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Harnessing miRNAs' S Milk-Derived Exosomes for Hair Loss Disorders: *In Vitro* Modulation of WNT Signaling and Dermal Papilla Proliferation

Daniela Pinto [1,*], Giorgia Mondadori [1], Monica Cozzi [1], Piero Tesauro [2], Martin Hintersteiner [3], Raúl López Domínguez [4], Esperanza de Santiago Rodríguez [4], Giammaria Giuliani [1] and Fabio Rinaldi [1]

[1] Giuliani SpA, Milan, Italy
[2] Studio Tesauro, Milan, Italy
[3] Evobiotix, Melide, CH
[4] GENYO, Centre for Genomics and Oncological Research: University of Granada, Andalusian Regional Government, Genomics Unit, PTS Granada, Avenida de la Ilustración 114, 18016 Granada, Spain
* Correspondence: dpinto@giulianipharma.com; Tel.: +39 0220541

Abstract

Androgenetic alopecia (AGA) and telogen effluvium (TE) are common hair loss disorders characterized by dysregulated hair follicle cycling and impaired dermal papilla cell function. Emerging evidences propose exosomes as key mediators of intercellular communication, largely via their microRNA (miRNA) cargo. Milk-derived exosomes (Mi-Exos) represent an accessible and biologically active source of regulatory miRNAs with therapeutic potential. This study evaluated the *in vitro* effects of bovine milk–derived exosomes (MEV-miRNAs) on human hair follicles. MEV-miRNAs were enriched in miRNA families (Let-7, miR-21, miR-30, miR-200, and miR-148/152) previously implicated in hair follicle regulation. Proliferation of hair follicle dermal papilla (HFDP) cell was assessed, and human hair follicles were cultured *ex vivo* to measure shaft elongation, and modulation of the WNT signaling pathway by qRT-PCR. MEV-miRNAs significantly increased HFDP cell viability after 24 hours compared with controls. Human hair follicles showed a non-significant trend toward increased elongation following treatment. Gene expression analysis revealed significant up-regulation of key WNT pathway components, including *WNT2*, *WNT5B*, *WNT10A*, *WNT11*, *MMP7*, *WISP1*, and *NKD1*, indicating activation of pro-regenerative signaling. Overall, MEV-miRNAs exhibit pro-proliferative and signaling-modulatory effects, supporting their potential as a novel therapeutic strategy for AGA and TE.

Keywords: exosome; microRNA; hair; androgenetic alopecia; telogen effluvium; WNT pathway

1. Introduction

Androgenetic alopecia (AGA), and telogen effluvium (TE), represent the prevalent forms of hair loss that affect diverse populations worldwide, highlighting a significant clinical challenge in dermatology. AGA, characterized by progressive thinning of hair predominantly in genetically predisposed individuals, is influenced by hormonal and genetic factors that primarily act through the androgen receptor and intricate signaling cascades such as the Wnt/β-catenin, the TGF-β, the bone morphogenetic protein (BMP), and the Hedgehog signaling pathway[1–3]. TE is characterized by diffuse hair loss occurring when a significant number of hair follicles transition prematurely into the telogen phase, often triggered by physiological or psychological stressors, hormonal changes, or nutritional deficiencies [4–6].

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Recent advancements have highlighted the significance of extracellular vesicles, particularly exosomes, in mediating intercellular communication and influencing hair follicle biology [7,8] and above all the role of the microRNAs (miRNAs) contained in extracellular vesicles such as exosomes.

Exosomes are a type of extracellular vesicles which originate from the invagination of endosomes membranes, which are then released into the extracellular space after fusion with the plasma membrane [9]. Their main role seems to be that of intercellular communication [10]. In fact, exosomes carry a wide variety of molecules in their lumen, from protein and lipids to nucleic acids. Among these, particularly relevant are miRNAs. Exosomes containing miRNA can influence functions and behavior of recipient cells through the regulation of gene expression [9,11]. Several types of cells can release exosomes, and therefore they can be found in numerous biological fluids [12]. A biological fluid particularly rich in exosomes is milk. Milk derived exosomes (Mi-Exos) are important for certain developmental processes in the newborn, including immune competence. Such effects have been also attributed to the large number of immunomodulatory miRNAs [13] in Mi-Exos. Mi-Exos have shown protective effects against inflammatory disorders such as necrotizing enterocolitis in premature infants, but it is believed that their protective roles may also include dermatological disorders [14]. Compared to liposomes and polymeric nanoparticles, exosomes possess high circulation capacity, superior penetration, cell membrane similarity and degradation resistance.

