

Identification of microRNAs involved in growth arrest and cell death in hydrogen peroxide-treated human dermal papilla cells

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Abstract. microRNAs (miRNAs) are small non-coding RNAs that regulate various biological processes by interfering with the translation of target genes. Several studies have suggested that miRNAs are involved in cellular responses to hydrogen peroxide (H₂O₂). Reactive oxygen species (ROS) are involved in hair malignancies, however, the H₂O₂-induced, miRNA-dependent regulatory mechanisms of human dermal papilla (HDP) cells are not fully understood. Our previous study demonstrated that changes in miRNA expression function to regulate growth arrest and apoptosis in UVB-irradiated HDPs. In the present study, miRNA expression was profiled in HDPs treated with H₂O₂. The transcriptome analysis of H₂O₂-treated HDPs enabled the identification of 68 differentially expressed miRNAs (62 were upregulated and 6 were downregulated) and 14,316 putative target genes of the miRNAs. Gene ontology (GO) analysis was utilized to verify that the putative target genes of the altered miRNAs were associated with H₂O₂-induced cell growth arrest and apoptosis. This bioinformatics analysis indicated that H₂O₂-response pathways involved in growth arrest and apoptosis were significantly affected. The identification of miRNAs and their putative targets may offer new therapeutic strategies for H₂O₂-induced hair follicle disorders.

Introduction

The dermal papilla (DP) describes the component of the hair follicle that is involved in hair growth and formation. During development of the hair follicle, the DP is generated by condensation of dermal mesenchymal cells. The DP exists at the base of hair follicles and regulates the hair cycle by providing key signals that control the timing and phase of hair follicle growth and formation (1,2). Anagen is the active growth phase of hair follicles, where the root divides rapidly and adds material to the hair follicle for rapid hair growth. DP stimulates the initiation of the anagen growth phase by secreting Fgf7/10 and TGFβ (3,4). DP-induced β-catenin/Wnt signaling sustains the anagen phase and DP-induced Notch/Wnt5a signaling induces hair follicle differentiation (5,6). The production of reactive oxygen species (ROS) in hair follicles results in hair developmental disorders, including graying and hair loss (7-9). One such example is alopecia, which is induced by a ROS-mediated reduction of hair growth (9,10).

Oxidative stress that results in crucial damage of DNA, proteins and lipids is implicated in several hair follicle disorders, including graying and hair loss (7-10). Oxidative stress is increased by the accumulation of ROS, including hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]), superoxide anion (O₂⁻) and the accumulation of reactive nitrogen species (RNS), including peroxynitrite (ONOO⁻). The generation of ROS and RNS is triggered by an imbalance between pro-oxidants and antioxidants. It has been identified that high levels of oxidative stress induces growth arrest, apoptosis and necrosis in cells (11). Mammalian cells have developed defense mechanisms to neutralize ROS, including antioxidant enzymes and non-enzymatic antioxidants (12,13). In hair follicles, low levels of ROS are generated in the mitochondria, and these act as crucial signaling molecules for hair follicle differentiation and morphogenesis (7). However, high levels of ROS directly damages cellular membranes, lipids, proteins and DNA (9). ROS levels increase with aging, which causes a decrease in the function and number of functional melanocyte cells in hair follicles (8). ROS-responsive microRNA (miRNA)

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expression regulates the cell cycle and apoptosis in a variety of cells (14-16).

miRNAs are short oligonucleotides, consisting of ~19-24 nucleotides (17). miRNAs repress the translation of their target genes by binding to partly complementary sequences in the 3' untranslated region of the target mRNA (18). miRNAs are involved in the control of diverse cellular processes, including cell growth, apoptosis, development, metabolism, stress adaptation, hormone signaling and differentiation (18-22). In hair follicles, a deficiency of miRNAs induced by the knockout of Dicer and Drosha blocks the anagen developmental phase by repression of the catagen phase (23). It has been hypothesized that the miRNA miR-31 is involved in hair follicle growth and hair fiber formation because it targets *Krt16*, *Krt17*, *Dlx3* and *Fgf10* (24). A recent study compared the miRNA expression profiles in balding and non-balding dermal papilla (25).

To the best of our knowledge, no previous studies have reported the miRNA expression profile in H₂O₂-treated DP. Therefore, in the present study, we analyzed changes in the miRNA expression profiles in DP alone and in DP that had been treated with H₂O₂. The target genes of significant miRNAs (those with >2-fold changes in expression) were predicted by an *in silico* prediction algorithm. Based on these data, we derive a model indicating that H₂O₂-specific miRNAs regulate ROS-responsive cellular functions.

Materials and methods

Cell culture. Human dermal papilla (HDP) cells were purchased from Cellbio Inc. (Seoul, Korea). HDPs were maintained as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin. HDPs were cultured in a humidified chamber with 5% CO₂ at 37°C.

Cell viability. HDPs (5x10³ cells) were plated in 96-well culture plates and treated with H₂O₂ for 24 h under the growth conditions described above. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed by adding 0.5 µg/ml MTT (Sigma-Aldrich) to the culture medium. Following 1 h of incubation under normal cell growth conditions, the growth medium was removed and 200 µl of dimethyl sulfoxide was added to each well. The absorbance was measured at 490 nm using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA).

Cell cycle analysis. The cell cycle was determined by flow cytometry via propidium iodide (PI) staining. Cell cycle analysis was performed as described previously (26).

RNA preparation and miRNA microarray. Total RNAs were extracted by RiboEX (GeneAll Biotechnology Co., Ltd., Seoul, Korea) and quantified by measuring the optical density ratio. The miRNA microarray (SurePrint G3 Human v16.0 miRNA 8x60K; Agilent Technologies, Santa Clara, CA, USA) was performed according to the manufacturer's instructions. miRNA was stained by pCp-Cy3 (Agilent Technologies), combined with T4 ligase (Agilent Technologies) and hybrid-

ized to a probe on the microarray. The microarray was imaged using the Agilent microarray scanner and digitized by Feature extraction. The digitized data were analyzed for fold change, miRNA potential target and gene ontology (GO) using Genespring GX version 11.5 (Agilent Technologies).

