa-miR-200b-3p and hsa-miR-215-5p included two genes: zinc finger E-box

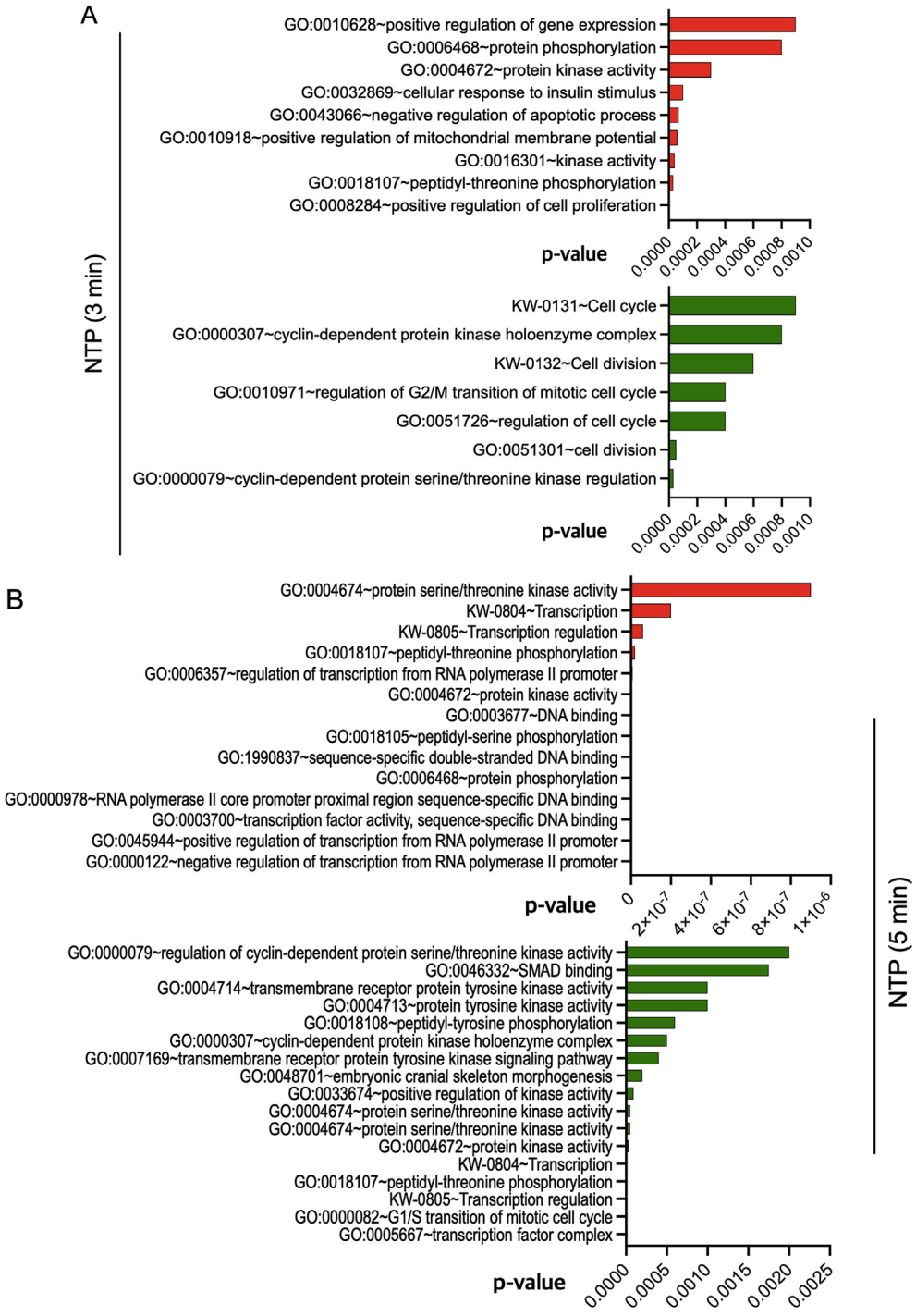

Fig. 5. GO analysis of NTP-exposed miRNAs. Top significantly enriched GO pathway categories under ontology of biological processes in NTP exposed groups compared with control groups (A) 3 min only, and (B) 5 min only. GO, gene ontology; NTP, non-thermal plasma.