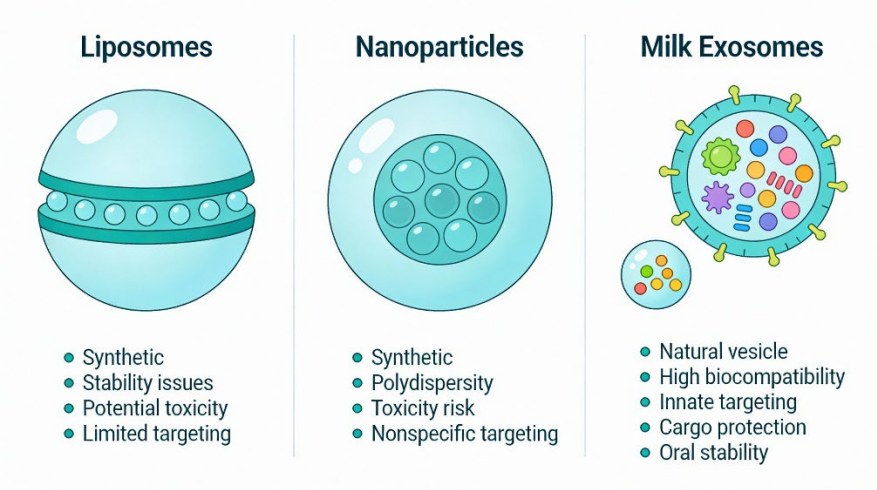

Figure 1. Milk Exosomes Versus Conventional Nanocarriers as Advanced Dermal Delivery Systems.

Among the various bioactive molecules contained within exosomes, miRNAs play a pivotal role in regulating gene expression and various cellular processes, including proliferation, differentiation, and apoptosis, all of which are critical in hair follicle dynamics [9,11,15,16]. Notably, Mi-Exos have emerged as a compelling avenue for research due to their relatively abundant composition of specific miRNAs and their demonstrated efficacy in various therapeutic contexts [2,7].

The notion that miRNAs derived from milk exosomes could potentially modulate hair follicle activity holds promise, particularly regarding alopecia. A recent study illustrated that colostrum-derived exosomes promote hair regeneration by influencing the transition from the telogen to the anagen phase, suggesting a critical role of these extracellular vesicles in hair follicle cycling [7]. Furthermore, mechanistic insights into how exosomal miRNAs can activate pathways such as Wnt/β-catenin have led to speculation regarding their therapeutic potential in treating AGA and related hair loss disorders [8,17].

Given this background, the present study aims to elucidate the *in vitro* effects of miRNAs derived from milk exosomes on hair loss disorders, specifically targeting AGA and TE. This work aimed at providing foundational data and is poised to pave the way for future translational applications in dermatological therapeutics.

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2. Materials and Methods

2.1. Exosomes Isolation and miRNA Characterisation

Lyophilized powders containing Exosomes derived from bovine milk (mEV-miRNAs) were obtained from Evobiotix SA, Switzerland and produced according to patent n. WO 2023/067490 A1.

Stock solutions were obtained by resuspending the dried exosomes in water and subsequent sterile filtration (0,22 μm).

2.2. RNA Extraction and Sequencing

Total RNA was extracted from six samples of lyophilized mEV-miRNAs, 15 mg each, using the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden), following the manufacturer's instructions. RNA concentration was determined using both a NanoDrop spectrophotometer and the Qubit Broad Range (BR) assay (Thermo Fisher Scientific).

Small RNA libraries were prepared using the QIAseq miRNA UDI Library Kit (QIAGEN). For each sample, 250 ng of total RNA was used as input material. Briefly, 3′ and 5′ adapters were sequentially ligated to mature miRNAs, followed by reverse transcription with incorporation of unique molecular identifiers (UMIs). Adapter and primer dilutions were optimized as follows: the 3′ adapter was diluted 1:5, the 5′ adapter 1:2.5, and the reverse transcription primer 1:5. Library amplification was performed using 22 PCR cycles, and unique dual indexes (UDIs) were applied to enable sample multiplexing.