Prediction of miRNA target genes and GO analysis. The putative target genes of significantly up and downregulated miRNAs were identified by the web tool TargetScan. Target gene prediction was performed on significant miRNAs with 50 context score percentile using the conserved and non-conserved database. The putative target genes were identified and sorted by GO of each gene.

Statistical analysis. Statistical significance was determined by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

H₂O₂ treatment decreases the viability of HDPs via cell cycle arrest or apoptosis. To determine the cytotoxicity of H₂O₂, we initially examined the viability and status of the G1 or G2/M population in HDPs. HDPs were treated with H₂O₂ (0-1,000 µM) and the resulting viability was measured using the MTT assay (Fig. 1A). Following 24 h of H₂O₂ treatment, a decrease in cell viability occurred in a dose-dependent manner. In the presence of 750 and 1,000 µM H₂O₂, a significant (P<0.05) decrease in cell viability to 71.23 and 50.60% of that in control cells, respectively, was observed (Fig. 1A). In the presence of 750 µM H₂O₂, HDPs exhibited a 21.07% reduction in G1 phase and an 11.66% increase in G2/M phase, compared with those of the control (Fig. 1B). These data suggest that H₂O₂ decreases cell viability of HDPs by inducing cell cycle arrest at the G2/M phase and cell death.

Identification of H₂O₂-induced changes in miRNA expression in HDPs. H₂O₂-induced changes in the miRNA expression profiles were analyzed using the Agilent miRNA microarray, which contains 1,368 probes that are able to detect 1,205 human miRNAs. The fluorescence intensity data for each sample was normalized by global normalization. To eliminate disorderly data, miRNA expression data were selected by requiring a present-flag for at least one of all samples. Using this method, 155 miRNAs were selected out of 1,205 human miRNAs detected by present-flag selection. To identify H₂O₂-regulated miRNAs, the refined data were compared for control HDPs grown under normal conditions and HDPs treated with 750 µM H₂O₂ for 24 h. The results demonstrated that the expression levels of 68 miRNAs were altered at least 1.5-fold in response to treatment with H₂O₂. The 68 miRNAs are presented in Fig. 2. Fold-change analysis revealed that 62 miRNAs are upregulated and 6 miRNAs are downregulated at levels of 1.5-fold or greater in control HDPs grown under normal conditions and HDPs treated with 750 µM H₂O₂ for 24 h (Table I).

Identification of H₂O₂-specific miRNA putative target genes and GO analysis. Our study identified 68 novel miRNAs that were significantly up or downregulated in response to H₂O₂ treatment. As miRNA functions as RNA interference during mRNA

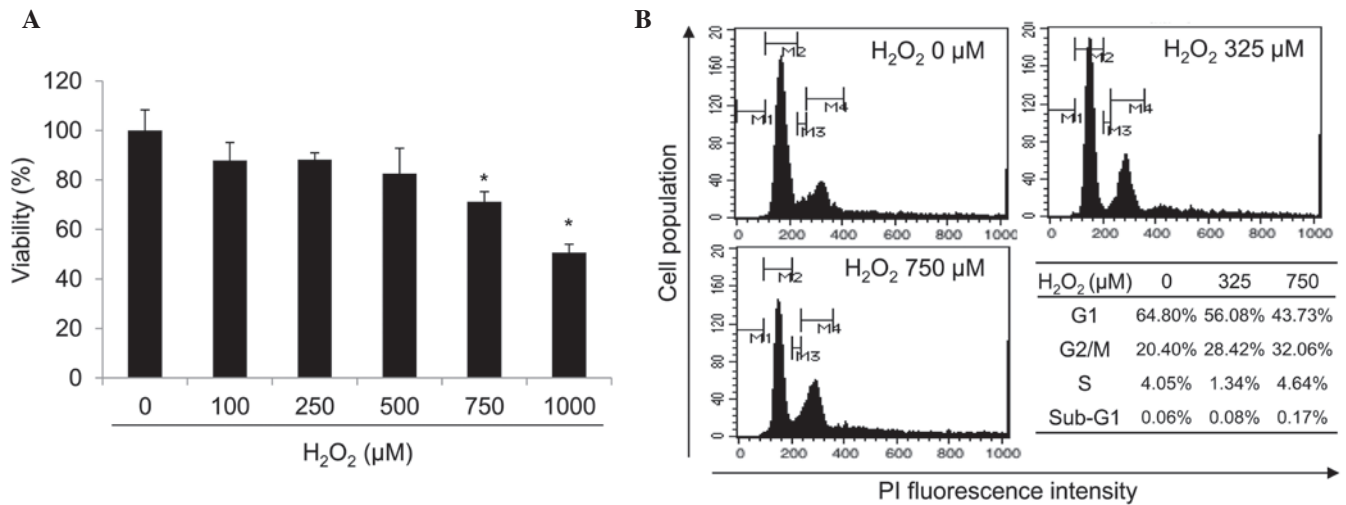


Figure 1. H₂O₂ repression of cell growth via cell cycle arrest and apoptosis in HDP cells. (A) Growth properties of H₂O₂-treated HDPs. HDPs (5x10³) were seeded in 96-well plates and treated with the indicated H₂O₂ concentrations, incubated for 24 h and growth properties were measured using the MTT assay. Cell viability (average ± SD) was determined from triplicate experiments (*P<0.05). (B) HDPs (2x10⁶) were seeded in a 60-mm culture dish and treated with 0-750 μM H₂O₂, incubated for 24 h and processed for flow cytometry by PI staining. Graphical representation of FACS data, bar graph represents percentage of total cell population in each cell cycle phase (± SD). H₂O₂, hydrogen peroxide; HDP, human dermal papilla; PI, propidium iodide.