binding homeobox 2 (*ZEB2*) and X-linked inhibitor of apoptosis (*XIAP*). ZEB2 is a critical protein for cancer cell growth and epithelial-mesenchymal transition (EMT) [45–47]. ZEB2 (previously known as SMAD-interacting protein-1 [SMADIP1 or SIP1]) and its mammalian paralog ZEB1 belong to the ZEB family within the zinc finger class of homeodomain transcription factors. As shown in Fig. 7A and B, ZEB1 expression was prominently high in melanoma, showing consistent upregulation at all stages of melanoma pathogenesis (Fig. 7C). Consistent with this, Kaplan–Meier survival analysis suggested that low ZEB1 levels could lead to a higher survival percentage (Fig. 7D). Therefore, we speculated that targeting ZEB2 through NTP exposure could suppress melanoma growth and metastasis. Several studies suggest the potential of ZEB1/2 to regulate cancer progression via the PI3K/AKT pathway, a critical survival, proliferation, and apoptosis-regulating pathway [48–50]. To better understand the mechanistic pathway involved in miRNA dysregulation, particularly the PI3K-AKT-ZEB1 axis, we next examined the protein and mRNA expression of AKT, phosphoinositide 3-kinase (PI3K), and ZEB1 along with the mRNA expression of cell cycle marker *CDKN2A*. While the protein expression levels of AKT, PI3K, and ZEB1 decreased in the 5 min NTP-exposed groups compared to the unexposed control group, they