Final libraries were quality-controlled and sequenced on an Illumina NextSeq 500 platform using paired-end sequencing (2 × 150 bp). Sequencing was performed at an average depth of approximately 25 million reads per sample.

2.3. Bioinformatic Analysis of Small RNA-seq Data

Raw sequencing data in FASTQ format were processed using a standardized small RNA pipeline. Initial quality control was performed with FastQC to assess read quality, adapter contamination, and length distribution. Adapter sequences were trimmed using Cutadapt, and reads with low quality or incorrect length were discarded.

Unique Molecular Identifiers (UMIs) were extracted from each read to eliminate PCR duplicates and improve quantification accuracy. Reads lacking valid UMIs were removed, as indicated by the percentage of lost reads in QC metrics.

High-quality reads were aligned to the Bos taurus reference genome using Bowtie (short-read aligner optimized for small RNAs). miRNA annotation was performed against the miRBase database (version 22.1) using the GeneGlobe/QIAGEN pipeline. Reads mapping to known miRNA loci were quantified, and the percentage of miRNA-mapped reads per sample was calculated.

Counts were normalized across samples (e.g., reads per million, RPM). Stringent filters were applied to retain high-confidence miRNAs based on read counts and annotation quality.

Ranked lists of miRNAs were generated for each sample (Top 20, Top 50, Top 100). Analysis were performed at Centre for Genomics and Oncological Research (GENYO).

2.4. Hair Follicle Dermal Papilla Cells Culture and Proliferation Assay (MTT Assay)

A primary cell culture of Hair Follicle dermal papilla (HFDP) cells was obtained from PromoCell (GmbH Sickingenstr. 63/65 69126 Heidelberg Germany). It was cultivated in 25 cm^2 cell culture flasks, in HFDPC medium (PromoCell) and incubated in humified 5% CO_2 atmosphere at 37 °C.

MTT assay was carried out according to the method of [18] with slight modifications. Briefly, HFDP cells were seeded in 96-well plates, at a density of 5 × 10^4 cell per well and incubated at 37 °C, 5% of CO_2. After overnight incubation, the medium was removed from the wells, and the cells were incubated with the mEV-miRNAs diluted in HFDPC medium at about 6 x 10^9 particles/mL. Untreated cells were used as control. Cells were then incubated for 24 h at 37 °C, 5% of CO_2. After incubation

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period, the medium was removed and 100 µL of MTT reagent (0.05 mg/mL) was applied to the cells. The plate was incubated for 3 hours in the dark at 37 °C, 5% of CO_2. Afterwards, the MTT solution was aspirated and 100 µL of dimethyl sulphoxide (DMSO) were added to dissolve purple formazan product. The solution was shaken in dark for 15 minutes at room temperature. The absorbance of the solutions was read at 570 nm and 630 nm (as reference) in a microplate reader (BioTek Instruments Inc., Bad Friedrichshall, Germany). The experiment was performed in triplicate. Data were expressed as cell viability percentage, compared to the control cells, as per following formula: % cell viability/ ctrl = (Abs sample / Abs ctrl) *100.

2.5. Human Hair Follicles Cultivation and Elongation Measurement

Human hair follicles were obtained from Studio Piero Tesauro (Milan, Italy) and according to the approval by the Ethical Independent Committee for Clinical, not pharmacological investigation in Genoa (Italy) and in accordance with the ethical standards of the 1964

Declaration of Helsinki. All of the volunteers signed the informed consent. Within the day from the excision, hair follicles coming from three different donors were isolated and cultivated *in vitro* in William's E medium supplemented with L-glutamine (2 mM), insulin (2 µg/mL), hydrocortisone (2 ng/mL) and antibiotic solution (penicillin/streptomycin 100 U/mL), in 24 well plates with 400 µL of medium and incubated in humified, 5% CO_2 atmosphere at 37 °C.

After overnight incubation, the basal medium of human hair follicles was substituted with basal medium enriched with mEV-miRNAs 6 x 10^{10} particles/mL, hair follicles cultivated in basal medium were used as control. The length of each hair follicle was measured with an eyepiece with micrometer at day 0 and every 24-48 hours interval, until the end of incubation day 4. Elongation is expressed in mm.

