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"Reverse Aging effects of Iris germanica L.-derived exosomes via identification of novel microRNAs and their gene regulatory functions"

Chaelin Park[1], Youngseok Kim[1], Seok Keun Cho[2], Bora Lee[2], Woorim Yang[3], Seong Wook Yang[3], Vincent Mutel[4], and Junoh Kim[1,*]

[1]Shinsegae International, Seoul; [2]Xenohelix Research Institute, Incheon; [3]Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul; [4]Tauderma SA, Conthey, Switzerland

1. Introduction

Plant-derived exosomes, which are nanosized (40-150nm) extracellular vesicles released via fusion of multivesicular bodies with plasma membranes, comprise of various bioactive components such as protein, lipid, mRNA, miRNA, DNA and metabolites [1,2]. Previous research has identified their notable roles in cell-cell communication, nutrient transportation, transmembrane signaling, and as efficient vehicles for drug therapy due to their nanosized and mobile features. Apart from animal-derived exosomes, plant-derived exosomes are presently being considered, and will continue to emerge as one of promising biological substances due to their outstanding combination of high-targeting, therapeutic properties of exosomes and excellent biocompatibility, high stability, low immunogenicity and biodegradable properties of being a plant origin [2]. In addition, a recent shift of interest towards a more personalized and precise medicine in the bioscience field are accentuating the roles of microRNA (miRNA) as key modulators in gene expression. In particular, miRNAs play the role of posttranscriptional regulators in gene expression through degradation or the blocking of the translation of target messenger RNAs for multiple purposes – differentiation, development, cell metabolism and defense against the environment [3,4]. As such, miRNAs carried by plant-derived exosomes are being valued for their ability to target specific cells and exert various physiological activities, which are capabilities that may eventually be beneficial to skin regeneration as well.

This research primarily focuses on identifying gene regulatory functions of novel miRNA in the exosome derived from the rhizomes of the *Iris germanica* plant, a conventional species known for their antioxidant, antimicrobial, anti-inflammatory effects in the skin [5]. Next-Generation Sequencing (NGS) analysis was conducted after isolating RNA from purified exosomes of *Iris germanica L.* rhizomes. The miRNA sequences were identified and quantified in replicative samples of Iris-derived exosomes, with several of them being uncovered as novel sequences in the plant genome. In addition, target genes selected based on their close relation with the 12 hallmarks of aging were analyzed by their miRNA expectation values for prediction miRNA

functions in human skin cells [6]. Moreover, in vitro and ex vivo studies were proceeded concurrently with the miRNA sequencing, suggesting that Iris-derived exosome could play a role in modulating the target gene expressions and provide beneficial effects to the human skin in a cross-kingdom manner. In essence, the researchers aim to evaluate the skin reverse-aging effects of Iris-derived exosome through multiple aspects, further outlining the principles of plant-derived exosomes and miRNA as potential and promising therapeutic agents in the cosmetic industry. The significance of this research further lies on the discovery of novel miRNA sequences of the *Iris germanica* L. plant, which has not been previously uncovered in the plant genome.

2. Materials and Methods

2.1 NGS Sequencing

Exosome Extraction and Library Construction

Exosomes were isolated using XENO-EVI Plant Kit (Cat. No. 93667873-PL), and their concentration was quantified using a nanoparticle tracking analyzer (NTA). After adjusting the exosome particle to $1.4x10^{10}$, total RNA was extracted from the purified exosomes using the XENOPURE™ RNA 200nt Kit (Cat.No.9366-SR). Library preparation was performed using the XENO-LIBERA kit (Cat. No. 9366-LBR1). 3' and 5' adapters were ligated to the ends of the RNA molecules, followed by reverse transcription to generate cDNA, which was subsequently amplified. The constructed libraries were sequenced using the NovaSeqX sequencing platform (Illumina). Raw sequencing data quality was assessed using Fast QC v0.11.9. Adapter sequences and low-quality 3' end bases (quality score<20) were trimmed using Cutadapt v4.4, retaining reads with lengths between 18 and 30 bases. Trimmed reads were aligned to the reference genome of *Iris pallida* (GenBank: GCA_029216955.1) using Bowtie v1.3.1. The aligned reads were further mapped to Viridiplantae non-coding RNA sequences downloaded from Rfam using Bowtie v1.3.1, and matching reads were removed from further analysis. The remaining reads were used for miRNA prediction using miRkwood and quantified with featureCounts. Predicted miRNAs were aligned to Viridiplantae miRNA sequences obtained from miRbase using BLAST v2.2.29 to assign them to known miRNA families. Potential target genes of the predicted miRNAs were then identified using psRNATarget, a plant small RNA target analysis server.

