INTRODUCTION

Flap necrosis is a serious complication following reconstructive surgery for soft tissue coverage, with treatment at times requiring a return to the operating room or prolonged wound care. Inadequate neovascularization and insufficient production of pro-angiogenic mediators are likely culprits for the poor healing. Injection of adipose-derived mesenchymal stem cells (ADSCs) into skin flaps has been shown by several investigators to increase flap survival and angiogenesis in various skin flap models. Recently, extracellular vesicles (EVs) released from ADSCs as a cell-free alternative were also found to improve flap viability after ischemia–reperfusion injury. Bai et al determined that exposure of parent ADSCs to oxidative stress via a low concentration of hydrogen peroxide (H₂O₂) enhanced the angiogenic effects of the EVs, but the mechanisms by which this occurs remain unclear.

The beneficial effects of mesenchymal stem cell (MSC)-based therapies have been proposed to be mediated predominantly by paracrine activity, to include soluble factors and EVs secreted from MSCs. Extracellular vesicles (EVs) secreted from adipose-derived mesenchymal stem cells (ADSCs) (ADSC-EVs) improve flap survival after ischemia–reperfusion injury. Exposure of parent ADSCs to oxidative stress has been shown to enhance this effect, but mechanisms are unclear. We aimed to determine whether angiogenesis-promoting protein and microRNA (miRNA) content is altered in EVs after preconditioning with hydrogen peroxide (H₂O₂ ADSC-EVs) and whether H₂O₂ ADSC-EVs can increase viability of random pattern skin flaps.

Methods: EVs secreted by human ADSCs were isolated after culture in EV-depleted medium ± H₂O₂. Nanoparticle tracking analysis determined size and concentration of purified EVs. Mass spectrometry and small RNA next-generation sequencing were performed to compare proteomic and miRNA profiles. ADSC-EVs, H₂O₂ ADSC-EVs, or vehicle were injected into random pattern skin flaps of BALB/c mice (4–5 mice per group). Viable and necrotic areas were measured on day 7, and tissues underwent histologic analysis.

Results: Angiogenic and antimicrobial protein content of EVs was altered with H₂O₂ preconditioning. Functional enrichment analysis identified constitutive photomorphogenesis 9 signalosome (known to direct vascular endothelial growth factor production) as the major enriched Gene Ontology term unique to H₂O₂ ADSC-EVs. Two miRNAs were increased, and 12 (including 10 antiangiogenic miRNAs) were reduced in H₂O₂ ADSC-EVs. Enhanced viability (P < 0.05) of flaps treated with H₂O₂ ADSC-EVs compared with vehicle corresponded to increased capillary density in the H₂O₂ group (P < 0.001).

Conclusion: Altered protein and miRNA content in ADSC-EVs after H₂O₂ pretreatment likely contributes to enhanced therapeutic effects on flap survival observed in preclinical models. (Plast Reconstr Surg Glob Open 2019;7:e2588; doi: 10.1097/GOX.0000000000002588; Published online 19 December 2019.)

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composed of different types of vesicles including exosomes (40–200 nm), which are of endosomal origin, and microvesicles (150–1,000 nm), which directly bud from the cell membrane. Because current isolation methods cannot physically separate exosomes from small microvesicles, the term “EVs” in this manuscript refers to vesicles in the size range of exosomes. MSCs have been shown to release EVs that can selectively package and transfer proteins, nucleic acids including microRNAs (miRNAs), and lipids to injured tissues which are capable of modulating gene expression and the biological activity of recipient cells. MiRNAs are an important class of highly conserved, small (~22 nucleotide), nonprotein-coding RNAs that regulate gene expression via degradation or translational repression of their targeted mRNA transcripts. They are significant modulators of numerous fundamental physiological and cellular processes including angiogenesis, and their dysregulation is implicated in several diseases.

