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Proteomic analysis of extracellular vesicles and conditioned medium from human adipose-derived stem/stromal cells and dermal fibroblasts

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ABSTRACT

Conditioned medium (CM) and extracellular vesicles (EV) from Adipose-derived Stem/stromal cells (ASC) and Dermal fibroblasts (DF) represent promising tools for therapeutic applications. Which one should be preferred is still under debate and no direct comparison of their proteome has been reported yet. Here, we apply quantitative proteomics to explore the protein composition of CM and EV from the two cell types. Data are available via ProteomeXchange (identifier PXD020219). We identified 1977 proteins by LC-MS/MS proteomic analysis. Unsupervised clustering analysis and PCA recognized CM and EV as separate groups. We identified 68 and 201 CM and EV specific factors. CM were enriched in proteins of endoplasmic reticulum, Golgi apparatus and lysosomes, whereas EV contained a large amount of GTPases, ribosome and translation factors. The analysis of ASC and DF secretomes revealed the presence of cell type-specific proteins. ASC-CM and -EV carried factors involved in ECM organization and immunological regulation, respectively. Conversely, DF-CM and –EV were enriched in epithelium development associated factors and -EV in Wnt signaling factors. In conclusion, this analysis provides evidence of a different protein composition between CM and EV and of the presence of cell type-specific bioactive mediators suggesting their specific future use as advanced therapy medicinal products.

Significance: The use of cell secretome presents several advantages over cell therapy such as the lower risks associated to the administration step and the avoidance of any potential risk of malignant transformation. The main secretome preparations consist in concentrated conditioned medium (CM) and extracellular vesicles (EV). Both of them showed well-documented therapeutic potentials. However, it is still not clear in which case it should be better to use one preparation over the other and an exhaustive comparison between their proteome has not been performed yet. The choice of the cell source is another relevant aspect that still needs to be addressed. In order to shed light on these questions we explored the protein composition of CM and EV obtained from Adipose-derived Stem/stromal Cells (ASC) and Dermal Fibroblasts (DF), by a comprehensive quantitative proteomics approach. The analysis showed a clear distinction between CM and EV proteome. CM were enriched in proteins of endoplasmic reticulum, Golgi apparatus and lysosomes, whereas EV contained a large amount of GTPases, ribosome and translation-related factors. Furthermore, the analysis of ASC and DF secretomes revealed specific biological processes for the different cell products. ASC secretome presented factors involved in ECM organization (hyaluronan and glycosaminoglycan metabolism) and immunological regulation (e.g. macrophage and IkB/NFkB signaling regulation), respectively. On the other hand, DF-CM and –EV were both enriched in epithelium development associated factors, whilst DF-CM in proteins involved in cellular processes regulation and -EV in Wnt signaling factors. In conclusion, our study shed a light on the different protein composition of CM and EV of two promising cell types, spanning from basic processes involved in secretion to specific pathways supporting their therapeutic potential and their possible future use as advanced therapy medicinal products.

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1. Introduction

Mesenchymal Stem/Stromal Cells (MSC) are non-hematopoietic multipotent stem cells located in the perivascular area of most vessels throughout the body. According to the International Society of Cell Therapy [1], MSC should meet the following criteria: spindle-shaped morphology, adherence to plastic, positivity for the mesenchymal markers CD105, CD73 and CD90, negativity for CD45, CD34, CD14 and HLA-DR, and, under appropriate stimuli, multi-lineage differentiation towards bone, cartilage and fat.

Over the years, MSC transplantation has offered promising therapeutic opportunities for a variety of diseases lacking effective treatments [2], such as degenerative pathologies affecting the nervous system (e.g. multiple sclerosis[3]and Alzheimer's disease [4]), cardiovascular [5] and immune [6–8] conditions. The original paradigm attributes MSC healing potential to their homing, self-renewal and multi-potency properties. With the discovery that MSC in situ engraftment and differentiation play only a marginal role in the success of cell therapy [9], over the years scientific awareness has increasingly grown that MSC therapeutic efficacy relies on the secretion of biologically active factors rather than cell replacement. Accordingly, the idea of harnessing the therapeutic potential of MSC secretome as an alternative to cell therapy has become more and more popular among the scientific community.