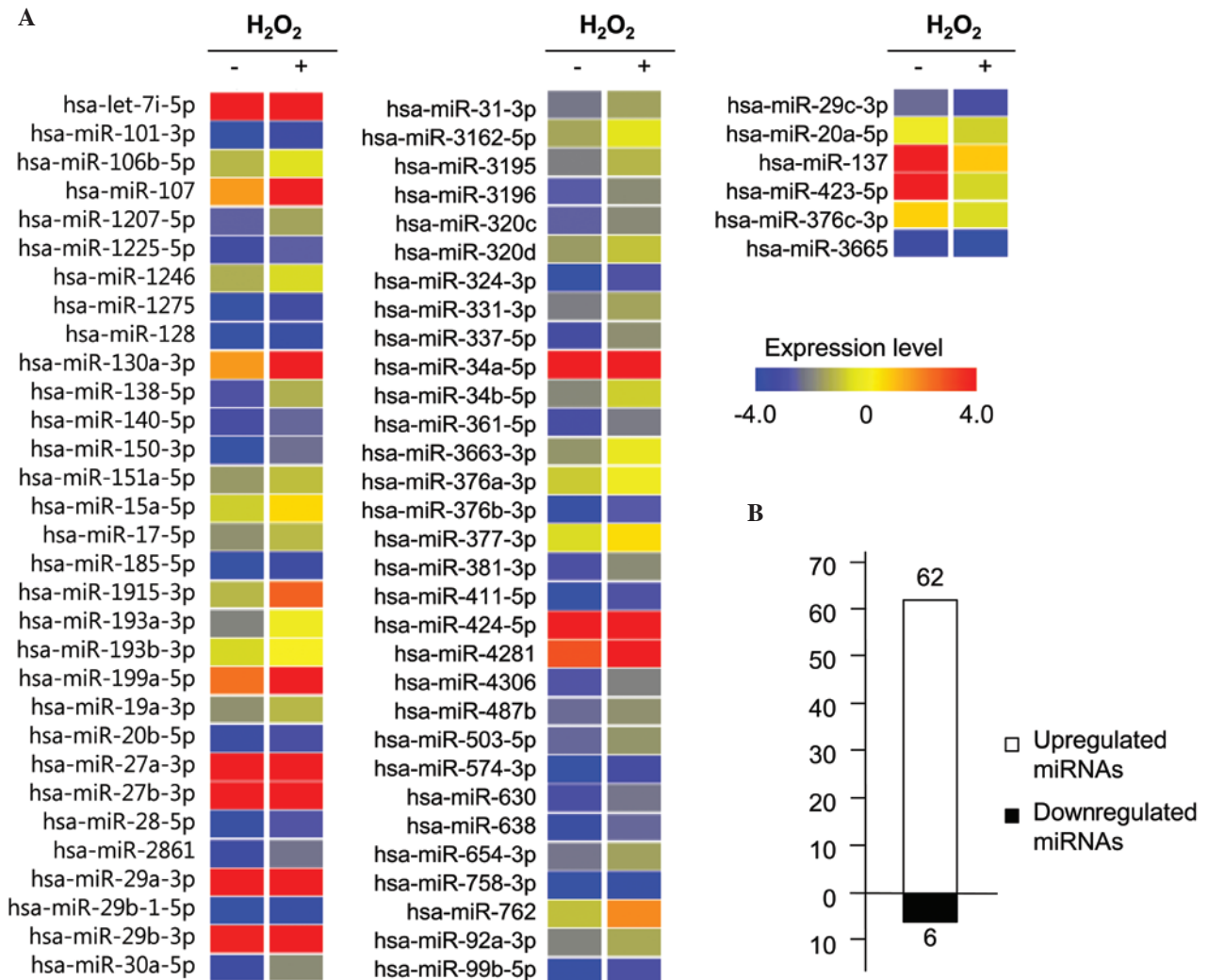


Figure 2. Changes in miRNA expression levels in H₂O₂-treated HDP cells. (A) Total RNA was extracted from control HDPs and HDPs treated with 750 μM H₂O₂ for 24 h. miRNA microarray was performed as described in Materials and methods. To obtain the fold change in expression levels, the fluorescence intensity of each miRNA was compared between normal HDPs and H₂O₂-treated HDPs. Changes in miRNA expression >2-fold are illustrated by heat map. Blue indicates a relatively low expression and red indicates a relatively high expression. (B) Up and downregulated miRNAs in response to H₂O₂ treatment were counted and represented by the bar graph. miRNA, microRNA; H₂O₂, hydrogen peroxide; HDP, human dermal papilla.

Table I. Up and downregulated miRNAs in H₂O₂-treated HDPs.