Results from several studies indicate that angiogenesis-related miRNAs and proteins are the main components in EVs to exert their pro-angiogenic function (reviewed in Bian et al). ADSC-EVs are enriched with specific protein and miRNA cargo molecules that simultaneously activate their related signal pathway to regulate the expression of angiogenic factors in endothelial cells. EV cargo can change in response to different microenvironments, for example, EVs secreted under hypoxic conditions have shown to contain increased expression of platelet-derived growth factor, epidermal-derived growth factor, fibroblast growth factor, and nuclear factor kappA signaling pathway proteins and pro-angiogenic miRNAs including miR-126, miR-130a, and miR-210.

The identity of which components of the EV proteome and miRNome responsible for the increased skin flap viability observed after H2O2 preconditioning of parent ADSCs has not been determined. Therefore, the aims of this study were to (1) use unbiased proteomics and next-generation sequencing approaches to comprehensively characterize the protein and miRNA content of EVs released from human ADSCs under normal culture conditions (control ADSC-EVs) and from ADSCs preconditioned with H2O2 (H2O2 ADSC-EVs) and (2) determine whether H2O2-induced changes in EV composition can increase viability of random pattern skin flaps in an established mouse model.

MATERIALS AND METHODS

Cell Culture

Human ADSCs (ATCC PCS-500-011; American Type Culture Collection, Manassas, VA) were expanded under standard culture conditions in a complete medium (Dulbecco’s Modified Eagle’s Medium (DMEM) High Glucose (ThermoFisher Scientific, Waltham, MA) containing 10% MSC-qualified fetal bovine serum (MilliporeSigma, Burlington, MA)/1×GlutaMAX/1×penicillin–streptomycin (ThermoFisher Scientific). These cells have been verified by ATCC to be multipotent, capable of differentiating into adipocytes, osteoblasts, and chondrocytes and analyzed using different cluster of differentiation (CD) markers to confirm that they meet MSC criteria as defined by the International Society for Cellular Therapy. For isolation of control ADSC-EVs and H2O2 ADSC-EVs, culture medium was replaced with DMEM High Glucose without Phenol Red/1×GlutaMAX (ThermoFisher Scientific) containing 5% exosome-depleted fetal bovine serum (Exo-FBS; System Biosciences [SBI], Palo Alto, CA) ± 50 µM H2O2 when cells (at passage 5–8) reached 60%–80% confluence. Cells were cultured for an additional 65 hours, and then the medium was collected and stored at −80°C until EV isolation.

Proteomic Analysis

Proteomic analysis of control ADSC-EVs and H2O2 ADSC-EVs was performed by SBI utilizing SBI’s ExoMS Total Exosome Protein Profiling Service. Briefly, EVs were isolated using an affinity purification strategy to remove free protein. Isolated EVs were lysed in a gel-loading buffer, and the protein concentration was determined by Qubit protein assay (ThermoFisher Scientific). EV proteins (10 µg) were processed for gel-based extraction and trypsinization to generate peptide libraries for liquid chromatography/mass spectrometry matrix-assisted laser desorption/ionization-time-of-flight and electrospray ionization-time-of-flight mass spectrometry. Half of the gel digest was analyzed by nano-LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75-µm analytical column at 350 nL/min; both columns were packed with Luna C18 resin (Phenomenex, Torrance, CA). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 70,000 full width at half maximum and 17,500 full width at half maximum for MS and MS/MS, respectively. The 15 most abundant ions were selected for MS/MS.

Peptide signatures were mapped to a database of known protein sequences (SwissProt Human) using Mascot (Matrix Science, Boston, MA). Mascot DAT files were parsed into Scaffold Proteome Software (Portland, OR) for validation, filtering, and to create a nonredundant list per sample. Data were filtered using 1% protein and peptide false discovery rate and requiring at least 2 unique peptides per protein. Normalized spectral abundance factor (NSAF) values were calculated based on the equation: NSAF = (SpC/MW)/Σ(SpC/MW), where SpC is the spectral counts, MW is the protein molecular weight in kDa, and N is the total number of proteins. NSAF values were used to approximate the relative abundance of proteins within a given sample, and relative abundance of a given protein between samples. Functional Gene Ontology (GO) enrichment analysis of identified proteins was performed using the Functional Annotation Clustering Tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resource 6.8) using default settings.