2.6. Hair Follicles' Taqman Array Analysis of WNT Signaling Pathway

At the end of incubation period, RNA was extracted from hair follicles according to the method described by Chomczynski and Mackey [19]. Complementary DNA (cDNA) was synthesized by reverse transcriptase using commercial kit "PrimeScriptTM RT Reagent Kit (perfect Real Time)" (TakaraBioInc., Japan). cDNA was then amplified in a TaqMan™ Array 96 – Well Plate (Study Name Human WNT Signaling Pathway, Appliedbiosystems Thermo Fisher Scientific 4391524) using the TaqMan™ Fast Advanced Master Mix (Appliedbiosystems Thermo Fisher Scientific 4444557) and amplification was measured using qRT-PCR. GAPDH was used as housekeeping gene. Each biological replicate was run in duplicate, and the obtained data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [20].

2.7. Statistical Analysis

Student's t-test was used for MTT assay, elongation, and qRT-PCR analyses (GraphPad Prism v. 10.4.1, GraphPad Software Inc.). Unpaired comparison of treatment means was analyzed with Welch's correction. P-values equal to or less than 0.05, 0.01, and 0.001 were considered significant.

3. Results

3.1. Identifying the miRNA fingerprint of mEV-miRNAs

The miRNA fingerprint of the mEV-miRNAs preparations was determined by RNA-extraction, deep sequencing and bioinformatics analysis. After filtering a list of the topmost abundant miRNAs identified as consensus over all samples was generated (Table 1) . This list of core miRNAs present in all bovine milk EV samples therefore represent something like the miRNA fingerprint of bovine milk derived extracellular vesicles.

Table 1. List of top 25 most abundant miRNAs in mEV-miRNAs.

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#	*miRNA*
1	Bta.Let.7.P2b2_5p
2	Bta.Let.7.P1d_5p
3	Bta.Mir.8.P1b_3p
4	Bta.Mir.8.P2b_3p
5	Bta.Mir.15.P2a_5p
6	Bta.Mir.30.P1b_5p
7	Bta.Mir.28.P2_5p
8	Bta.Let.7.P2a3_5p
9	Bta.Let.7.P2a1_5p
10	Bta.Let.7.P2a2_5p
11	Bta.Let.7.P2c3_5p
12	Bta.Mir.26.P4_5p
13	Bta.Mir.26.P1_5p
14	Bta.Mir.320.P1b_3p
15	Bta.Let.7.P1c_5p
16	Bta.Mir.26.P2_5p
17	Bta.Mir.191_5p
18	Bta.Mir.423_5p
19	Bta.Let.7.P2b1_5p
20	Bta.Mir.30.P1a_5p
21	Bta.Mir.21_5p
22	Bta.Mir.320.P1a_3p
23	Bta.Mir.29.P2d2_3p
24	Bta.Mir.29.P2b_3p
25	Bta.Let.7.P2c1_5p

3.2. Proliferative Activity on HFDP Cells

The proliferation assay showed that cultivating HFDP cells with medium enriched with mEV-miRNAs for 24 hours significantly increased their viability (+24.27%) compared to control cells (Fig. 2). This data suggests hair growth promoting effects of mEV-miRNAs.

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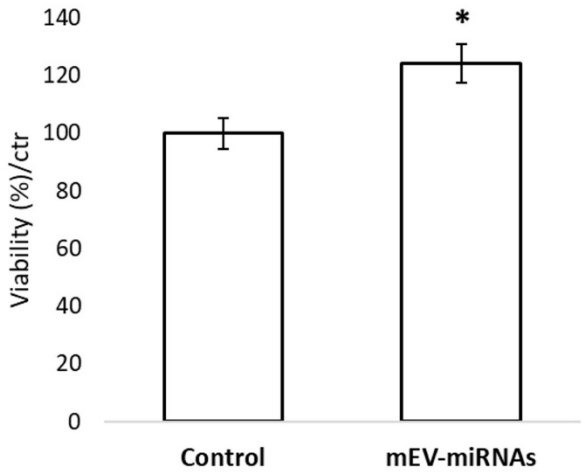

Figure 2. Percentage of viability of HFDP cells subjected to treatment with mEV-miRNAs 6 × 10^9 particles/mL for 24 hours. Columns represent mean value of three independent analyses and error bars represent SEM. Asterisks indicate statistically significant difference compared to control (*=p<0.05).