Dermal fibroblasts (DF) are the major cell type in the human dermis. They are responsible for the synthesis and remodeling of extracellular matrix proteins and during wound healing they also modulate the functions of immune cells and other surrounding cells through paracrine mechanism [10]. Recently, in the context of cell therapy, DF have started to be considered a convenient alternative to MSC, since they possess common characteristics (i.e. positivity to the same mesenchymal markers and multi-differentiative potential towards mesodermal lineage) and are able to exert anti-inflammatory, immunomodulatory and regenerative actions [11,12]. The therapeutic potential being similar, it should be noted that DF -compared to MSC- are easier to isolate and expand in vitro. Besides their canonical role in skin regeneration and wound healing, our recent investigation also showed that DF release pro-osteogenic factors [13].

Cell-free approaches present several advantages over cell therapy. At first, the potential risks associated to the administration step are lower, as the injection of soluble factors and/or submicron particles batters down the occurrence of thrombosis and other cardiovascular events such as arrhythmia. Then, with cell-free preparations, the potential risk of malignant transformation and tumorigenesis is almost excluded. Moreover, unlike cell-based therapeutics, cell-free products do not require the otherwise mandatory steps of isolation/thawing and in vitro expansion just before administration. Indeed, cell secretome can be produced in advance and stored until use, thus shortening the times and enlightening the procedures needed for the treatment. At last, different researches demonstrated that the lyophilization/recontistitution steps do not affect the quality nor the action of the final product [14–16], with clear advantages in terms of handling and storage.

As previously demonstrated for cell-based therapy [17], several evidences suggest that cell source should be carefully considered too for secretome preparations based on the different clinical applications [13,18]. Within MSC, differences accounting for peculiar cell behaviors in vivo are recognized both among distinct harvesting sources (e.g. bone marrow MSC have a higher osteogenic and chondrogenic potential than adipose-derived ones, which in turn are more efficient in adipogenesis and show a faster growth rate [19]) but also among different subsets of the same population (e.g. $CD146^+$ MSC have a greater potency than $CD146^-$ cells in terms of cartilage protection [20]).

Moreover, we already demonstrated substantial differences between MSC isolated from adipose tissue (ASC) and dermal fibroblasts, regarding both the composition of their secretome [13,21] and their in vivo action [22].

Here, we want to go deeply in the characterization of whole secretome and extracellular vesicles (EV) derived from ASC and DF. We chose to analyze the whole CM, still retaining the vesicular fraction, rather than EV-depleted one, as it represents a more common secretome formula, whose therapeutic potential has been demonstrated in several preclinical models [23]. Furthermore, this cell-free product is currently in phase I and II clinical trials for diverse applications [16]. With this aim, we apply a comprehensive quantitative proteomics approach to explore the protein composition of whole secretome and EV obtained from the two cell systems. This effort is greatly facilitated by the significant technological advances in the field of proteomics during the last two decades. However, despite this potential, the knowledge of the secreted proteins in mediating MSC function is still very limited. LC-MS/MS is currently the preferred approach, since it allows a huge range of molecules to be covered, the most intriguing aspect being to discover new secreted factors that are therapeutically relevant [24].

2. Materials and methods

2.1. ASC and DF isolation and maintenance

Primary cell cultures were obtained from waste tissues deriving from aesthetic or prosthetic surgery performed at IRCCS Istituto Ortopedico Galeazzi, according to the procedure PQ 7.5.125, version 4. Written informed consent was obtained from all donors. In order to avoid gender-related variability, only female donors were selected for this study. Following well-established isolation procedures [13], 3 ASC (mean donor age:50 ± 21 y/o) and 3 DF (mean donor age: 39 ± 12 y/o) populations were obtained. In one case both cell types were successfully harvested from the same donor, a 26 y/o female who underwent abdominoplasty. Briefly, ASC were isolated from adipose tissue samples following digestion with 0.75 mg/ml type I collagenase (250 U/mg, Worthington Biochemical Corporation, Lakewood, NJ, USA) and filtering of the stromal vascular fraction. DF were obtained from fragmented abdominal dermis after digestion with 0.1%type I collagenase. Cells were cultured in DMEM supplemented with 10% FBS (GE Healthcare, Life Science), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Secretome collection, CM and EV preparation