RNA Sequencing Analysis

Next-generation sequencing (NGS) of control ADSC-EVs and H2O2 ADSC-EVs was performed by SBI utilizing SBI’s Exosome RNA NGS Service. Briefly, EVs were
isolated using SBI’s ExoQuick precipitation method, and RNA was extracted using the SeraMIR Exosome RNA Purification Column Kit (SBI). Measurement of small RNA concentration was determined using a Bioanalyzer 2100 instrument with the Agilent Small RNA Kit (Agilent Technologies, Santa Clara, CA). Small RNA libraries were constructed with the CleanTag Small RNA Library Preparation Kit (TriLink Biotechnologies, San Diego, CA). The final purified library was quantified with the High Sensitivity DNA Kit (Agilent Technologies). The libraries were pooled, and the 140–300-bp region was size selected on an 8% TBE (tris-borate-ethylenediaminetetraacetic acid) gel. The size-selected library was quantified with the High Sensitivity DNA 1000 Screen Tape Kit (Agilent Technologies) and the TailorMix HT1 qPCR Assay (SeqMatic, Fremont, CA), followed by a NextSeq High Output single-end sequencing run at SR75 using NextSeq 500/550 High Output v2 Kit (Illumina, San Diego, CA) at a depth of 5–15 million reads per sample. Data analysis was conducted using the Banana Slug Exosome RNA-seq Analysis platform using the human reference GRCh37/hg19 assembly (UCSC Genome Bioinformatics, Santa Cruz, CA). Differentially expressed miRNAs were identified using the DESeq package and reported as fold change >2 with significance considered at P < 0.01 (adjusted for multiple comparisons).

To determine the biological significance of differentially expressed EV miRNAs, TargetScan Release 7.2 was used to generate a list of predicted miRNA targets. A cutoff of ≤ –0.3 cumulative weighted context ++ score was used to exclude weak predictions. GO enrichment analysis of predicted target genes was performed using DAVID. A total of 14 male BALB/c mice (9–12 weeks old, Charles River Laboratories, Wilmington, MA) were used. Cranially based, dorsal 4 cm × 2 cm random pattern skin flaps beginning 1 cm caudal to the occipital neckline were raised in mice deeply anesthetized under isoflurane inhalation. To help create a reliable ischemic gradient from the flap base, axial vessels (if present) were crushed and a medical grade silicone sheet (approximately 0.13 mm thick) was inserted to separate the flap and avoid neovascularization from the bed. The flap was sutured in place with 4-0 nylon and left without a dressing. Animals then received intradermal injections (300 µL total volume) of control ADSC-EVs (3 × 10^10 particles; n = 4 mice), H_2O_2 ADSC-EVs (3 × 10^10 particles; n = 5 mice), or vehicle (n = 5 mice) at approximately 10 points throughout the proximal, middle, and distal regions of the flap. Although H_2O_2 stimulation increased the number of EVs 2.5 ± 0.4-fold (n = 3 batches), particle number was adjusted to inject equal numbers of EVs per group. Buprenorphine was administered subcutaneously (0.05 mg/kg) during surgery and orally (0.4 mg/kg mixed in 2 g/kg of hazelnut spread) every 12 hours for 2 days.

On day 7, mice were euthanized and flaps were photographed with a ruler in the field of view to enable calibration. Flap survival was determined grossly based on color, texture, and overall appearance. Sizes of viable and necrotic areas were measured using digital image analysis by 2 observers (ImageJ software, National Institutes of Health). A tissue biopsy (1 cm^2) was taken 3 mm proximal to the necrotic margin on the viable side and processed for histology.

**Histologic Analysis**

Tissues were fixed in formalin and embedded in paraffin, and serial sections (5 µm) were stained with hematoxylin–eosin. The number of capillaries per high-power field (400× magnification) were counted in a minimum of 10 fields per section by 2 blinded investigators and averaged.

