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MicroRNA and mRNA cargo of extracellular vesicles from porcine adipose tissue-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) are clinically useful for cell-based therapy, but concerns regarding their ability to replicate limit their human application. MSCs release extracellular vesicles (EVs) that mediate at least in part the paracrine effects of the parental cells. To understand the molecular basis of their biological properties, we characterized the RNA cargo of EVs from porcine adipose-tissue derived MSCs. Comprehensive characterization of mRNA and miRNA gene expression using high-throughput RNA sequencing (RNA-seq) revealed that EVs are selectively enriched for distinct classes of RNAs. For example, EVs preferentially express mRNA for transcription factors (e.g. MDFIC, POU3F1, NRIP1) and genes involved in angiogenesis (e.g. HGF, HES1, TCF4) and adipogenesis (e.g. CEBPA, KLF7). EVs also express Golgi apparatus genes (ARRB1, GOLGA4) and genes involved in TGF-β signaling. In contrast, mitochondrial, calcium signaling, and cytoskeleton genes are selectively excluded from EVs, possibly because these genes remain sequestered in organelles or intracellular compartments. RNA-seq generated reads for at least 386 annotated miRNAs, but only miR148a, miR532-5p, miR378, and let-7f were enriched in EVs compared to MSCs. Gene ontology analysis indicates that these miRNAs target transcription factors and genes that participate in several cellular pathways, including angiogenesis, cellular transport, apoptosis, and proteolysis. Our data suggest that EVs transport gene regulatory information to modulate angiogenesis, adipogenesis, and other cell pathways in recipient cells. These observations may contribute to development of regenerative strategies using EVs to overcome potential complications of cell-based therapy.

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1. Introduction

Mesenchymal stromal/stem cells (MSCs) are undifferentiated non-embryonic stromal cells with multi-lineage potential reflecting their stem cell-like properties. Their ability to differentiate into a broad spectrum of mesenchymal cell lineages and their immunomodulatory properties offer therapeutic avenues for both tissue repair and regeneration (Charbord, 2010; Jiang et al., 2002). Importantly, MSCs can be isolated from a variety of tissues, including the stromal vascular fraction of subcutaneous adipose tissue, which is easily accessible and often abundantly available.

Considerable experimental evidence shows that delivery of MSCs can lead to structural and functional improvement of many organs and tissues (Lee et al., 2014). In line with these observations, we have previously shown in porcine renovascular disease that adipose tissue-derived MSCs improved stenotic kidney function and structure after renal revascularization (Ebrahimi et al., 2013; Eirin et al., 2012) and improved function in the non-revascularized stenotic-kidney (Zhu et al., 2013). Furthermore, several clinical studies have shown that MSCs are

Abbreviations: MSCs, mesenchymal stromal/stem cells; EVs, extracellular vesicles; RNA-seq, RNA sequencing; FACS, fluorescence activated cell sorting; RPKM, reads per kilobasepair per million mapped reads; HGF, hepatocyte growth-factor; HES, hairy-and-enhancer of split; TCF, T-cell factor; CEBPA, CCAAT/enhancer binding protein alpha; KLF, Kruppel-like factor; ARRB, arrestin beta; IFT, intraflagellar transport; GOLGA, Golgi autoantigen, golgin subfamily A; ENG, endoglin; MRPL11, mitochondrial ribosomal protein L11; TSFM, translation elongation factor, mitochondrial; COX5A, cytochrome C oxidase subunit 5A; S100A11, S100 calcium binding protein A11; CANT1, calcium activated nucleotidase 1; RCN1, reticulocalbin 1, EF-hand calcium binding domain; ACTA2, actin, alpha 2, smooth muscle, aorta; MYO1C, myosin IC; POU, Pit-Oct-Unc; JARID2, Jumonji, AT Rich Interactive Domain-2; PRC2, Polycomb repressive complex-2; PEG3, paternally expressed-3; FZD3, frizzled-class-receptor-3; ZFP91, zinc-finger-protein-91; Gas7, growth-arrest specific gene-7; CCNG2, S/G2 cyclin, cyclin G2; LIN28B, lin-28 homolog-B; HMGA2, high-mobility-group AT-hook-2; IGF2BP1, insulin-like growth factor-2 mRNA binding protein-1.