2.2 miRNA function analysis

EV Isolation

A crude extract was obtained from fine powder prepared from "ABIO Materials". A filtered enzyme solution (0.4 % cellulose R10, 0.4 % macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM CaCl2, 0.1 % BSA, 1x PIs, 5 μM MG132, and 1 mM PMSF) was added at a ratio of 40 mL per 1 g of sample, and the mixture was incubated for 2 hours at 30 rpm. The sample was then filtered through two layers of Miracloth. Then, the solution was centrifuged at 100x g for 10 min followed by 4,000 rpm for 10 min at 4 °C. After carefully collecting the supernatant, it was subjected to high-speed centrifugation at 13,000 rpm for 10 min at 4 °C. This step was repeated until the supernatant became clear. EVs were subsequently isolated using the XENO-EVI kit (Cat. 9366-EVI-30) according to the manufacturer's

instructions. EVI buffer was added to the prepared samples at a ratio of 15 mL (sample) to 4 mL (EVI buffer). The tubes were inverted five times and incubated at room temperature for 1 hour. After incubation, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the EV-containing pellet was resuspended in 100 µL of 0.1 µm-filtered 1× PBS. Next, EV samples (20µL) were diluted with 950µL of 0.1µm-filtered 1xPBS. The diluted solution was filtered again through 0.1 µm filter, after which NTA was performed using the ZETAVIEW® TWIN (PMX-220) system. The particle concentration (particles/mL) was analyzed using data provided by the ZetaView software.

Transmission electron microscopy (TEM)

EV samples (10µL) were used for TEM imaging. A drop of the sample was placed on a Formvar-carbon-coated grid for 15 seconds. The droplet was removed, and a drop of 1 % uranyl acetate was applied for 30 seconds. The stain was then removed using filter paper, followed by a brief wash with a drop of distilled water. The samples were imaged using the HT7800 TEM (HITACHI) at the Yonsei Biomedical Research Institute, Yonsei University college of medicine.

Immunoblot analysis

Lysates (powder) and EVs were mixed with sodium dodecyl sulfate (SDS) sample buffer and boiled at 95 °C for 10 minutes. Proteins were separated by SDS-PAGE using 8–13% gradient gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, California, USA). Membranes were blocked with 5% skimmed milk in PBST and incubated overnight at 4 °C with primary antibodies. The antibodies used to detect exosomal marker proteins included α-AGO1 (Agrisera), α-HSP90 (Agrisera), α-HSP70 (Agrisera), α-BiP (Agrisera), α-PEN1 (Agrisera), and α-ACT (Agrisera). Detection was performed using a secondary HRP-conjugated anti-rabbit IgG antibody (Abcam) and an enhanced chemiluminescence (ECL) system.

Cell Culture and miRNA transfection

Hs68 cell lines were cultured in Dulbecco′s Modified Eagle′s Medium (Welgene, #LM 001-05, South Korea) supplemented with 10% fetal bovine serum (Gibco, #16000-044, USA), 100 U/mL penicillin and 100 µg/ml streptomycin (Gibco, #15140122, USA) at 37°C in 5% CO2. All cells were tested for mycoplasma contamination using a mycoplasma detection polymerase chain reaction (PCR) test (Bionics, South Korea). For transfection, all miRNA oligonucleotides were obtained from Bioneer Co., Ltd. (Korea) and dissolved in DEPC-treated water at a final concentration of 100 µM. 135 µL of Opti-MEM was mixed with 6 µL of Lipofectamine RNAiMAX (ThermoFisher Scientific, Pittsburgh, PA, USA) and 300 nM of synthesized novel miRNAs, then incubated at room temperature for 20 minutes. The resulting transfection complex was added to the cells and incubated for 72 hours. Control groups were treated with Lipofectamine alone to eliminate transfection-related bias. For RNA extraction, TRIzol (Invitrogen) was used for total RNA extraction.