**Statistical Analysis**

Data are reported as mean ± standard error of the mean. GO enrichment analysis in DAVID used a modified Fisher’s exact test to determine whether genes were enriched in the annotation categories, and enrichment statistics were adjusted for multiple hypothesis testing by the Benjamini correction. All other statistical analyses were performed using SigmaPlot 11.2 software (Systat Software, San Jose, CA) with P < 0.05 considered significant. Comparisons among groups (viability measurements and capillary density) were made using 1-way analysis of variance (ANOVA) followed by multiple comparison testing (Holm-Sidak method) to assess differences between individual pairs of means among the groups.
RESULTS

Protein Content of ADSC-EVs and H2O2 ADSC-EVs

To analyze the protein content of EVs produced by ADSCs with or without H2O2 stimulation, MS analysis of the proteome of control ADSC-EVs and H2O2 ADSC-EVs was performed. As demonstrated in the Venn diagram (Fig. 1), 68 proteins were common to both groups, 40 proteins were found only in control ADSC-EVs, and 18 proteins were found only in H2O2 ADSC-EVs. Functional GO enrichment analysis using DAVID software revealed that approximately 87% of identified proteins in both groups were associated with the GO term extracellular exosome (Benjamini P value control ADSC-EVs = 2.64 × 10^−55, H2O2 ADSC-EVs = 1.47 × 10^−46). Altogether, 40%–50% of identified proteins were classified as secreted proteins. The most highly enriched biological processes common to both groups were extracellular matrix organization and keratinocyte differentiation (Tables 1 and 2). Enriched GO biological processes unique to control ADSC-EVs included innate immune response and phagocytosis (Table 3). Interestingly, the major enriched GO term unique to H2O2 ADSC-EVs that remained significant after the adjustment for multiple hypothesis testing using the conservative Benjamini correction was constitutive photomorphogenesis 9 (COP9) signalosome, which directs the production of vascular endothelial growth factor (Table 4).

Seventeen proteins identified in both groups that were altered >2-fold in H2O2 ADSC-EVs are listed in Table 5. Twelve (71%) of these proteins are involved in angiogenesis, and 8 (47%) have antimicrobial properties. Angiogenesis-promoting proteins enriched in H2O2 ADSC-EVs included transforming growth factor-beta-induced protein ig-h3, inter-alpha-trypsin inhibitor heavy chain H2, periostin, and pentraxin-related protein PTX3.

MiRNA Content of ADSC-EVs and H2O2 ADSC-EVs

A total of 495 and 454 known miRNAs were identified in control ADSC-EVs and H2O2 ADSC-EVs, respectively.