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well tolerated and have an excellent safety record (Lalu et al., 2012; Mathiasen et al., 2009). Notwithstanding preclinical efficacy and safety in ongoing clinical trials, challenges remain in clinical applications as reports have documented that MSCs may promote tumor growth, malformation, or micro-infarctions (Kunter et al., 2007). Hence, safe and effective alternatives for their application are desired.

Recent data suggest that extracellular vesicles (EVs) released from MSCs mediate their paracrine effect by transferring proteins, lipids, and genetic material to target cells (Lai et al., 2011; Yeo et al., 2013). Furthermore, experimental studies have shown that MSC-derived EVs emulate the effect of MSCs in various experimental models, stimulating cell proliferation and repair (Bruno et al., 2012; Herrera et al., 2010). Yet, safe and effective application of this therapy requires a thorough characterization of their molecular content.

Pigs are very effective disease models in biomedical research, particularly for translating findings to humans. The porcine model mimics several characteristics of human physiology, allowing deeper insight into clinically-relevant pathogenic mechanisms and developing regenerative strategies to ameliorate disease progression (Swindle et al., 2012). In this study, we addressed the molecular basis for the therapeutic potential of porcine MSC-derived EVs. We comprehensively characterized the mRNA and miRNA expression profile of EVs derived from porcine adipose tissue-MSCs using high-throughput RNA sequencing (RNA-seq) analysis. One key finding is that EVs from porcine MSCs are selectively enriched for distinct classes of mRNAs and miRNAs compared to the MSCs that produce them. Our results provide a molecular basis for understanding the therapeutic potential of EVs derived from MSCs.

2. Methods

2.1. MSC and EV Characterization and Culture

Autologous MSCs were collected from abdominal fat (5–10 g) of 4 female domestic pigs. Adipose tissue was digested in collagenase-H for 45 min, filtered, and cultured for 3 weeks in advanced MEM medium (Gibco/Invitrogen) supplemented with 5% platelet lysate (PLTmax, Mill Creek Life Sciences, Rochester, MN) in 37°/5% CO2. The 3rd passage was collected and kept in Gibco Cell Culture Freezing Medium (Life Technologies) at −80 °C for in-vitro phenotype/function analysis. We avoided the use of any animal products (beyond porcine MSCs) in our cell culture procedures to approximate a clinical-grade tissue culture product. Cellular phenotype was examined in-vitro with immunofluorescent staining of porcine MSCs positive for CD90, CD44, and CD105, as previously described (Ebrahimi et al., 2013; Eirin et al., 2012; Zhu et al., 2013) and consistent with our experience with human MSCs (Dudakovic et al., 2014a,b).

EVs were isolated from supernatants of 10×10^6 MSCs and cultured for 48 h in advanced MEM medium without supplements. After centrifugation at 2000 g, cell-free supernatants were ultra-centrifuged at 100,000 g for 1 h at 4 °C, washed in serum-free medium containing HEPES 25 mM and submitted to a second ultracentrifugation. EVs were collected and characterized based on the expression of both microvesicle (ß1-integrins, CD73, and CD40) and exosome (CD9 and CD81) markers using fluorescence activated cell sorting (FACS) (Hulsmans and Holvoet, 2013).

2.2. RNA Sequencing & Bioinformatic Analysis

RNA sequencing and bioinformatic analysis was performed as previously described (Dudakovic et al., 2014b). The following Annotation Sources were used to establish porcine libraries: UCSC Genome Browser assembly ID: susScr3; Sequencing/Assembly provider ID: Swine Genome Sequencing Consortium Sscrofa10.2; Assembly date: Aug. 2011; GenBank Assembly ID: GCA_000003025.4; NCBI Genome information: NCBI genome/84 (*Sus scrofa*); NCBI Assembly information: NCBI assembly/304498 (Swine Genome Sequencing Consortium Sscrofa10.2); BioProject information: NCBI BioProject: 13421; Gene sets: NCBI & Ensembl.