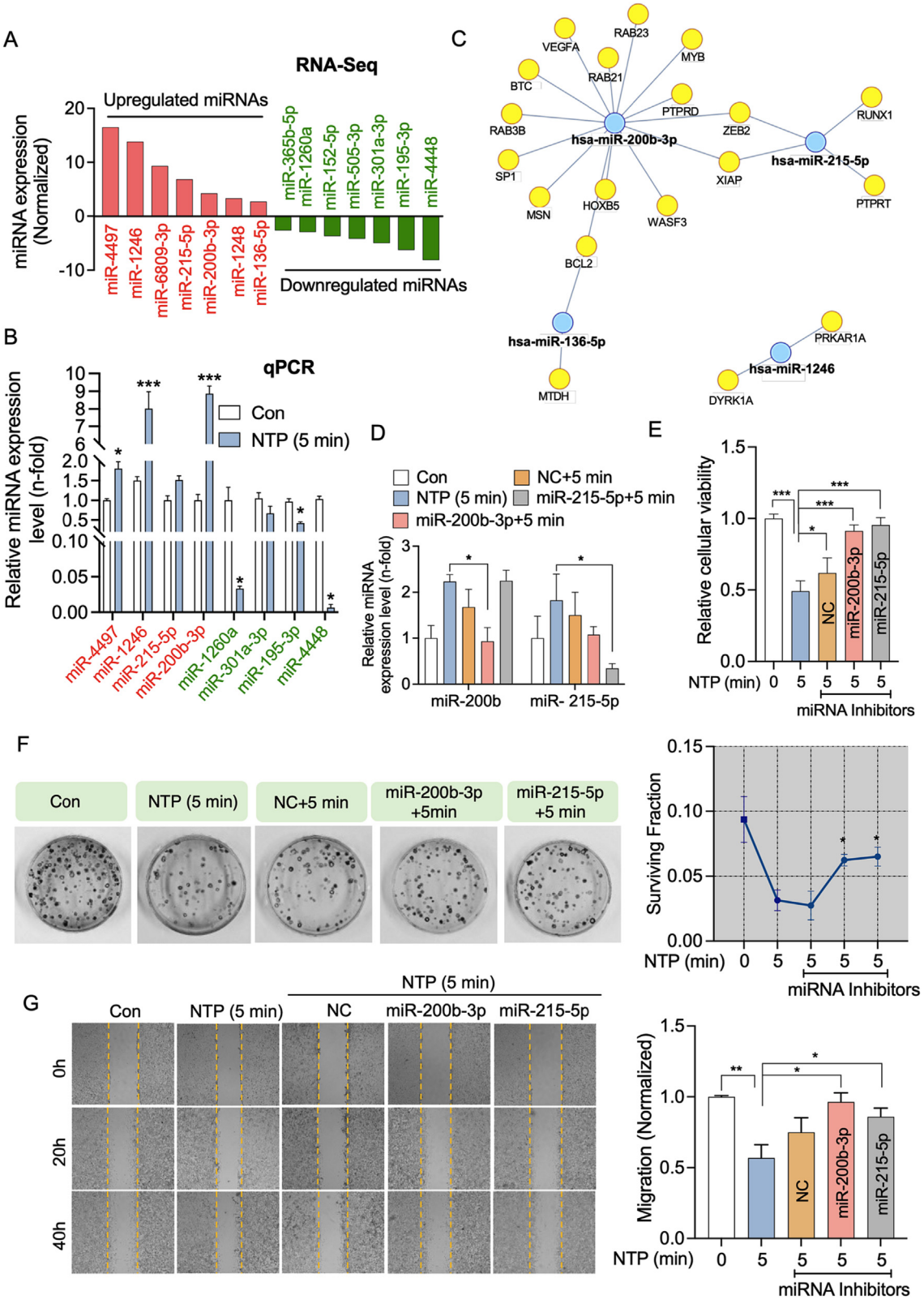

Fig. 6. NTP-induced cellular growth suppression is mediated through miR-200b-3p and miR-215-5p. (A) Bar graph showing top upregulated (red) and downregulated (green) identified from RNAseq analysis in 5 min NTP-exposed groups, (B) qRT-PCR analysis of upregulated (red) and downregulated (green) miRNAs in control and 5 min NTP-exposed SK-2 cell line, (C) Network analysis of upregulated miRNAs using mienturnet. (D) qRT-PCR analysis of the expression of miR-200b-3p and miR-215-5p levels in SK-2 cells following NTP-exposure where these miRNAs were inhibited. (E) Cellular viability of SK-2 cells after 5 min NTP treatment in the presence or absence of miR-200b-3p or miR-215-5p inhibitors. (F) Representative images and survival fraction graph illustrating the clonogenic assay conducted on SK-2 cells after NTP exposure in the presence or absence of given miRNAs inhibitors. (G) Representative images and graphs indicating cell migration analysis under similar conditions as panel F. Statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) was determined using Sidak's or Tukey's multiple comparison tests with one or two-way ANOVA. NC, normal control; NTP, non-thermal plasma.