TABLE 1. Functional Annotation Clustering Analysis of Control ADSC-EV Proteome

| Term | Count | % | P       | Benjamini P |
|------|-------|---|---------|-------------|
| GO:0005615 extracellular space | 58 | 54.7 | 6.73E-37 | 7.14E-35 |
| GO:0005576 extracellular region | 52 | 49.1 | 2.05E-26 | 1.09E-24 |
| UP_KEYWORDS secreted | 53 | 50 | 8.49E-25 | 1.96E-23 |
| UP_KEYWORDS signal | 51 | 48.1 | 2.62E-10 | 2.01E-08 |
| UP_KEYWORDS glycoprotein | 50 | 47.2 | 2.14E-09 | 8.23E-07 |
| Functional annotation cluster 2 enrichment score: 6.93 | | | | |
| GO:0018149 peptide cross-linking | 10 | 9.4 | 1.91E-11 | 5.65E-09 |
| GO:0030216 keratinocyte differentiation | 11 | 10.4 | 3.63E-11 | 8.04E-09 |
| GO:0031424 keratinization | 7 | 6.6 | 4.85E-07 | 7.16E-05 |
| GO:0008544 epidermis development | 8 | 7.5 | 9.47E-07 | 1.20E-04 |
| Functional annotation cluster 3 enrichment score: 5.55 | | | | |
| UP_KEYWORDS extracellular matrix | 13 | 12.3 | 8.11E-09 | 4.86E-07 |
| GO:0030198 extracellular matrix organization | 12 | 11.3 | 3.59E-08 | 6.35E-06 |
| GO:0005518 collagen binding | 5 | 4.7 | 4.75E-04 | 0.0083 |
| Functional annotation cluster 4 enrichment score: 5.49 | | | | |
| GO:0010951 negative regulation of endopeptidase activity | 13 | 12.3 | 1.11E-11 | 4.93E-09 |
| GO:004867 serine-type endopeptidase inhibitor activity | 9 | 8.5 | 1.25E-07 | 1.44E-05 |
| UP_KEYWORDS protease inhibitor | 9 | 8.5 | 2.37E-07 | 6.83E-06 |
| Functional annotation cluster 5 enrichment score: 4.54 | | | | |
| GO:0044267 cellular protein metabolic process | 8 | 7.5 | 8.56E-06 | 8.42E-04 |
| Functional annotation cluster 6 enrichment score: 4.4 | | | | |
| GO:0002576 platelet degranulation | 8 | 7.5 | 3.47E-06 | 3.84E-04 |
| GO:0031093 platelet alpha granule lumen | 6 | 5.7 | 1.60E-05 | 2.60E-04 |
| GO:000577 fibrinogen complex | 3 | 2.8 | 0.0011 | 0.0128 |
| Functional annotation cluster 7 enrichment score: 3.87 | | | | |
| GO:0007155 cell adhesion | 12 | 11.3 | 1.28E-04 | 0.0075 |
| Functional annotation cluster 8 enrichment score: 3.54 | | | | |
| UP_KEYWORDS antimicrobial | 7 | 6.6 | 1.92E-05 | 2.33E-04 |
| GO:0050832 defense response to fungus | 4 | 3.8 | 6.06E-04 | 0.0221 |
| GO:0041024 defense response to bacterium | 6 | 5.7 | 0.0021 | 0.0543 |
| Functional annotation cluster 9 enrichment score: 3.15 | | | | |
| GO:0016209 antioxidant activity | 5 | 4.7 | 5.65E-06 | 2.60E-04 |
| GO:00003029 response to reactive oxygen species | 5 | 4.7 | 9.67E-05 | 0.0071 |
| Functional annotation cluster 10 enrichment score: 3.04 | | | | |
| GO:0051092 positive regulation of NFkB transcription factor activity | 7 | 6.6 | 1.77E-04 | 0.0092 |
| GO:0006954 inflammatory response | 10 | 9.4 | 5.76E-04 | 0.0219 |
| UP_KEYWORDS innate immunity | 7 | 6.6 | 0.0022 | 0.0150 |

NFκB, nuclear factor kappaB.
Using a cutoff of >2-fold change and \( P < 0.01 \), 2 miRNAs were increased and 12 miRNAs (including 10 anti-angiogenic miRNAs) were reduced in \( \text{H}_2\text{O}_2 \) ADSC-EVs (Table 6). Evaluation of putative target mRNAs using TargetScan Release 7.2 identified 980 predicted gene targets of these differentially expressed miRNAs. Functional GO enrichment analysis of constructed gene sets in DAVID identified the most highly enriched biological processes as transcription from RNA polymerase II promoter and embryonic skeletal system morphogenesis (Table 7).

**Flap Survival and Capillary Density**

On postoperative day 7, survival area was significantly larger in skin flaps of mice treated with \( \text{H}_2\text{O}_2 \) ADSC-EVs compared with vehicle \(( P = 0.04, \text{ANOVA; Figs. 2 and 3})\). Increased capillary density was observed in the \( \text{H}_2\text{O}_2 \) ADSC-EVs.
ADSC-EV group compared to that in the other groups (\(P < 0.001\), ANOVA; Figs. 4 and 5). Increased vessel diameter was also apparent in tissues treated with \(\text{H}_2\text{O}_2\) ADSC-EVs (Fig. 5).