3.3. Elongation of Human Hair Follicles

Cultivating human hair follicles in medium enriched with mEV-miRNAs and measuring their elongation, further showed a tendency to growth promotion. Indeed, the experiment performed on the first two donors showed a tendency to up-regulate their shaft elongation, although data are not statistically significant (Fig.3).

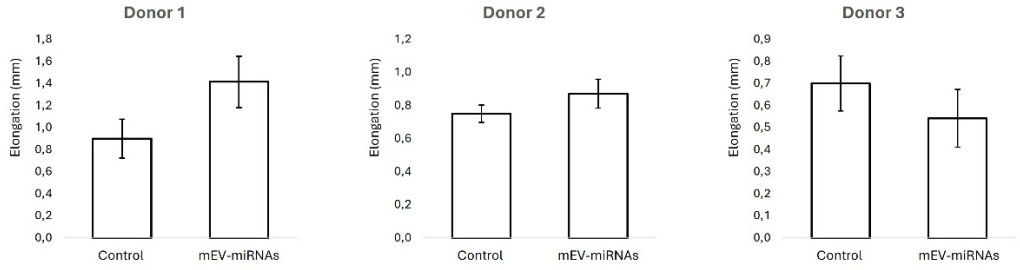

Figure 3. Elongation of human hair follicles subjected to treatment with mEV-miRNAs 6 x 10^{10} particles/mL for 4 days. Columns represent mean value of at least six independent measurement of different hair follicles and error bars represent SEM.

3.4. WNT Signaling Pathway Differentially Expressed Genes

The TaqMan™ Array Human WNT Pathway 96-well Plate (Applied Biosystems™) includes 92 assays targeting genes linked to WNT signaling and four assays for endogenous reference genes. This array focuses on genes belonging to the WNT family of proteins involved in cell-to-cell communication, key modulators of the WNT pathway, and WNT-associated genes involved in pathway regulation, downstream signaling, and development. Fig. 4 shows average value of three biological replicates of significantly regulated genes compared to control samples. Genes which were not significantly different from the control sample are not shown.

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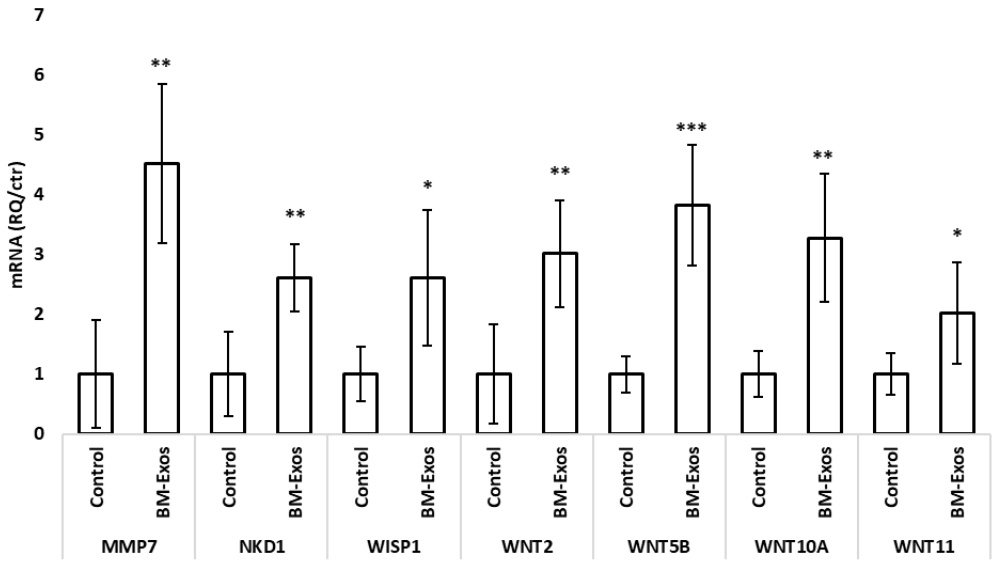

Figure 4. Gene expression analysis of WNT signaling pathway genes determined by qRT-PCR of hair follicles treated with mEV-miRNAs 6 x 10^{10} particles/mL for 4 days. Columns represent mean value of three independent samples and error bars represent SD. Asterisks indicate significant difference compared to control hair follicles (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