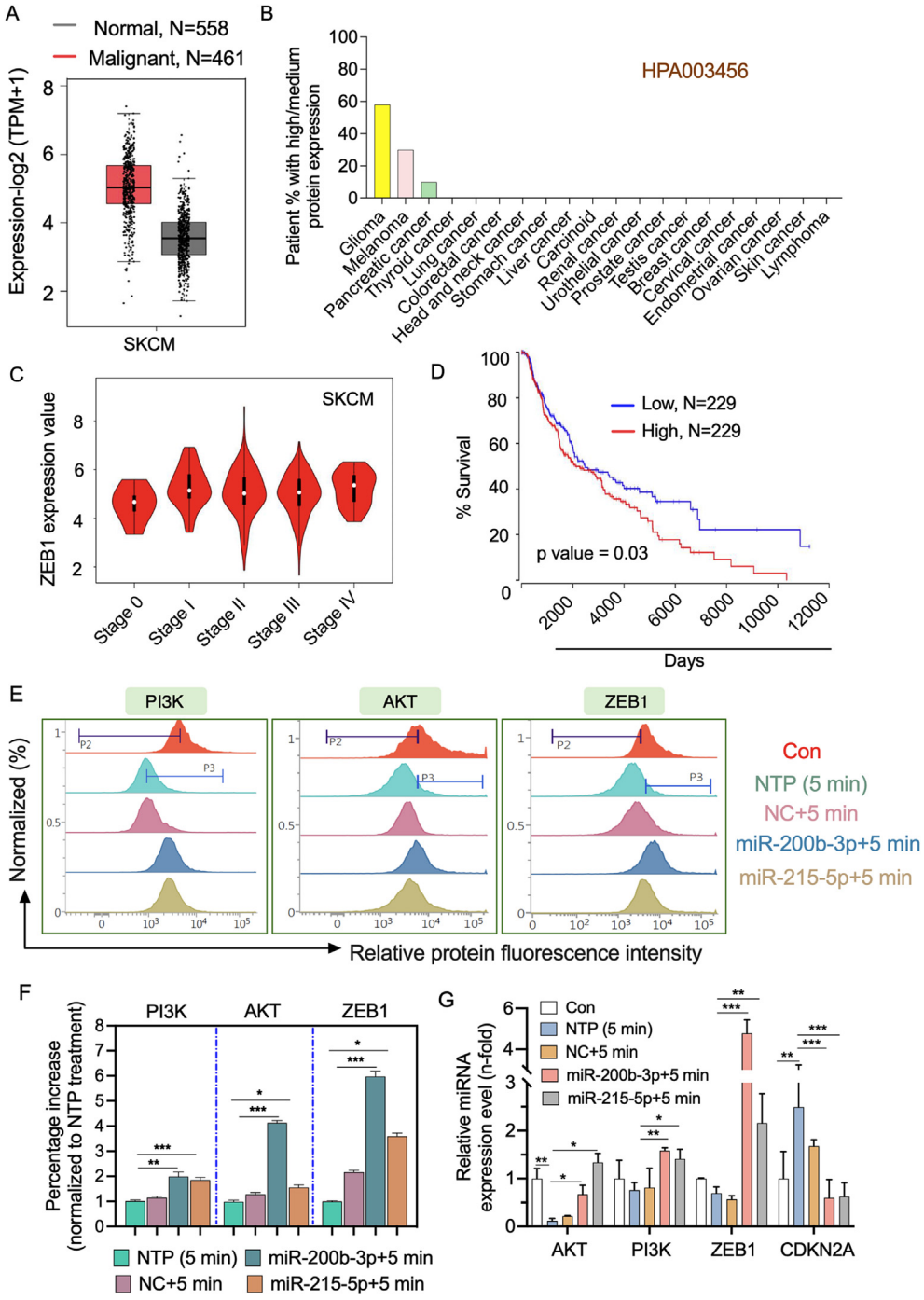

Fig. 7. PI3K-AKT-ZEB axis in melanoma promotes cellular progression. (A) Expression of ZEB2 in normal skin and melanoma tissues obtained using protein atlas database (B) Protein expression levels of ZEB2 in Skin Cutaneous Melanoma (SKCM) samples analyzed by GEPIA2. (C) Stage-wise expression of ZEB2 was retrieved in melanoma, and a representative plot is shown using GEPIA2. (D) Graph showing survival analysis of SKCM samples with high and low ZEB2 levels was retrieved using OncoLnc webtool. (E, F) Flow cytometry analysis of the protein expression of AKT, PI3K, and ZEB1 in SK-2 cells in the presence or absence of miRNAs inhibitors following NTP treatment. Quantification of data has been shown in the representative graph in panel F. P3 gating is used to compare miR inhibitor groups with NTP treatment. (G) qRT-PCR analysis of the expression of AKT, PI3K, ZEB1, and CDKN2A under similar conditions as panel E. PI3K, phosphoinositide 3-kinases; SKCM, skin cutaneous melanoma; GEPIA2, gene expression profiling interactive analysis; ZEB1, Zinc finger E-box-binding homeobox 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CASP9, Caspase-9; Statistical significance (*p < 0.05, **p < 0.01 and ***p < 0.001) was determined using Tukey's multiple comparison tests with two-way ANOVA.