RT-qPCR

Total RNA (1µg) was reverse-transcribed into cDNA using the XENO cDNA Synthesis Kit (XENOHELIX, Korea). Quantitative real-time PCR (RT-qPCR) was performed using a 2× PCR Mixture (XENOHELIX, Korea) to evaluate the expression of miRNA target genes. ACTIN was used as a housekeeping gene for normalization. All reactions were performed in triplicate and independently repeated three times.

2.3 In vitro experiment

Fibroblast Cell Culture

Human dermal fibroblasts (HDFs) from 3 healthy donors (32, 36 and 37 years, females) and tested negative for HIV, HCV, HBV and COVID were isolated and cultured in DMEM medium (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were expanded and banked at a passage where the population doubling levels (PDL) were estimated to be 12-14. Cells at this stage are regarded as pre-senescent or young cells [7].

Senescence Induction

HDFs (individually from each donor) were (at PDL 12) induced to senescence by treatment with 100 ng/mL doxorubicin plus 100 ng/mL IGF1 (senescence-induced drugs) for 14 days. Fifteen days after initial cell plating, cells were stained for SA-β-Gal (beta-galactosidase) activity to confirm the cells underwent senescence. After the confirmation of senescence, cells from 3 donors were pooled HDFp (equal ratios) and grown for 7 days either in presence or absence of 100 ng/mL doxorubicin plus 100 ng/mL IGF1. The following day, exosomes were added simultaneously with doxorubicin plus IGF1 and cells were further incubated for 7 days. In all cases, extracts were readded every 48 h. Fifteen days after initial cell plating, cells were lysed in lysis buffer provided in the RNA extraction kit and stored in -80°C until RNA was extracted. The exosome effect was tested at 3 concentrations along with untreated control and 10 genes were tested. Next, SA-β-gal staining was performed using the Senescence Cells Histochemical Staining Kit (Sigma Aldrich, Cat No. CS0030) according to the manufacturer's instructions. Briefly, cells were washed with phosphate buffered saline (PBS) and fixed at room temperature for 5 min then incubated in Staining Working-Gal Activity Assay Solution at 37°C for 16h. Images were acquired under the phase contrast microscope (Nikon Ts2-FL) [7].

RNA extraction & qPCR

Total RNA from the cell lysates was extracted according to the manufacturer's protocol using Total RNA extraction kit (Machery Nagel/Bio-rad). The amount of total RNA extracted was quantified using NanoDrop One (Thermofisher). A ratio of ~2.0 is generally accepted as pure for RNA. Total RNA was proceeded with reverse transcription(RT) to cDNA using RT Reagent kit using the Aria MX PCR system. Resulting cDNA was amplified by PCR using corresponding gene specific primers. Quantitative real-time PCR(qPCR) reactions were performed using SYBR Green Supermix kit according to Aria MX PCR system. Acquired data was analyzed through Aria MX analysis software. The mRNA expression of the genes of interest was calculated according to the delta-delta Ct method Normalized data were presented as fold increase over untreated negative control with +/- Standard Deviation (SD). Statistical significance was performed by Student's T test. Synthesized primers were tested for finding the right annealing temperatures and specific amplifications. Once standardized, all the primers were used on experimental samples [7].