| A, Upregulated | | | | | | | | |
|-----------------|-------------|-------|------------------|-------------|-------|-----------------|-------------|-------|
| miRNA | Fold change | Chr | miRNA | Fold change | Chr | miRNA | Fold change | Chr |
| hsa-let-7i-5p | 1.52 | chr12 | hsa-miR-19a-3p | 1.52 | chr13 | hsa-miR-361-5p | 1.86 | chrX |
| hsa-miR-101-3p | 1.91 | chr1 | hsa-miR-20b-5p | 1.56 | chrX | hsa-miR-3663-3p | 2.73 | chr10 |
| hsa-miR-106b-5p | 1.59 | chr7 | hsa-miR-27a-3p | 2.13 | chr19 | hsa-miR-376a-3p | 1.65 | chr14 |
| hsa-miR-107 | 1.55 | chr10 | hsa-miR-27b-3p | 1.56 | chr9 | hsa-miR-376b-3p | 2.39 | chr14 |
| hsa-miR-1207-5p | 2.08 | chr8 | hsa-miR-28-5p | 2.17 | chr3 | hsa-miR-4281 | 2.15 | chr5 |
| hsa-miR-1225-5p | 1.62 | chr16 | hsa-miR-2861 | 2.29 | chr9 | hsa-miR-4306 | 1.65 | chr13 |
| hsa-miR-1246 | 1.60 | chr2 | hsa-miR-29a-3p | 1.60 | chr7 | hsa-miR-487b | 1.51 | chr14 |
| hsa-miR-1275 | 1.75 | chr6 | hsa-miR-29b-1-5p | 2.14 | chr7 | hsa-miR-503-5p | 1.66 | chrX |
| hsa-miR-128 | 1.54 | chr2 | hsa-miR-29b-3p | 3.26 | chr1 | hsa-miR-574-3p | 2.01 | chr4 |
| hsa-miR-130a-3p | 1.63 | chr11 | hsa-miR-30a-5p | 3.01 | chr6 | hsa-miR-630 | 1.66 | chr15 |
| hsa-miR-138-5p | 2.84 | chr3 | hsa-miR-31-3p | 1.58 | chr9 | hsa-miR-638 | 2.33 | chr19 |
| hsa-miR-140-5p | 1.55 | chr16 | hsa-miR-3162-5p | 2.00 | chr11 | hsa-miR-654-3p | 1.63 | chr14 |
| hsa-miR-150-3p | 3.95 | chr19 | hsa-miR-3195 | 1.81 | chr20 | hsa-miR-758-3p | 3.00 | chr14 |
| hsa-miR-151a-5p | 1.52 | chr8 | hsa-miR-3196 | 1.67 | chr20 | hsa-miR-762 | 2.34 | chr16 |
| hsa-miR-15a-5p | 1.76 | chr13 | hsa-miR-320c | 1.52 | chr18 | hsa-miR-92a-3p | 1.51 | chr13 |
| hsa-miR-17-5p | 1.56 | chr13 | hsa-miR-320d | 1.52 | chr13 | hsa-miR-99b-5p | 2.01 | chr19 |
| hsa-miR-185-5p | 1.64 | chr22 | hsa-miR-324-3p | 2.38 | chr17 | hsa-miR-377-3p | 1.55 | chr14 |
| hsa-miR-1915-3p | 2.81 | chr10 | hsa-miR-331-3p | 1.51 | chr12 | hsa-miR-381-3p | 2.03 | chr14 |
| hsa-miR-193a-3p | 3.65 | chr17 | hsa-miR-337-5p | 2.52 | chr14 | hsa-miR-411-5p | 2.47 | chr14 |
| hsa-miR-193b-3p | 1.56 | chr16 | hsa-miR-34a-5p | 2.04 | chr1 | hsa-miR-424-5p | 1.72 | chrX |
| hsa-miR-199a-5p | 1.61 | chr1 | hsa-miR-34b-5p | 2.19 | chr11 | | | |

| B, Downregulated | | | | | | | | |
|------------------|-------------|-------|----------------|-------------|-------|-----------------|-------------|-------|
| miRNA | Fold change | Chr | miRNA | Fold change | Chr | miRNA | Fold change | Chr |
| hsa-miR-29c-3p | -1.51 | chr1 | hsa-miR-137 | -1.50 | chr1 | hsa-miR-376c-3p | -1.56 | chr14 |
| hsa-miR-20a-5p | -1.51 | chr13 | hsa-miR-423-5p | -1.57 | chr17 | hsa-miR-3665 | -2.36 | chr13 |

miRNAs, microRNAs; H₂O₂, hydrogen peroxide; HDPs, human dermal papilla cells.

translation, we predicted that H₂O₂-specific miRNAs may regulate all or a number of the H₂O₂-response genes. Therefore, the target genes of H₂O₂-specific miRNAs were analyzed using the bioinformatics target gene prediction program TargetScan. For target gene analysis, default parameters were utilized, 50 context score percentile in the conserved and nonconserved database. H₂O₂-induced miRNAs targeted 14,046 genes; H₂O₂-repressed miRNAs targeted 6,019 genes. To identify the cellular functions of the putative target genes, GO analysis was performed, which is a method that categorizes the genes according to the cellular function classified for a standard for each gene. As illustrated in Fig. 3, GO analysis identified the following cellular functional activities: for molecular function, catalytic (17.01%), nucleic acid binding transcription factor (6.67%), enzyme regulator (2.62%), molecular transducer (2.61%), binding (67.63%) and transporter (3.45%); for biological process, signaling (7.29%), biological adhesion (1.65%), multicellular organismal (4.11%), cellular (18.52%), metabolic (13.96%), cellular component organization or biogenesis (0.63%), immune system (0.01%), biological regulation (14.89%), establishment of localization (6.16%), localization

(6.21%), response to stimulus (7.15%), single organism (15.35%) and developmental (4.06%); for cellular component part, cell (25.45%), membrane (10.69%), organelle (1.93%), extracellular region (0.75%), extracellular matrix (0.2%), organelle (15.01%), membrane-enclosed lumen (1.05%), cell junction (0.96%), extracellular matrix (0.77%), membrane (14.3%), cell (25.45%), extracellular region (2.82%) and synapse (0.61%). All of these were implicated in UVB-mediated responses in HDPs. These GO annotations provided comprehensive information on the function of H₂O₂-regulated transcripts in HDPs. TargetScan was used to predict the gene targets of the top five miRNAs that demonstrated the greatest increase or decrease in expression levels. The putative miRNA target genes were sorted into cell cycle, apoptosis and cell growth, and proliferation-related GO (Tables II and III). Cell cycle-related GO included cell cycle (GO:0007049), cell cycle arrest (GO:0007049), negative regulation of cell cycle (GO:0045786) and regulation of cell cycle (GO:0051726). Apoptosis-related GO included apoptotic process (GO:0006915), apoptotic signaling pathway (GO:0097190), cell death (GO:0008219), death (GO:0016265),