were upregulated or unaffected in the NTP-exposed groups treated with miRNA inhibitors (Fig. 7E, F, and S2D). This was further confirmed by qRT-PCR analysis of the respective markers. The downregulation of *AKT*, *PI3K*, and *ZEB1* and upregulation of *CDKN2A* gene expression was observed in the 5 min NTP-exposed and NC (+5 min NTP) groups, whereas the groups pretreated with miRNA inhibitors followed by NTP-exposure demonstrated a reversed trend of gene expression. These data suggest the induction of cellular senescence and suppression of the PI3K-AKT-ZEB1 pathway following NTP treatment (Fig. 7G). Overall, these findings indicated that exposing melanoma cells to NTP upregulates miR-200b-3p and miR-215-5p expression, which could lead to melanoma growth inhibition via the PI3K-AKT pathway.

Since the NTP-ZEB1 axis could involve more NTP-regulated miRNAs, we further investigated the miRNAs downregulated by NTP exposure and used them as input to derive an enriched net-

work of interactions using the Mienturnet tool. As shown in Fig. 8A, the output network revealed enriched miRNA clusters belonging to hsa-miR-19a-3p, hsa-miR-129-5p, hsa-miR-340-3p, and hsa-miR-18a-3p. Additionally, we reviewed the literature to ascertain the clinical significance of the expression and survival of miRNAs in SKCM. Fig. 8B–G shows the survival plots of the respective miRNAs in SKCM samples using The Cancer Genome Atlas database. These plots indicate that the NTP-mediated downregulation of these miRNAs may lead to a higher survival rate and suppression of melanoma progression. These miRNAs mainly targeted the cell cycle and p53 signaling pathways (Fig. 3). Overall, NTP exposure may function by upregulating (miR-200b-3p-ZEB axis) or downregulating miRNAs, causing melanoma cell death and growth inhibition (Fig. 8H).