3. Results

3.1 NGS Sequencing

Exosome Extraction

Table 1. Exosome Quantification of *Iris germanica*

Sample No.	Exosome particles (particles/ml)	RNA Concentration (ng/μl)
1	2.8×10^{10}	42.4
2	2.8×10^{10}	43.4
3	2.8×10^{10}	39.4

Library Construction

Table 2. Library Construction of *Iris germanica*

Sample No.	Concentration (ng/μl)	Molarity (nM)	Size(bp)	Adapter index	Adapter index sequence
1	41.6	546	143	SRI4	TGACCA
2	44.6	585	147	SRI5	ACAGTG
3	40.7	534	142	SRI6	GCCAAT

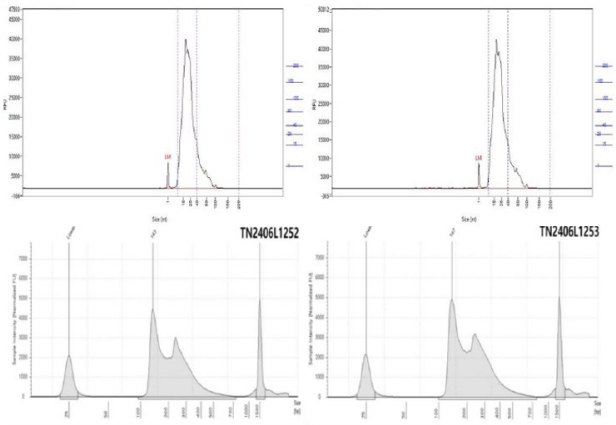

Figure 1. Electrophoresis results of each exosomal RNA isolation

Table 3. Sequencing Quality

Sample	Total reads	Yield base	≥Q20 Bases(%)	≥Q30 Bases(%)	Read length
Total	148,895,840	11,167,188,000	90.04	83.68	1x75

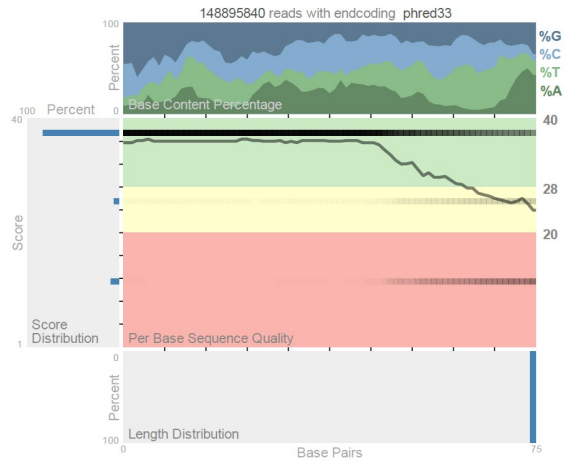

Figure 2. Quality of sequencing data of total 3 rhizome samples

Table 4. Pre-processing of raw data (18 to 30 base pair)

Sample No.	Raw Data		Trimmed Data		
	# of reads	Yield (base)	# of reads	Yield (base)	≥Q30 Bases(%)
1	28,465,939	2,134,945,425	4,960,426	112,698,742	97.28
2	31,098,458	2,332,384,350	5,683,262	128,500,458	97.39
3	29,532,987	2,214,974,025	5,210,950	117,815,222	97.33

Table 5. miRNA target gene analysis (expectation≥3)

Sample No.	Number of Target Genes	Target Genes
1	41.6	IGF1R, TOP2A, CAV1, COL4A1, FGF7, DNMT3A, HMOX1
2	44.6	IGF1R, TOP2A, CAV1, COL4A1, FGF7, DNMT3A, HMOX1, VCAN, SIRT1, SOD2
3	40.7	IGF1R, TOP2A, CAV1, COL4A1, FGF7, DNMT3A, VCAN, BNC2, CD44

Table 6. miRNA target gene analysis with human transcript (expectation≥2)

Sample No.	Number of Target Genes	Identical Target Genes
1	69	ASB6, ASB6, CCDC176, CEP41, CEP63, CREBL2, CUX1, EEA1, ERGIC1, F2RL2, GAS7, GPC4, LUC7L3, MAOA,
2	87	NMNAT2, NRIP3, NTM, PDK3, PHOX2A, PLEKHM1, POLH, PPM1E, PRKCA, PTPN3, RICTOR, SCML2, SHC3,
3	72	SLC11A1, TMEM18, TMTC3, TNNI1, UBE4B, UHRF1BP1L, USP20, USP20, VAX1, YTHDC1, ZBTB47, ZNF599