Sequencing RNA libraries were prepared according to the manufacturer's protocol (TruSeq RNA Sample Prep Kit v2, Illumina). In brief, poly-A mRNA, purified from total RNA using oligo dT magnetic beads, was fragmented at 95 °C for 8 min, eluted from the beads and primed for first strand cDNA synthesis. RNA fragments were copied into first strand cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen), while second strand cDNA synthesis was done using DNA polymerase-I and RNase-H. A single AMPure XP bead (Agencourt) clean-up step purified the double-stranded cDNA. Then, cDNA ends were repaired and phosphorylated using Klenow, T4 polymerase, and T4 polynucleotide kinase followed by a single AMPure XP bead clean-up. Blunt-ended cDNAs were modified to include a single 3′ adenylate (A) residue using Klenow exo- (3′ to 5′ exo minus). Paired-end DNA adaptors (Illumina) with a single "T" base overhang at the 3′ end were immediately ligated to the 'A tailed' cDNA population. Unique indexes, included in the standard TruSeq Kits (12-Set A and 12-Set B) were incorporated at the adaptor ligation step for multiplex sample loading on the flow cells. The resulting constructs were purified by two consecutive AMPure XP bead clean-up steps. The adapter-modified DNA fragments were enriched by 12 cycles of PCR using primers included in the Illumina Sample Prep Kit. The concentration and size distribution of the libraries were determined on an Agilent Bioanalyzer DNA 1000 chip. A final quantification using Qubit fluorometry (Invitrogen) was performed to confirm sample concentrations.

Libraries were loaded onto flow cells at concentrations of 8–10 pM to generate cluster densities of 700,000/mm^2 following the standard protocol for the Illumina cBot and cBot Paired-end cluster kit version 3. Flow cells were sequenced as 51×2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HCS v2.0.12 data collection software. Base-calling was performed using Illumina's RTA version 1.17.21.3. The mRNA-Seq data were analyzed using the MAPRSeq v.1.2.1 system for RNA-sequencing data analysis (http://bioinformaticstools.mayo.edu/research/maprseq/), the Bioinformatics Core standard tool, which includes alignment with TopHat 2.0.6 (Kalari et al., 2014; Kim et al., 2013) and gene counts with the featureCounts software (Liao et al., 2014). The miRNA-Seq data were analyzed using CAP-miRSeq v1.1 (Sun et al., 2014). Normalization and differential expression analysis were performed using edgeR 2.6.2 (Robinson et al., 2010).

2.3. mRNA Expression Analysis

Expression values for each gene were normalized to 1 million reads and corrected for gene length (reads per kilobasepair per million mapped reads, RPKM). Genes with RPKM > 0.1, fold-change (EVs/MSCs) > 1.4 and p values < 0.05 (EVs vs. MSCs, Student's t-test) were classified as genes enriched in EVs (Dudakovic et al., 2014b). Genes with RPKM > 0.1 and fold-change (EVs/MSCs) < 0.7 were considered excluded from EVs. Functional annotation clustering analysis was performed using DAVID6.7 database (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a,b) to obtain a ranking of primary gene ontology categories for the up-regulated (enriched) and down-regulated (depleted) genes.

2.4. miRNA Expression Analysis

miRNA expression levels (normalized total reads) in EVs and MSCs, as well as the fold-change enrichment in EVs or MSCs were calculated. We used miRDB (Version 6.2) to predict target genes of miRNA with fold-change > 1.4 and p-values < 0.05 (Student's t-test), using a target prediction score ≥ 80 (Wang, 2008). Gene ontology analysis was performed using DAVID6.7.

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