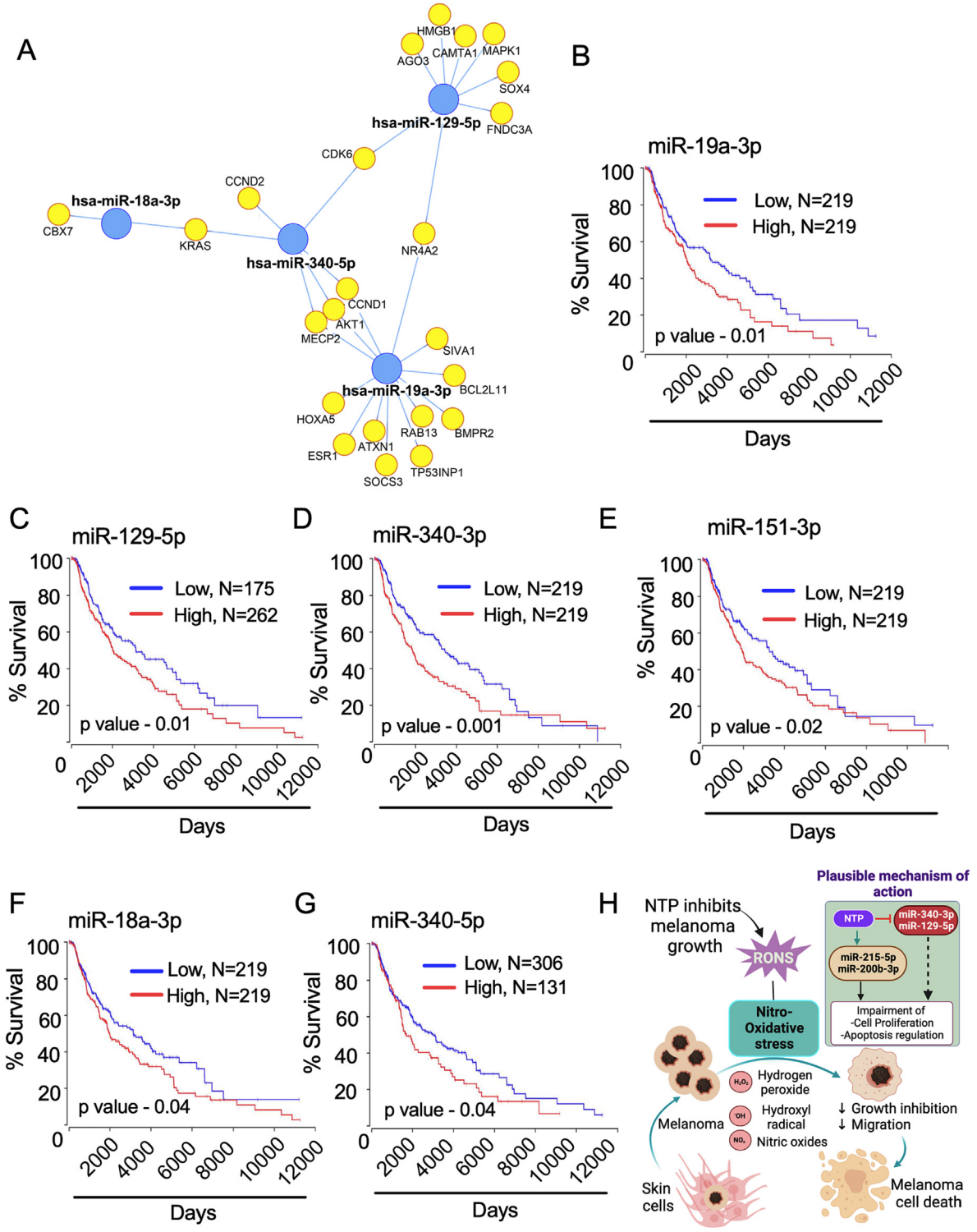

Fig. 8. Analysis of downregulated miRNAs and their clinical relevance in SKCM patient survival. (A) Network enrichment of downregulated miRNAs, (B-G) Survival analysis plots of SKCM samples with high and low levels of hsa-miR-19a-3p, hsa-miR-129-5p, hsa-miR-340-3p, hsa-miR-151-3p, hsa-miR-18a-3p, and hsa-miR-340a-5p, respectively. (H) An illustration depicting the plausible action of NTP for melanoma growth inhibition. SKCM, skin cutaneous melanoma.

Discussion

Untreated melanoma has a high propensity to spread if not treated in its early stages, making it one of the most dangerous malignancies affecting humans. Therefore, there has been an increased interest in finding a simple, selective, and effective method to treat melanoma. Given the recent reports on using NTP in *in vitro* and *in vivo* cancer models, little is known about the molecular players contributing to its anticancer mechanism. In our previous studies, cancer cells were more susceptible to NTP-induced RONS production than non-cancerous cells, and NTP treatment caused apoptotic cellular responses, mostly in cancer cells [26,51]. Cells exposed to NTP typically show a considerable increase in intracellular RONS levels [25,33]. The molecular network responsible for NTP-induced oxidative or nitrosative stress, which preferentially induces cell death in tumor cells over healthy cells, remains poorly understood [25,26]. In the present study, we investigated how NTP modulates miRNA networks in melanoma cells. miRNAs have gained enormous attention as their importance in various diseases is increasingly revealed [7,52]. Because miRNAs control gene expression before and after transcription, they are frequently employed in high-throughput screening tools to identify important genes that could be used as molecular targets for various interventions. Despite extensive studies on the primary genes linked to melanoma and emerging therapies, disease-free survival remains low. Improving our understanding of treatment targets and discovering new targets or therapeutics are required to overcome this challenge and enable efficient melanoma suppression.