3.2 miRNA funtion analysis

Exosome Characterization

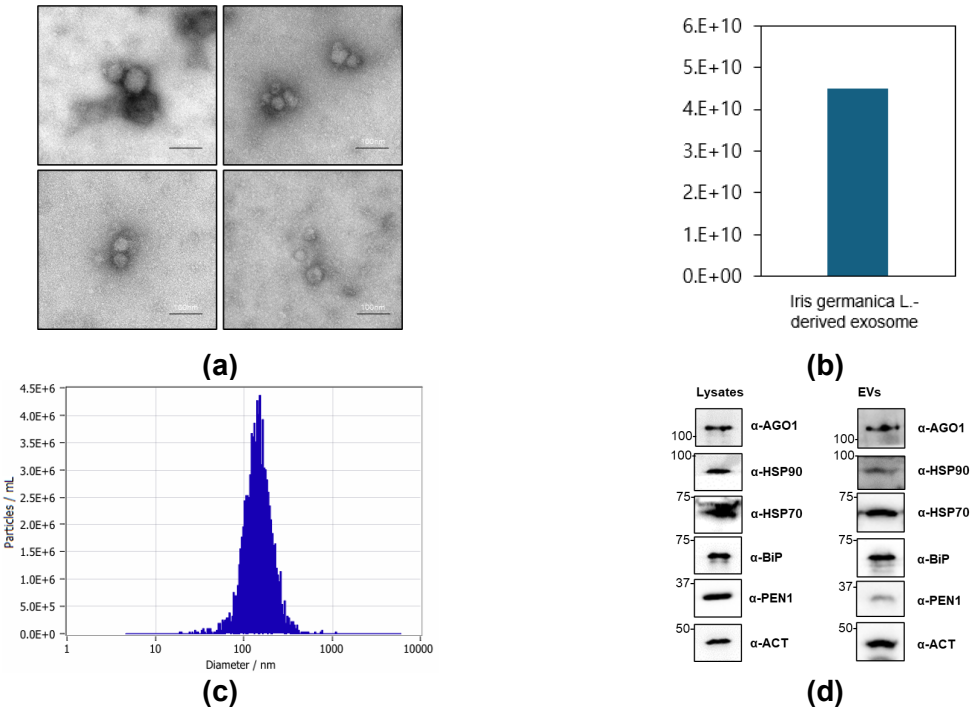

Figure 3. Exosome characterization (a) TEM (b,c) NTA and (d) Western blot analysis