**DISCUSSION**

The use of stem cell-derived EVs has gained much attention as an emerging approach for therapeutic angiogenesis in ischemic diseases.\(^{14}\) In fact, EVs are currently being tested in clinical trials in patients with acute ischemic stroke, type 1 diabetes mellitus, and macular holes.\(^{6}\) Recently, Bai et al determined that preconditioning ADSCs with a low concentration of \(\text{H}_2\text{O}_2\) improved the pro-angiogenic properties of secreted EVs in vitro and in vivo in a model of skin flap transplantation, suggesting that \(\text{H}_2\text{O}_2\) may induce EVs to exhibit an enhanced pro-angiogenic capacity for cell-free therapeutic applications.\(^{7}\) In this study, comprehensive proteomic and miRNA

| Term | Count | % | \(P\) | Benjamini \(P\) |
|------|-------|---|-----|----------|
| Functional annotation cluster 1 enrichment score: 1.75 | | | |
| GO:0008180 COP9 signalosome | 3 | 16.7 | 4.79E-04 | 0.0088 |
| UP_KEYWORDS nucleotide binding | 6 | 33.3 | 0.0125 | 0.2309 |
| UP_KEYWORDS ATP binding | 5 | 27.8 | 0.0243 | 0.3474 |
| Functional annotation cluster 2 enrichment score: 1.73 | | | |
| UP_KEYWORDS Ubl conjugation | 6 | 33.3 | 0.0103 | 0.3019 |
| UP_KEYWORDS isopeptide bond | 5 | 27.8 | 0.01218 | 0.2729 |
| Functional annotation cluster 3 enrichment score: 1.59 | | | |
| UP_KEYWORDS acetylation | 10 | 55.6 | 6.51E-04 | 0.0654 |
| UP_KEYWORDS methylation | 5 | 27.8 | 0.0080 | 0.3401 |
| GO:0098699 cell–cell adhesion | 6 | 33.3 | 0.0103 | 0.3019 |
| GO:0098641 cadherin binding involved in cell–cell adhesion | 3 | 16.7 | 0.0301 | 0.8627 |
| Functional annotation cluster 4 enrichment score: 1.28 | | | |
| GO:0006413 translational initiation | 3 | 16.7 | 0.0083 | 0.5560 |
| GO:0044822 poly(A) RNA binding | 5 | 27.8 | 0.0235 | 0.9050 |
| Functional annotation cluster 5 enrichment score: 1.26 | | | |
| GO:0005576 extracellular region | 8 | 44.4 | 3.64E-04 | 0.0089 |
| GO:0005615 extracellular space | 6 | 33.3 | 0.0064 | 0.0905 |
| GO:0010951 negative regulation of endopeptidase activity | 3 | 16.7 | 0.0065 | 0.7210 |

**TABLE 5. Proteins Identified in Both Control ADSC-EVs and \(\text{H}_2\text{O}_2\) ADSC-EVs that Were Altered (>2-Fold Change) in \(\text{H}_2\text{O}_2\) ADSC-EVs**

| Proteins Upregulated in \(\text{H}_2\text{O}_2\)-ADSC-EVs | Abbreviation | MW (kDa) | UniProtKB Acc. #* | Fold Change | Function | Reference |
|----------------------------|-------------|---------|----------------|-------------|----------|----------|
| Histone H4 | H4_HUMAN | 11 | P62805 | 4.35 | Major antimicrobial peptide on skin released from sebaceous glands | 31 |
| Transforming growth factor-beta-induced protein igf3 | BGF3_HUMAN | 75 | Q15582 | 3.59 | Collagen-binding extracellular matrix protein; involved in vascular remodeling; pro-angiogenic | 26 |
| Inter-alpha-trypsin inhibitor heavy chain H2 | ITIH2_HUMAN | 106 | P19823 | 2.63 | Serum protein that promotes hyaluronan-mediated angiogenesis in tissue injury | 27 |
| Filaggrin-2 | FILA2_HUMAN | 248 | Q5D862 | 2.39 | Essential for normal keratinocyte differentiation; C-terminal fragments are antimicrobial peptides | 32, 33 |
| Periostin | POSTN_HUMAN | 93 | Q15063 | 2.39 | Extracellular matrix protein that promotes angiogenesis and tissue repair | 28 |
| Thrombospondin-1 | TSP1_HUMAN | 129 | P07996 | 2.32 | Potent endogenous inhibitor of angiogenesis | 34 |
| Pentraxin-related protein PTX3 | PTX3_HUMAN | 42 | P26022 | 2.22 | Pattern recognition molecule of innate immune system; promotes angiogenesis after stroke | 29, 30 |
| Annexin A5 | ANXA5_HUMAN | 36 | P08758 | 2.09 | Inhibits tumor angiogenesis; probe for apoptosis | 35 |