In this study, human melanoma cell lines were exposed to NTP and evaluated for growth inhibition. NTP exposure for 3 and 5 min effectively suppressed melanoma cell growth, as evidenced by the reduction in cell viability, clonogenic potential, and the expression of pro-survival and cell cycle regulatory genes, including *c-MYC, Ki67, AKT, CDKN2A, p53,* and *CASP9* (Fig. 1). Our findings agree with previous reports indicating that the NTP-mediated inhibition of melanoma and other cancers is most likely via apoptosis or cell cycle dysregulation. NTP-mediated anticancer activity mainly depends on generating reactive species, resulting in oxidative stress [25,28,33]. In this study, we identified NTP-regulated miRNAs using high-throughput RNA-seq. Bioinformatics analysis of NTP-exposed melanoma cells yielded many differentially regulated miRNAs (82 upregulated and 66 downregulated miRNAs after 5 min of NTP exposure) (Fig. 2). To the best of our knowledge, this is the first report to directly identify differentially regulated miRNAs in melanoma cells after exposure to soft-air jet plasma.

miRNAs have been implicated in the occurrence, development, diagnosis, and therapy of cancer [53]. Similar to genes, miRNAs can be classified as oncogenic or tumor suppressive. Oncogenic miRNAs can increase the malignancy and growth of cancer cells and are associated with poor prognosis. For instance, miR-424-5p can cause anoikis resistance and improve the migration capacity of cancer cells [54]. miR-424-5p downregulation is important in cancer therapy as it is a possible target for cancer treatment [54,55]. In this study, we observed the downregulation of miR-424-5p and miR-324-5p after 3 min of NTP exposure (Fig. 3). Prolonged NTP exposure downregulates the expression of other oncogenic miRNAs, such as miR-18a, miR-19a, miR-92, and miR-340. The increased expression of these miRNAs enhances tumorigenic potential by altering the cell cycle and apoptotic pathways [56–60]. Certain miRNAs, such as miR-34a, are downregulated and act as tumor suppressors in various cancers [61]. In this study, NTP exposure led to the upregulation of proapoptotic miRNAs such as miR-34, miR-17, and miR-184 [62–66]. In addition, we observed the consistent upregulation of miR- 200b-3p, miR-215-5p, miR-708, miR-335, and miR-622. Interestingly, the inhibition of miR-200b-3p and miR-215-5p prior to NTP exposure induced a rescue effect in SK-2 cells (Figs. 6 and 7). Our results showed a remarkable increase in the viability, migration, and colony-forming ability of melanoma cells in the miRNA-inhibited groups, in contrast to the NTP-treated group, confirming the tumor-suppressive role of miR-200b-3p and miR-215-5p in these cells. In our RNA-seq data, we observed modulation in the "cell cycle," "cellular proliferation," "regulation of transcription," and "p53 signaling" as key processes. Subsequent GO and network analyses revealed that the differentially expressed miRNAs involved in cancer progression target several transcription factors (TFs) (Figs. 4 and 5). EMT-TFs are considered potential downstream targets of miRNAs in cancer metastasis. Network analysis revealed the convergence of the upregulated miRNAs in the ZEB family of TFs (Fig. 6).