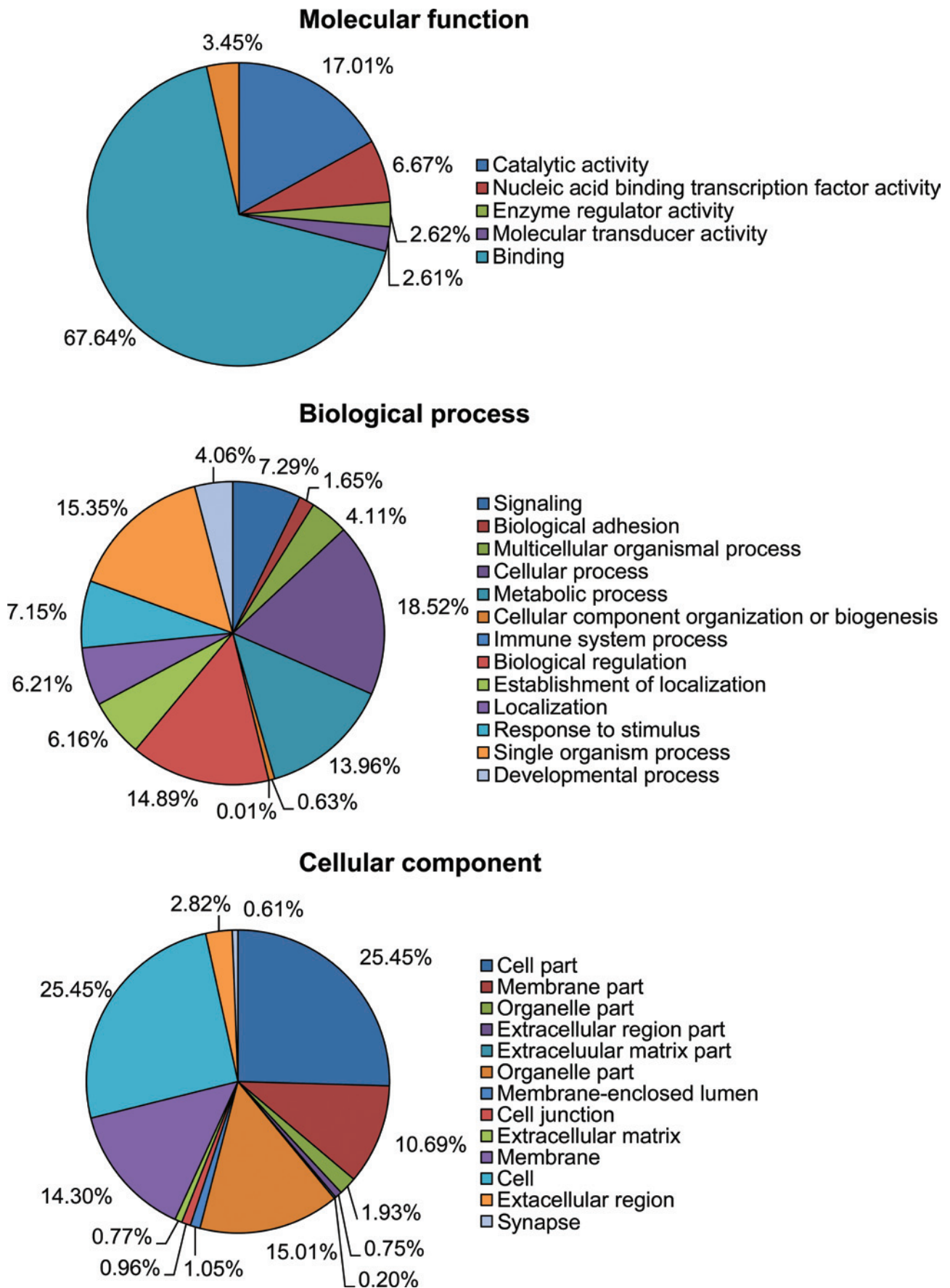


Figure 3. GO enrichment analysis of the target genes of up and downregulated miRNAs. Putative target genes of H₂O₂-specific miRNAs were identified by TargetScan and the cellular functions were predicted by GO analysis. The charts represent the GO analysis performed by Genespring GX. GO, gene ontology; miRNAs, microRNAs; H₂O₂, hydrogen peroxide.

Table II. Predicted target genes of the top five most upregulated miRNAs in H₂O₂-treated HDPs.