4. Discussion

In this study we investigated the potential of efficacy of miRNAs contained in mEV-miRNAs to influence hair growth and to treat diseases like AGA and TE. Recent studies have acknowledged that exosomes from various sources can promote hair growth by enhancing cell proliferation and regulating gene expression in hair follicle dermal papilla (HFDP) cells [1,4]. As an example, recent research has explored the potential of exosome from different sources for hair growth and treatment of hair loss. Exosomes deriving from *Leuconostoc holzapfelii* isolated from human scalp successfully increased cell proliferation, migration and regulated gene expression of HFDP cells *in vitro* [21]. Kim et al. [7] showed that exosomes derived from colostrum induced proliferation of HFDP cells and rescued dihydrotestosterone-induced arrest of follicle development *in vitro*, in addition they showed that the same exosomes stimulated dorsal hair re-growth in mice at comparable levels of minoxidil. Another study also showed that exosomes from bovine colostrum (but not exosomes from mesenchymal stem cells) were able to increase the growth of hair follicles cultivated *in vitro* [22].

Most importantly, miRNAs have found to be essential for hair follicle development in embryos and are necessary for post-natal growth, as they regulate proliferation and apoptosis [23]. For example, in hair follicle stem cells miR-205 is reported to promote stem cell expansion during early HF development. On the contrary miR-125b is considered a repressor of HF stem cell differentiation, required for anagen onset [23].

In the HF hair matrix miR-31 promotes proliferation via modulating the activity of BMP and FGF signaling pathways, as well as via changes in the expression of structural proteins (keratin 16 and keratin 17). On the contrary, miR-214 has an inhibitory effect on hair growth mediated by targeting the activity of the Wnt and Shh signaling pathways. miR-24 and miR-205 are involved in hair follicle differentiation and protection from hair follicle regression (catagen) associated apoptosis, respectively [23].

Specific miRNAs have been found crucial in hair loss disease including AGA and TE [24,25]

In this study we used exosomes deriving from bovine milk (Bos Taurus). This source for the isolation of exosomes was chosen primary due to its mammalian nature and hence conservation of essential signaling pathways, its accessibility and scalability. More specifically, the miRNAs derived

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from Bos Taurus, were verified to be orthologs of miRNAs from Homo sapiens (https://mirgenedb.org/), allowing them to influence function and behavior of human recipient cells.

The miRNA content characterization showed that the MEV-miRNAs mainly contained miRNAs from five families: Let-7 family (Bta-Let-7-P2a3_5p, Bta-Let-7-P2b1_5p, Bta-Let-7P1d_5p, and Bta-Let-7-P2c3_5p); miR-21 family (Bta-miR-21_5p); miR-30 family (Bta-miR-30-P1b_5p, Bta-miR-30-P1d_5p); miR-200 family (Bta-miR-8-P1a_3p, Bta-miR-8-P1b_3p); and miR-148/152 Family (Bta-Mir-148-P1_3p).

Previous studies have shown that some of these were highly expressed in hair follicles of Mus Musculus, such as miR-21 and many miRNAs belonging to Let-7 family [26]. miR-21 controls hair follicle growth in cashmere goats, being highly expressed in telogen phase [27] and an *in vitro* study has showed that a long non-coding RNA (lncRNA) may arrest the progression of AGA by down-regulating miRNA-21 and TGF-β1 in Hair Follicle Stem cells (HFSC) and at the same time up-regulating the WNT/β catenin pathway [28]. The role of miRNAs belonging to Let-7 family in the regulation of hair follicles growth cycle in cashmere goat has been investigated by few studies, suggesting that miR-let7a has a regulatory function in this cycle, in particular its targets IGF-1R, C-myc, and FGF5 proteins [29]. miR-let-7b has been studied in alpaca instead, where it seems to negatively regulate TGFβR I, which in turn inhibits the elongation of hair in the dorsal region of alpaca [30]. Another study from Liu et al. [31], have shown that miR-let-7b regulates the growth of alpaca through a downregulation of ectodysplasin A. Some miRNA belonging to miR30 family have shown correlations to hair pathologies, for instance miR-30b/d was one of the most significantly associated gene to alopecia areata (AA), since its expression is significantly reduced in hair follicles of AA patients [32].