| Proteins Downregulated in \(\text{H}_2\text{O}_2\)-ADSC-EVs | Abbreviation | MW (kDa) | UniProtKB Acc. # | Fold Change | Function | Reference |
|----------------------------|-------------|---------|----------------|-------------|----------|----------|
| Plakophilin-1 | PKP1_HUMAN | 83 | Q13835 | −5.56 | Structural component of desmosomes important for cell–cell adhesion | 36 |
| Vitamin D-binding protein | VDBP_HUMAN | 53 | P02774 | −2.65 | Inhibits angiogenesis; anti-endothelial activity | 37 |
| Apolipoprotein B-100 | APOB_HUMAN | 516 | P04114 | −2.65 | Contains cryptic host defense peptides | 38 |
| Lactotransferrin | TRFL_HUMAN | 78 | P02788 | −2.51 | Inhibits angiogenesis in colon tumor model; iron-binding antimicrobial protein | 39, 40 |
| Serotransferrin | TRFE_HUMAN | 77 | P02787 | −2.51 | Inhibits angiogenesis in pancreatic tumor model; iron-binding antimicrobial protein | 41, 42 |
| Alpha-fetoprotein | FETA_HUMAN | 69 | P02771 | −2.51 | Modulates expression of VEGF; pro-angiogenic | 43 |
| Fatty acid-binding protein 5 | FABP5_HUMAN | 15 | Q01469 | −2.09 | Promotes angiogenesis and activates IL6/STAT3/VEGFA pathway | 44 |
| Dermcidin | DCD_HUMAN | 11 | P81605 | −2.09 | Antimicrobial peptide | 45 |
| Hornerin | HORN_HUMAN | 282 | Q80Y3 | −2.08 | Protein in skin that contains cationic antimicrobial peptides; pro-angiogenic | 46, 47 |

*UniProtKB Acc. #, UniProt Knowledgebase Accession Number.
analyses revealed that exposure to an \( H_2O_2 \)-induced oxidative stress microenvironment altered the expression of several angiogenic and antimicrobial proteins and miRNAs in ADSC-EVs. We also verified that \( H_2O_2 \) ADSC-EVs increased microvascular density and viability of random pattern skin flaps in vivo.

Functional enrichment analysis of proteins found only in \( H_2O_2 \) ADSC-EVs identified COP9 signalosome as the primary enriched GO term unique to \( H_2O_2 \) ADSC-EVs. The COP9 signalosome is a highly conserved multimeric protein complex with kinase activity that cooperates with the ubiquitin/26S proteasome system in the regulation of stability of important cellular proteins. The COP9 signalosome has been shown to direct the production of vascular endothelial growth factor, a major mediator of angiogenesis, thereby promoting angiogenesis. Upregulation of proteins involved in this complex therefore may be a mechanism by which \( H_2O_2 \) ADSC-EVs enhance angiogenesis.

Angiogenesis is well known to be a complex process that is regulated by a variety of angiogenic stimulators and inhibitors. \( H_2O_2 \) pretreatment resulted in the upregulation and downregulation of several pro-angiogenic and antiangiogenic proteins and miRNAs (listed in Tables 5 and 6), thereby influencing this intricate balance. Angiogenesis-promoting proteins enriched in ADSC-EVs after \( H_2O_2 \) stimulation included the extracellular matrix proteins periostin and transforming growth factor-beta-induced protein ig-h3, and pentraxin-related protein PTX3 and inter-alpha-trypsin inhibitor heavy chain H2, previously shown to promote wound healing and vascular remodeling. Of the 14 differentially expressed miRNAs, 10 are considered to be antiangiogenic and all were downregulated in \( H_2O_2 \) ADSC-EVs, consequently increasing angiogenesis via release of vascular endothelial growth factor, a major mediator of angiogenesis, thereby promoting angiogenesis.
Both groups contained known pro-angiogenic miRNAs that were not differentially expressed such as abundant levels of miR-21. MiR-21 has been shown to have antibacterial, anti-inflammatory, and proliferative roles in a diabetic infected wound model and has been proposed as a promising intervention target for the treatment of refractory wounds such as diabetic foot ulcers.