| miRNA | Target genes and functions | | |
|-----------------|--|---|---|
| | Cell cycle | Apoptosis | Cell growth and proliferation |
| hsa-miR-150-3p | - | - | - |
| hsa-miR-193a-3p | PSRC1, JUB, CCNT2, CCPG1, DLGAP5, PAPD5, SIAH1, TSC1, CABLES2, SMC1A, ERBB2IP, SEP3, KLK10, PTEN, CCND1, NEDD9, NF1, NF2, SUFU, CTF8, SASS6, E2F6, SENP5, VASH1, SPECC1L, CADM1, APPL1, GAS1, ING1, IRF1, FOXO4, ATM | BCL2L10, DIDO1, WDR92, RFFL, ZNF346, CADM1, RNF144B, GAS1, TNFRSF21, IL1A, JAK2, MCL1, MLL, OSM, SH3GLB1, PDCD2, DRAM, PSEN1, BAG1, BCL2L2, RTKN, ATXN1, ELMO2, SIAH1, TIAL1, TNFAIP3, TNFRSF1B, TRAF1, PHLDA2, DYRK2, AIFM2, TNFSF9, TNFRSF10B, SQSTM1, EBAG9, TP53INP1, ARHGEF6, PPM1F | BMPR2, TDGF1, KAZALD1, PPP1R9B, WISP1, EBAG9, SOCS6, CIAO1, NET1, ENOX2, TNFSF13B, FLT1, FLT3, HOXD13, IGFBP5, KIT, KRAS, NEDD9, NODAL, OSM, CCDC88A, PRKCCQ |
| hsa-miR-29b-3p | PPM1D, CDC23, CCND2, CCNF, UBA3, CCNT2, AURKB, HDAC4, DCLRE1A, SUPT5H, TACC1, PTP4A1, CDC7, GSG2, SMPD3, RCC2, PTEN, NEDD9, ZAK, XRN1, CDK2, CDK6, HTATIP2, CGREF1, STAG2, SEP9, DBF4, MAPRE2, E2F7, SLC5A8, AHR, VASH1, MAPRE1, CLASP2, SEP6 | FEM1B, HTATIP2, IL24, SLC5A8, AHR, FOXO3, PPP1R13B, SIRT1, ZNF346, CECR2, BIRC2, FAS, IL2RA, MCL1, SH3GLB1, ZAK, CYCS, DIABLO, DUSP22, BIRC6, BAK1, ATXN1, CIDEC, SGK1, ISG20L1, TNFAIP3, TNFRSF1A, TRAF5, FAM130A1, CASP7, AIFM2, BMF, RABEP1, TP53INP1, TRAF4, SLK | BIRC6, PURA, TRAF5, VEGFA, EPC1, CDC7, CREG1, WISP1, CCND2, MORF4L2, CDK2, MORF4L1, CHRNA7, IFNG, IGF1, LIF, LIFR, NDN, NEDD9, PDGFRB, ING3, PPP2CA |
| hsa-miR-30a-5p | TBRG1, CCNK, CCNA1, BCL10, LMLN, UBA3, CCNT2, CCNE2, SEP7, NEK4, TFDP1, TSC1, UBE2I, PTP4A1, EVI5, MIS12, SUV39H2, CHAF1B, CDC7, RECK, RGS2, RCBTB1, NEK1, NF1, PNN, RBM5, STAG2, DBF4, ESCO1, ESCO2, DDX11, E2F3, EPHB2, CLASP2, SEP6, CD2AP, NSL1, MTBP, INCENP, JAG2, KIF11, RHOB | BCL2L11, EDAR, NLRP3, RFFL, TICAM1, PRUNE2, TRIM35, SIRT1, RNF144B, TCTN3, GJA1, CECR2, SH3KBP1, HTT, HIP1, IL1A, IL2RA, IL17A, MAP3K5, MLL, MNT, BCL2L15, NAIP, DDIT4, C8orf4, AVEN, PTGER3, BIRC6, TRIB3, ATXN1, SOX9, ACTC1, TFDP1, TIA1, CASP3, UNC5C, TNFSF9, TNFRSF10D, TNFRSF10B, BCL10, ATG12, EBAG9, DLG5, ARHGEF6, ATG5 | C19orf10, BIRC6, BNC1, SOX9, VIPR1, CAMK2D, CDC7, CDCA7, CUL3, SOCS1, CREG1, OCS3, EBAG9, CFDP1, ENOX2, ADRA1D, ADRA2A, ADRB2, DDX11, TLX1, IL7, JAG2, KRAS, LIFR, LYN, MAFG, NOV, PDGFRB, PPP2CA |

Table II. Continued.

| miRNA | Target genes and functions | | |
|----------------|--|---|---|
| | Cell cycle | Apoptosis | Cell growth and proliferation |
| hsa-miR-758-3p | PARD6G, CCNG1, AURKB, MPHOSPH1, DLGAP5, DMTF1, BMP7, NEK4, TACC1, TP53BP2, VHL, WEE1, PPAPDC1B, RBL1, PPP1CB, NUSAP1, WWOX, ZAK, CDKN1B, KHDRBS1, STAG2, FOXN3, SGOL1, E2F6, CLASP2, PDS5A, CD2AP, CADM1, EID1, APPL1, LIN9, STRN3, ING1, LIG4, MCM3, MCM6 | MALT1, UNC5D, CTSB, RYBP, CADM1, APAF1, PAK1, ZAK, KRT20, APTX, IFT57, TNFRSF19, BCL2L1, ATXN1, PERP, UBE2Z, BNIP2, TIA1, TIAL1, TP53BP2, C10orf97, ACTN4, RABEP1, EBAG9, BAG2, BRE | SLAMF1, STAT5B, KLF5, BLZF1, CDC2L5, EBAG9, SOCS6, NAMPT, CDKN1B, CFDP1, ENOX2, TCFL5, FGF7, ID4, IGF1, ILK, LIFR, NDN, CRIM1, POU3F2 |

miRNAs, microRNAs; H₂O₂, hydrogen peroxide; HDPs, human dermal papilla cells.