Target Gene Analysis

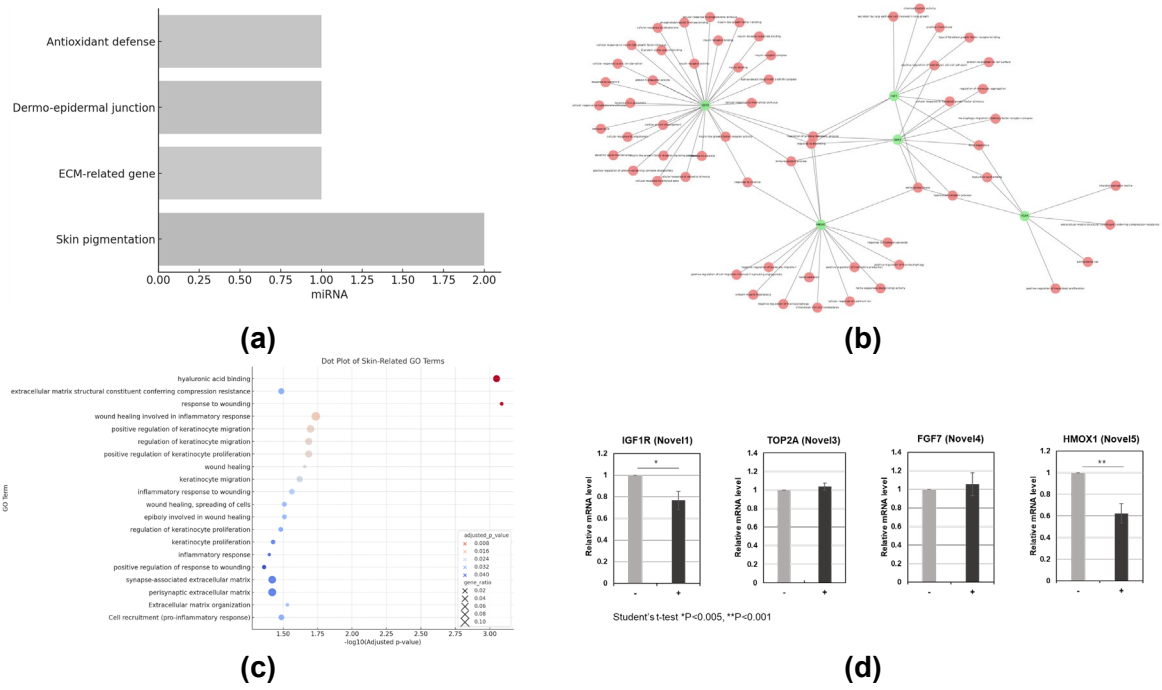

Figure 4. (a) miRNA expression folds and skin efficacies of the target genes (b,c) GO analysis of the selected target genes (d) Relative miRNA levels of target genes IGF1R, TOP2A, FGF7, HMOX1 in Hs68 cells

3.3 In vitro experiment

Senescence Induction

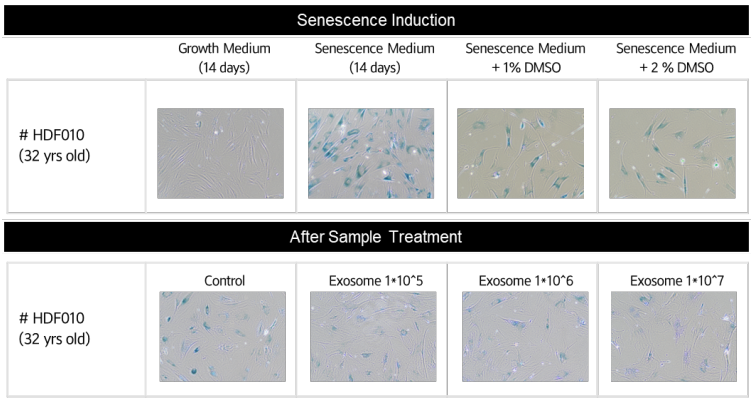

Figure 5. (a) β-Gal results after senescence and sample treatment in HDF010 (32y) cells