Results from this study also suggest that ADSC-EVs may benefit wound healing and infection prevention of skin flaps through their antimicrobial peptide content. Twenty-four antimicrobial peptides and proteins important in innate immunity were identified in ADSC-EVs, including dermcidin, lipocalin-1, lysozyme C, neutrophil defensin 1, prolactin-inducible protein, psoriasin (S100A7), calprotectin (S100A8/A9), histone H4, lactotransferrin, and hornerin, to name a few. These peptides are known to have activities against various Gram-positive bacteria.

Fig. 3. Representative images of skin flaps injected with vehicle (A), control ADSC-EVs (B), and H2O2 ADSC-EVs (C) (elevated to display the underside) on postoperative day 7. Note the increased area of viable tissue within the flap treated with H2O2 ADSC-EVs (C).

Fig. 4. Quantitative analysis of capillary density. *P < 0.05 vs vehicle and control ADSC-EVs.

Fig. 5. Representative hematoxylin–eosin-stained sections of skin flaps injected with vehicle (A), control ADSC-EVs (B), and H2O2 ADSC-EVs (C). Top panels are magnified views of corresponding boxes in bottom panels. Scale bar = 50 µm. Blood vessels are denoted by *.
and Gram-negative bacteria, viruses, and fungi, and likely are responsible for the antimicrobial properties of MSCs observed by us and others. Some of these peptides have been identified in sweat EVs, suggesting a role in skin immunity. Although it has been postulated that MSC-EVs contain active agents with potential antimicrobial effects, a paucity of data exists regarding the antimicrobial peptide cargo of ADSC-EVs and how it may be affected by different microenvironments. In general, antimicrobial peptides and proteins were reduced in H2O2 ADSC-EVs. However, histone H4 and filaggrin-2, whose peptides are important in antimicrobial defense of the skin, were upregulated in H2O2 ADSC-EVs. Interestingly, pentraxin-related protein PTX3, which was also upregulated in H2O2 ADSC-EVs, is an essential soluble pattern recognition molecule of the innate immune system and a key regulator of angiogenesis that exerts dual functions involving both antimicrobial resistance and tissue repair.

Although the therapeutic benefits of EVs seem highly encouraging in preclinical models, technical challenges currently exist in this exciting new field that must be resolved before EV-based therapeutics may be incorporated into clinical practice. One of the major challenges is the efficient production of EVs in a clinically applicable scale. Other obstacles include a lack of consensus (1) on the best method to isolate and purify EVs, (2) on the compositions of EVs from different sources or different culture conditions, and (3) as to how to quantitate the EVs. Nevertheless, Mendt et al in conjunction with Codika Biosciences have recently developed a process for the production of Good Manufacturing Practice-grade EVs derived from MSCs. Using a Quantum bioreactor culture system, they demonstrated successful Good Manufacturing Practice-grade production, scalability, EV stability, and consistent in vitro and in vivo efficacy in preclinical models of pancreatic cancer. In addition, EVs can be lyophilized and stored at 4°C without compromising stability for 36 months and are easily reconstituted, thereby demonstrating great promise as an “off-the-shelf” therapeutic.

CONCLUSIONS

ADSC-EVs hold immense potential as an allogeneic, “off-the-shelf” cell-free therapeutic for skin flap transplantation offering the benefits of stem cell therapy while representing a theoretically safer alternative. The broad repertoire of angiogenic and antimicrobial proteins and miRNAs in ADSC-EVs, which are altered after H2O2 preconditioning, likely contributes to their favorable therapeutic effects observed on skin flap survival. Further investigation is needed to determine the relative importance of particular miRNAs and proteins and their affected signaling pathways in flap recovery.

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