Table III. Predicted target genes of the top five most downregulated miRNAs in H₂O₂-treated HDPs.

| miRNA | Target genes and functions | | |
|-----------------|--|--|---|
| | Cell cycle | Apoptosis | Cell growth and proliferation |
| hsa-miR-376c-3p | JUB, CCNB3, CCNT2, NOLC1, MTSS1, SIAH2, AURKA, VHL, HMGA2, CHAF1B, ERBB2IP, PTEN, NPAT, PAFAH1B1, GMNN, XRN1, MPHOSPH8, SPIN1, POLS, LZTS1, CHEK2, SASS6, GADD45A, MAPRE1, KANK1, SASH1, APPL1, ANXA1, HPGD, SEP14, MN1 | EGLN3, RFFL, ACVR1C, PARP4, CNTN4, GADD45A, DAPK2, TNFAIP8, SGMS1, ARF6, MCL1, APIP, GULP1, RHOT1, DUSP22, ROCK1, ATXN1, SHB, SIAH2, ACTC1, PHLDA2, FASTKD3, CASP3, CASP7, AIFM1, ATG12, CD5L | MARK4, CXCL5, BMPR2, SSR1, STAT5A, TGFBR1, TNFSF4, HMGA2, SOCS2, SOCS6, CD86, CD47, MORF4L2, DNAJA2, CFDP1, TNFSF13B, TBC1D8, SOCS4, FGF7, FLT1, FLT3, RBM9, ANXA1, IL2, CXCL10, KRAS, NOV, IL17RB |
| hsa-miR-423-5p | JUB, CCNF, PKMYT1, MPHOSPH1, DMTF1, SUV39H1, TACC1, DBF4B, BIN3, MAPK3, MAPK4, PTCH1, RAP1A, UPF1, TSPYL2, NF2, PAFAH1B1, CINP, SUFU, PFDN1, RBM5, CDKN1A, HMG20B, TXNIP, SPIN1, RCC1, FOXN3, LZTS1, PMF1, E2F7, KCTD11, SNF1LK, DAB2IP, CYLD, DDX11, E2F2, EP300, | BCL2L11, IL24, DIDO1, RFFL, CLU, ACVR1C, DAP, DEDD2, DOCK1, E2F2, EIF5A, EP300, ERN1, PTK2B, FOSL2, CYFIP2, CIDEB, HIPK2, SAP30BP, CARD10, HTT, KCNIP3, HIP1, BIRC5, IAPP, IL17A, LTBR, MDM4, MLL, NGFR, NDUFA13, PDCD1, RNF216, PPARD, PRKCG, BAG1, BCL2L1, RTKN, | C19orf10, RAC2, SHC1, STAT5A, STAT5B, TGFBR2, VEGFB, PROK1, CUL3, CDC2L5, FGF18, NRP1, SOCS3, SCGB3A1, S1PR2, TAOK2, CIAO1, HTRA3, NTN1, CLU, ADM, CSF1, ADRA1D, KCTD11, CTF1, ADRA2A, DDX11, DHPS, S1PR3, PTK2B, FOXO1, FLT3LG, RBM9, HOXC10, VSX2, IGF1, IGF1BP6, |

Table III. Continued.

| miRNA | Target genes and functions | | |
|----------------|---|--|--|
| | Cell cycle | Apoptosis | Cell growth and proliferation |
| hsa-miR-423-5p | PDS5A, NSL1, SGSM3, BIRC5, INCENP, IRF1, MCM7, MN1, NBL1, NBN, SEP3, SEP8 | NOD2, BNIP1, BOK, PRDX2, CACNA1A, BCL2L14, BIRC7, CASP2, PLA2G6, TRAF7, AIFM2, LGALS12, RIPK1, TNFSF12, TNFRSF14, TNFRSF10D, SQSTM1, NOL3, BMF, DNAJA3, TAOK2, NTN1, BRE | KRT6A, MAFG, ODC1, PDGFRB, C9orf127, IL17RB, CCDC88A, PRKCQ |
| hsa-miR-3665 | - | - | - |
| hsa-miR-20a-5p | PARD6B, JUB, RUNX3, CDC23, PCAF, BCL10, CCND2, CCNG2, RNF8, CCNT2, PKMYT1, ZNF830, NEK9, KIF23, RASSF2, RB1CC1, SEP7, CDC25A, DLEC1, DMTF1, PAPD5, BMP2, STK11, TACC1, BUB1, VHL, WEE1, PTP4A1, SUV39H2, MAP9, PPP6C, ERBB2IP, MAPK1, MAPK4, PCNP, PTEN, RAD17, RAP1A, RB1, RBL1, RBL2, CCND1, TIPIN, SEP6, NPAT, PAFAH1B1, FZR1, ZAK, XRN1, CHAF1A, CDKN1A, TXNIP, CETN2, SEP2, CIT, TUSC2, SNF1LK, CYLD, E2F1, E2F3, FANCD2, C11orf82, HEPACAM, MAPRE1, MAPRE3, SEP9, PDS5A, SASH1, CLASP1, RABGAP1, GAK, NSL1, APPL1, FBXO5, CKAP2, PDCD4, C13orf15, GRLF1, RACGAP1, BIRC5, TMPRSS11A, IRF1, MCC, MCM3, NBL1 | NOD1, FASTK, EGLN3, NLRP3, E2F1, EIF4G2, C11orf82, CARD8, ACIN1, FAIM2, SULF1, RYBP, SGMS1, MAGI3, CKAP2, GJA1, TNFRSF21, PDCD4, BIRC5, APP, MCL1, MDM4, MAP3K5, BCL2L15, OSM, SH3GLB1, PDE1B, ZAK, RHOT1, BCAP29, PTGER3, BCL2, PERP, ISG20L1, BNIP2, TNFAIP3, SLTM, C10orf97, RNF34, FXR1, CASP2, PLA2G6, CASP7, CASP8, CUL1, AIFM2, TNFRSF10D, TNFRSF10B, IER3, SQSTM1, SGPL1, TAX1BP1, BCL10, RABEP1, ATG12, DEDD, CD5L, STK17B, TP53INP1, ATG5, LITAF, TRAF4, SLK | PURA, BCL2, CXCL5, BMPR2, SSR1, TBX3, TGFB2, TSG101, CAMK2D, ITCH, CDCA7, BRMS1L, CUL3, CCND2, S1PR2, SOCS6, ARHGEF11, DNAJA2, CFDP1, HOXB13, MORF4L1, TBC1D8, CHRNA7, S1PR3, ERBB3, HEPACAM, FGF4, FGF7, LRP12, IGFBP7, LIF, LIFR, OSM, DERL2, CRIM1, ING3, TIPIN, PPP2CA |
| hsa-miR-29c-3p | PPM1D, CDC23, CCND2, CCNF, UBA3, CCNT2, AURKB, HDAC4, DCLRE1A, SUPT5H, TACC1, PTP4A1, CDC7, SMPD3, RCC2, PTEN, NEDD9, ZAK, XRN1, | FEM1B, HTATIP2, IL24, SLC5A8, AHR, FOXO3, PPP1R13B, SIRT1, ZNF346, CECR2, BIRC2, FAS, IL2RA, MCL1, SH3GLB1, ZAK, CYCS, DIABLO, DUSP22, | BIRC6, PURA, TRAF5, VEGFA, EPC1, CDC7, CREG1, CCND2, MORF4L2, CDK2, MORF4L1, CHRNA7, RBM9, IFNG, IGF1, LIF, LIFR, NDN, NEDD9, |