qPCR gene analysis

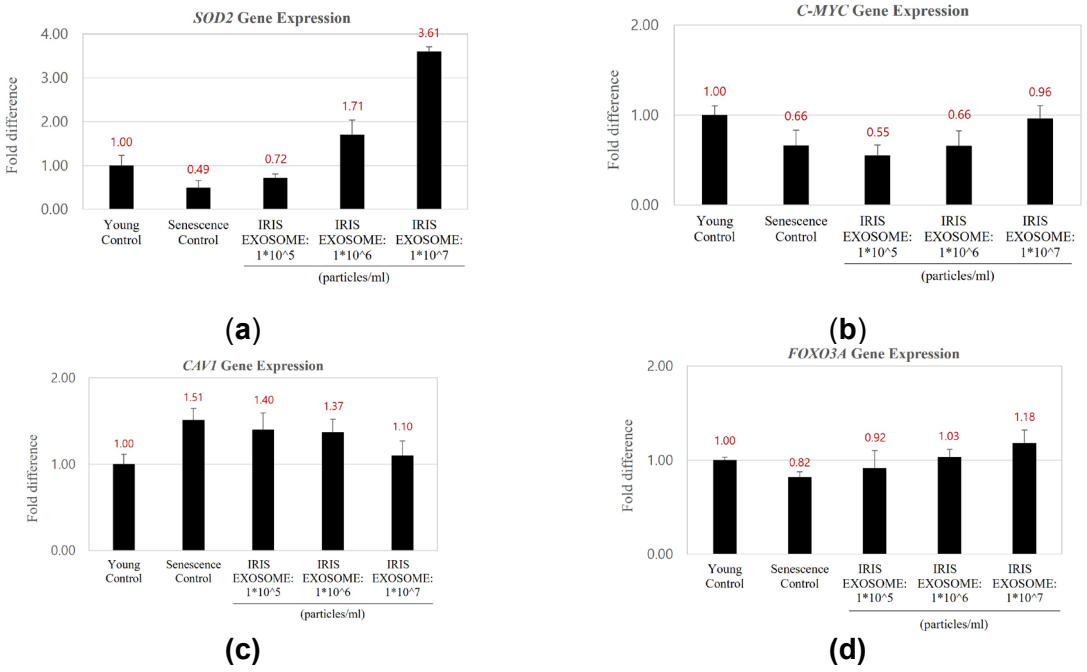

Figure 6. qPCR gene expression of target genes (a) SOD2 (b) C-MYC (c) CAV1 and FOXO3A

Target Gene	Reverse Aging	Iris-derived Exosome	Keyword	Function
c-MYC	▲	▲	Pluripotency TF	- c-MYC is one of the Yamanaka factors essential for reprogramming somatic cells into induced pluripetent stem cells (iPSCs) by promoting cell proliferation and maintaining pluripotency.
SOD2	▲	▲	Antioxidant defense	- Improvement of endogenous antioxidant defenses - Protect from heme oxidation (dark circles reduction)
SIRT1	▲	▲	Cellular Longevity	- Sirt1 is a longevity gene that plays a crucial role in extending lifespan, enhancing stress resistance, reducing inflammation, and maintaining skin health by promoting collagen synthesis and protecting against UV-induced damage
FOXO3A	▲	▲	Cellular Longevity	- FOXO3A is a longevity gene that helps extend lifespan by enhancing stress resistance and cell repair. In the skin, it protects against aging by reducing oxidative damage and supporting cell survival.
FGF7	▲	▲	Cell Proliferation	- FGF7 (Fibroblast Growth Factor 7) plays key role in skin regeneration by promoting the proliferation and migration of keratinocytes, aiding in wound healing, and enhancing the repair of damaged skin.
CAV1	▼	▼	Dermis Thickness	- CAV1 (Caveolin-1) regulates skin aging by influencing the thickness of dermal cells; it helps maintain dermal integrity and prevents thinning of the skin, which is associated with aging.

Figure 7. Summary of regulations of four target genes related to the reverse aging of skin

4. Discussion

In this research, isolated and purified exosome particles were quantified using NTA, with concentrations of each of the 3 samples being adjusted to $2.8x10^{10}$ particles/ml. The concentration of total RNA extracted was also quantified, with each of the samples being within the range of 39.4~43.4 ng/µl, as shown in Table 1. miRNA library was then constructed, with each of the sample with an adapter index sequence of TGACCA, ACAGTG, GCCAAT, as listed in Table 2. The subsequent sequencing results and the quality of the data are identified in Table 3 and Figure 2. The average number of reads from the trimmed data of total three rhizome samples was 5,284,879,000 and the quality was measured with average of 97.3% bases greater than or equal to Q30.

miRNA target gene analysis was performed and genes were selected based on their expectation values, with lower expectation values indicating higher similarity and potential interaction between miRNA and its target gene. The predicted target genes of miRNAs of each of the rhizome samples are listed in Table 5. The following target genes of IGF1R, TOP2A, CAV1, COL4A1, FGF7, and DNMT3A were consistently identified across all three rhizome samples, highlighting their significance of presence throughout the *Iris germanica* L. rhizome. Other unique target genes of significance were identified to be HMOX1 (sample no.1), VCAN, SIRT1, SOD2 (sample no.2) and VCAN, BNC2, CD44 (sample no.3). Several miRNA sequences of the target genes were shown to be part of the novel miRNA family (Novel 1,2,3,4,5,6,7,8,10,11) in the plant species. The predicted target genes of miRNA in relation to the human transcript in each of the rhizome samples are listed in Table 6. The average number of target genes from all three samples was 76 and the following target genes of SLC11A1, ZNF599, USP20, ERGIC1, YTHDC1 and 33 other more were identically identified across all three rhizome samples.