The results from the present study indicate that the miRNAs contained within these mEV-miRNAs significantly enhanced the proliferation of HFDP cells over 24 hours of incubation, demonstrating a marked difference compared to the untreated control. This is consistent with findings where exosomes derived from bovine colostrum were also shown to stimulate HFDP cell proliferation and mitigate the inhibiting effects of dihydrotestosterone on hair follicle development *in vitro* and *in vivo* [3,4]. Notably, while we observed a tendency towards increased elongation of human hair follicles upon treatment with mEV-miRNAs, though not statistically significant, such trends echo previous reports linking exosomal delivery of miRNAs to hair follicle growth acceleration [33].[It is important to notice that the time during which we measured hair follicles elongation was limited to four days, it is possible that longer incubation periods may allow observation of significant increased elongation.

The analysis of genes linked to WNT signaling pathway of hair follicles cultivated in the presence of mEV-miRNAs showed significantly increased expression of *WNT2*, *WNT5B*, *WNT10A*, and *WNT11*. It has been previously shown that *WNT2* and *WNT10A* are important for hair follicle growth and development, being activators of the canonical WNT pathway. *WNT2* is involved in hair follicle development process and plays a pivotal role in hair follicle morphogenesis and hair length regulation [34], while *WNT10A* plays important role in the development of ectodermal appendages, it was shown that it is switched on during early-anagen in bulge hair follicle stem cells, suggesting that it is required for regenerating the outer root sheath during the human hair cycle [35,36]. Previous studies have shown that a *WNT10A* variant was associated with short anagen hair (SAH) and patients with loss of function mutation in *WNT10A* suffer from alopecia or hypotrichosis [36,37]. *WNT5B* seems to be ligand of the non-canonical WNT pathway and often considered an antagonist to β-catenin signaling [38] although some studies have found association between *WNT5B* mRNA up-regulation and hair growth [39]. Reddy et al. [40] showed that *WNT11* is expressed in certain type of cells of the outer root sheath and dermal sheath and they suggest it as a candidate for the signals that control the movements of outer root sheath cells during anagen. Another study showed that *WNT11* was significantly up-regulated in anagen hair and down-regulated in catagen hair of cashmere goats [41].

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Another significantly up-regulated gene in hair follicles cultivated with mEV-miRNAs was MMP7, which encodes for matrilysin. This protein is a direct target of WNT signaling pathway and it seems to control the extracellular matrix modelling of hair follicles [42,43]. WNT1-inducible signaling pathway protein 1 (WISP1) is a secreted multicellular protein that activates a variety of downstream signaling of various biological processes, from cell proliferation, adhesion and differentiation to wound healing and tissue repair [44]. Finally, NKD1 was also significantly up-regulated in this study, this is considered antagonists and negative-feedback regulators of WNT pathway [45] and its increased expression may be due to the activation of the WNT signaling pathway.

5. Conclusions

In conclusion, our study provides the first foundational *in vitro* evidence supporting the use of mEV-miRNAs and their miRNA cargo as a promising therapeutic modality for various hair loss disorders, including androgenetic alopecia and telogen effluvium. The multifaceted effects observed, from promoting HFDP cell proliferation to modulating critical signaling pathways, underscore the potential of exosomal therapies in the dermatological landscape. Future investigations should focus on *in vivo* validations and the exploration of specific miRNA contributions to enhance our understanding of therapeutic potentials.

Author Contributions: Conceptualization, D.P. and F.R.; methodology, D.P., G.M., M.H., R.L.D., E.R. ; investigation, G.M., M.C., P.T. R.L.D., E.R.; resources, M.H and G.G.; data analysis and curation, D.P., G.M., M.H. R.L.D., E.R.; writing—original draft preparation, D.P., G.M. and M.H..; writing—review and editing, D.P. and F.R.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Independent Committee for Clinical, not a pharmacological, investigation in Genoa (Italy), protocol code n. 2018/3, date of approval 27 February 2018.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors have conflicts of interest to declare concerning this study. F.R. serves as a consultant for Giuliani S.P.A., and D.P., G.M. and M.C. are employed by Giuliani S.P.A. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval for the version to be published.

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