ZEB can stimulate EMT and play a critical role in cell migration and invasion [67]. In colorectal cancer (CRC) cells, the tumor suppressor death domain-associated protein (DAXX) has been shown to inhibit ZEB1 by modifying E-cadherin expression to prevent tumor cell invasion and proliferation. Importantly, ZEB2 can activate EMT to induce chemoresistance. The PI3K/AKT pathway, a downstream pathway of ZEB, induces EMT by reducing E-cadherin levels, resulting in the development of cisplatin resistance in non-small cell lung cancer cells [68]. This pathway promotes tumor cell survival, proliferation, invasion, and metastasis while inhibiting apoptosis [69]. An aberrantly activated PI3K pathway has been reported to target ZEB1 activation via various signaling cascades in numerous cancer types [70,71]. We also observed that ZEB1 was highly expressed in melanoma (SKCM) samples and is present in all stages. As expected, the survival rate was poor (Fig. 6). Overall, ZEB1/2 mediates EMT and is a potential therapeutic target for cancer treatment. In our study, the consistent upregulation of miR-200b, miR-215, and miR-708 observed might cause ZEB1 inhibition, while some ZEB2-targeting miRNAs such as miR-187, miR-335, and miR-622 were also upregulated. Growth suppression via the PI3K-AKT-ZEB1 axis in the current study was collectively evidenced by the notable reduction in *AKT* levels (Fig. 1) and GO analysis suggesting the enrichment of the PI3K-AKT pathway (Fig. 5). Additionally, protein and gene expression analysis of SK-2 cells *in vitro* supported the theory that the inhibition of miR-200b-3p and miR-215-5p resulted in upregulated or unaffected expression levels of PI3K, AKT, and ZEB1 compared to those in the NTP-exposed group, suggesting suppression of the tumor progression pathway. Moreover, it is believed that miR-200c exerts an inhibitory impact on TGF-β-mediated EMT by downregulating both ZEB1 and ZEB2 proteins [72,73]. Consistent with this observation, RNA-Seq analysis indicated that the number of differentially regulated miRNAs significantly increased with increased duration of NTP exposure. GO analysis revealed that the "cell cycle," "apoptosis," and "p53 signaling" processes showed the highest increase in the number of differentially regulated miRNAs. Further analysis revealed the downregulation of several miRNAs that regulate cell growth and apoptosis, including miR-19a, miR-129, miR-340, miR-151, and miR-18a. The upregulation of these miRNAs may lead to poor survival in patients with SKCM (Fig. 8). Collectively, network analysis revealed many key TFs involved in cancer progression (Figs. 4–8). Therefore, it can be postulated that NTP exposure performs a dual function: upregulating miRNAs that target oncogenes involved in EMT and migration and downregulating miRNAs that dysregulate cell cycle arrest and apoptosis. Overall, this study showed that NTP inhibits melanoma growth, possibly by regulating the aforementioned miRNAs. However, additional studies are necessary to better understand the underlying mechanisms.

Conclusions

Melanoma is the most aggressive form of skin cancer, necessitating a better understanding and availability of therapeutic modalities. This study highlights the complex interplay between NTP and regulatory miRNAs in melanoma cells. Importantly, the variable miRNA expression patterns in melanoma during redox stress may provide a good foundation for subsequent functional studies to uncover potential oncogenic or tumor-suppressive miRNAs. Additionally, our mechanistic evaluation demonstrated that dysregulated miRNAs, particularly the upregulated miR-200b-3p and miR-215-5p, modulated melanoma growth and cell cycle progression via the PI3K-AKT-ZEB axis. This study provides a new direction for the treatment of melanoma using NTP. The elucidation of plasma-induced miRNAs provides a basis for tailoring NTP treatments with precision based on their unique molecular profiles. The identified miRNAs can be explored as potential diagnostic markers and simultaneously targeted in combination with NTP treatment using a multimodal therapeutic approach, thereby amplifying treatment effectiveness. Furthermore, beyond its immediate application, this study contributes to a broader understanding and advancement of the molecular mechanisms involved in NTP-mediated melanoma suppression. Considering the preliminary nature of our investigation, more work is necessary to identify the direct target genes of other differentially regulated miRNAs and their *in vivo* implications in the clinical and research domains.

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Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Pradeep Bhartiya: Conceptualization, Methodology, Software, Writing – original draft. **Apurva Jaiswal:** Conceptualization, Methodology, Software, Writing – original draft. **Manorma Negi:** Conceptualization, Methodology, Software. **Neha Kaushik:** Conceptualization, Methodology, Software, Writing – original draft, Writing – review & editing, Funding acquisition. **Eun Ha Choi:** Writing – review & editing, Funding acquisition. **Nagendra Kumar Kaushik:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2024.02.022.

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