Next, target gene analysis results of the Iris-derived exosomes have highlighted the significance of novel miRNA sequences present in the exosome. The miRNA folds related to the target genes with skin efficacies of antioxidant defense, dermo-epidermal junction, ECM and skin pigmentation are represented in Figure 4a. GO (gene ontology) analysis outlining the biological functions and relationship of the HMOX1, VCAN, CD44, IGF1R and FGF7 genes is further represented in Figure 4b. The gene ratio of skin-related GO terms is shown in Figure 4c, and the relative miRNA levels of the four genes in Hs68 cells are represented in figure 4d. IGF1R (cell growth and pigmentation control), VCAN (ECM-related and anti-inflammation), CD44 (hyaluronic acid maintenance and anti-inflammation), HMOX1 (anti-oxidation) and FGF7 (keratinocyte growth factor) target genes were shown to be expressed between 0.5~1 levels.

In the in vitro study, primary HDF from 3 healthy donors in which a stable, persistent arrest of cell proliferation called cellular senescence was induced by exposure for several days to the anti-cancer drug Doxorubicin combined with insulin-like growth factor 1 (IGF1). The induction of the senescent status was assessed in these 3 individual donor cells by monitoring the β-galactosidase induction which is a hallmark of senescence, compared to the same cells kept in a medium preserving their young phenotype. The model was then used to assess the expression of 10 selected genes mRNA by RT-qPCR following the treatment of a senescence-induced pool of HDF cells from the same individual donors with several concentrations of the 3 exosomes. These genes were SOD2, FGF7, PPM1E, CAV1, COL4A, c-MYC, DNMT3A, SIRT1, FOXO3A and TNFα. In parallel, the expression of these genes was also assessed in the same pool of HDF from the same individual donors maintained in a quiescent young status. Finally, the effect of a treatment with 3 concentrations of the exosomes was visualized using the β-galactosidase staining of a senescence-induced pool of HDF from the same individual

donors. As shown in figure 5, the senescence level represented by the blue staining is shown to reduce with Iris-derived exosome concentrations of $1x10^5$, $1x10^6$ and $1x10^7$ in HDF010 (32y donor cells). Moreover SOD2 (anti-oxidation and pluripotency transcription factor), C-MYC (cell metabolism, differentiation, proliferation), CAV1 (dermis thickness), FOXO3A (anti-inflammation, cell longevity) gene expressions are shown to be regulated in an increasing/decreasing pattern under same exosome concentrations. The summary of the gene expression levels, along with SIRT1 (cell longevity) and FGF7 (cell proliferation), is outlined in figure 7. Whereas genes relatively beneficial to the human skin regeneration was upregulated with Iris-derived exosomes, CAV1, in which the over-expression may contribute to skin aging, was shown to be down-regulated.

5. Conclusion

In conclusion, the *Iris germanica* L.-derived exosomes were shown to have several novel miRNA sequences that previously have not been discovered in the plant genome. The miRNA sequences were further shown to regulate key target genes (IGF1R, TOP2A, FGF7 and HMOX1) that were selected based on their connection with cell longevity and reverse aging, according to the 12 hallmarks of aging by Lopez et al [6]. Furthermore, in vitro studies of Iris-derived exosomes have provided additional evidence through the reduction of beta-galactosidase signals after senescence induction via doxorubicin. The up and down-regulation of target genes (SOD2, C-MYC, CAV1, FOXO3A) and the consistent trends represented through qPCR gene analysis have delineated the Iris-derived exosome as potential cosmetic ingredient aiding in reversing skin aging symptoms. This research has suggested the possibility of major roles of the plant miRNA in skin regeneration, and the researchers aim to extend the preliminary results to human skin transcripts and 3D skin models for concrete evidence.

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