Table III. Continued.

| miRNA | Target genes and functions | | |
|----------------|---|---|-------------------------------|
| | Cell cycle | Apoptosis | Cell growth and proliferation |
| hsa-miR-29c-3p | CDK2, CDK6, HTATIP2, GREF1, STAG2, SEP9, DBF4, MAPRE2, FOXN3, E2F7, SLC5A8, AHR, VASH1, MAPRE1, CLASP2, SEP6, PPP1R13B, MCC, MLF1, NASP | BIRC6, BAK1, ATXN1, CIDEC, SGK1, ISG20L1, TNFAIP3, TNFRSF1A, TRAF5, FAM130A1, CASP7, AIFM2, BMF, RABEP1, TP53INP1, TRAF4, SLK | PDGFRB, ING3, PPP2CA |

miRNAs, microRNAs; H₂O₂, hydrogen peroxide; HDPs, human dermal papilla cells.

programmed cell death (GO:0012501), regulation of apoptotic process (GO:0042981), regulation of cell death (GO:0010941), regulation of execution phase of apoptosis (GO:1900117) and regulation of programmed cell death (GO:0043067). Cell growth-related and cell proliferation-related GO included positive regulation of cell proliferation (GO:0008284), regulation of cell growth (GO:0001558), regulation of cell proliferation (GO:0042127) and regulation of growth (GO:0040008).

Discussion

ROS, such as H₂O₂, are generated as reactive byproducts of cellular metabolism in the mitochondria. The intracellular level of ROS is distinctly regulated by the cellular antioxidant system, including non-enzymatic and enzymatic antioxidants (27). The ROS level is increased in response to environmental stresses, including UV irradiation, toxic chemicals, heat and even high glucose concentrations (27,28). High levels of ROS induce cell cycle arrest, senescence and apoptosis due to ROS damage of cellular membranes, lipids, proteins and DNA (7-10). In the present study, and in others previously, it has been identified that H₂O₂ induces growth arrest in HDPs (Fig. 1) (29-31). The H₂O₂-induced growth arrest occurs later within the cascade of events activated in response to H₂O₂ treatment. Previous investigations identified H₂O₂-responsive miRNAs, including miR-34 and miR-145, which also are implicated in the ROS-responsive pathway (32,33). Therefore, miRNA appears to be required for H₂O₂-dependent growth arrest.

The present study identified 68 miRNAs that were regulated by H₂O₂ in HDPs (Fig. 2). miR-193-3p and miR-29b increased in H₂O₂-treated HDPs and induced apoptosis by targeting MCL-1 (34,35). MCL-1 is a BCL-2 family member that is involved in mitochondria-dependent intrinsic apoptosis (36). MCL-1 represses apoptosis by preventing the formation of mitochondrial membrane potential (36). Our data, together with those of Lin *et al* (15), demonstrated that miR-193a-3p increased in response to ROS and subsequently induced cell death. A previous study revealed that miR-30a-5p was upregulated by H₂O₂, it repressed autophagy by targeting beclin-1 and eventually induced apoptosis (37). In the present study, it was demonstrated that miR-20a-5p and miR-423-5p

were repressed by H₂O₂ in HDPs (Table I). miR-20a-5p had multiple target genes, including *BNIP2*, *APP*, *ASK1* and *TNKS2*. Therefore, miR-20a-5p regulated proliferation, migration, invasion and inflammation by the regulation of its target genes (38-41). miR-424-5p targeted *p21Cip1/Waf1*, which functions in proliferation and G1 phase transition (42).

Numerous studies have demonstrated that ROS, including H₂O₂, induces intrinsic apoptosis (43-45). H₂O₂-induced apoptosis is regulated by mitochondrial membrane permeability, which is regulated by the BCL-2 family (36). The anti-apoptotic BCL-2 family, including *BCL2L10*, *BCL2L11*, *BCL2L2* and *BCL10* were predicted as targets of miRNAs that were upregulated by H₂O₂ (Table II). The pre-apoptotic BCL-2 family members *BOK* and *BAK1* were predicted as targets of miRNAs that were downregulated by H₂O₂ (Table III). Cell cycle regulating proteins, such as cyclins and CDKs, function during each phase of the cell cycle (G1, S, G2 and M) (46). CCNA1 and CDK2 are required for the regulation of the G2/M phase (47,48). The results of the present study predict that hsa-miR-30a-5p and hsa-miR-29b-3p target *CCNA1* and *CDK2*. These data suggest that H₂O₂-mediated growth arrest and cell death in HDPs is associated with the changes in expression of specific miRNAs.

Bioinformatics analysis of miRNA expression profiles, miRNA target genes and the GO of target genes provided a more holistic view of the underlying cellular mechanisms that occur in response to H₂O₂-induced growth arrest and apoptosis. The identification of miRNAs and their putative targets may offer new therapeutic strategies for H₂O₂-induced hair follicle disorders, such as hair loss.

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