ASC and DF were used at passage IV-V and IV respectively, in order to avoid phenotypic changes that may occur in long-term cultures and affect secretome composition. CM and EV were prepared following consolidated/standardized protocols [13,21,22,25,26]. Upon reaching 80%–90% confluence, cells were washed with PBS, incubated for 1 h in phenol- and serum-free medium, complete with L-glutamine and antibiotics, and cultured in the same conditions for 72 h. Cells were daily monitored and no macroscopic signs of suffering (e.g. detaching) was ever observed. Preliminary data confirm that cell metabolic activity, assessed by MTT assay (Sigma-Aldrich, St. Louis, MO, USA) through standard procedures [27], is maintained during the whole starving period (Supplementary Table S1). Moreover, nutrient deprivation does not induce senescence, as shown by β-Galactosidase staining (Cell Signaling Technology, Danvers, MA, USA) (Supplementary Fig. S1). Conditioned media were then collected and centrifuged at 2500*g* for 15 min at 4 °C to remove dead cells, large apoptotic bodies and debris. For CM production, an aliquot of the collected media was concentrated by 40–50 times through centrifugation at 4 °C, 4000 *g*, using Amicon Ultra 15 ml filters (Millipore, Burlington, MA, USA) with 3 kDa MWCO (molecular weight cut-off). In parallel, EV isolation from the remaining conditioned media was accomplished by means of ultracentrifugation at 100,000 *g* for 2 h at 4 °C. The obtained pellet was then resuspended in PBS to remove residual aggregates of macromolecules and ultracentrifuged once again under the same conditions. After the second ultracentrifugation step, EV pellets were resuspended in convenient volumes of PBS (30 μl/10^7 donor cells). In order to assess particle size

and concentration, EV samples were analyzed by NanoSight NS300 (Malvern PANalytical, Salisbury, UK). Measurements were acquired under the quality criteria of 20–120 particles/frame, concentration of 10^6-4 × 10^9 particles/ml and valid tracks > 20%. Data were analyzed by the in-build software. The protein content of CM and EV samples was assessed through the Bio-Rad Protein Assay (Bio-Rad, Milan, Italy) following standard procedures.

2.3. nLC-MS/MS, data analysis and validation by Western Blot

CM and EV samples from ASC and DF were analyzed by differential proteomics. 20 μg of total proteins from each sample, corresponding to a mean volume of 42.1 ± 15.5 μl for CM specimens and 67.3 ± 24.1 μl for EV ones, were in-solution digested using Filter Aided Sample Preparation (FASP) protocol, as reported in literature [28]. Aliquots of the samples containing tryptic peptides were desalted using StageTip C18 (Thermo Fisher Scientific, Bremen, Germany) and analyzed by nLC-MS/MS using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source (ProxeonBiosystems, Odense, Denmark) and a nUPLC Easy nLC 1000 (ProxeonBiosystems, Odense, Denmark). Peptide separations occurred on a homemade (75 μm i.d., 15 cm long) reverse phase silica capillary column, packed with 1.9-μm ReproSil-Pur 120 C18-AQ (Dr. Maisch HPLC GmbH,Ammerbuch-Entringen, Germany). A gradient of eluents A (distilled water with 0.1% v/v formic acid) and B (acetonitrile with 0.1% v/v formic acid) was used to achieve separation (300 nl/min flow rate). After 5 min at 2% of B, the acetonitrile phase was increased up to 40% B in 83 min, followed by a wash step at 90% of B. Full scan spectra were acquired with the lock-mass option, resolution set to 70,000 and mass range from m/z 300 to 2000 Da. The ten most intense doubly and triply charged ions were selected and fragmented. All MS/MS samples were analyzed using Mascot (version 2.6, Matrix Science) search engine to search the human_proteome 20190508 (95,915 sequences, 38,065,794 residues). Searches were performed with the following settings: trypsin as proteolytic enzyme; 2 missed cleavages allowed; carbamidomethylation on cysteine as fixed modification; protein N-terminus-acetylation, methionine oxidation as variable modifications; mass tolerance was set to 5 ppm and to 0.02 Da for precursor and fragment ions, respectively. To quantify proteins, the raw data were loaded into the MaxQuant [29] software version 1.6.1.0. Label-free protein quantification was based on the intensities of precursors. The experiments were performed in technical triplicates. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [30] partner repository with the dataset identifier PXD020219 and https://doi.org/10.6019/PXD020219.

To validate nLC-MS/MS data, the differential abundance of selected molecules in ASC and DF samples was confirmed by Western Blot. Briefly, 10 μg CM or EV derived from 1.5 × 10^6 cells were separated in a 4%–15% polyacrylamide gel (Bio-Rad, Milan, Italy), transferred to a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) and probed for the expression of ENPP1 (#2061, Cell Signaling Technology, Danvers, MA, USA), LGALS3BP and CTHRC1 (ab67353 and ab256458 respectively, Abcam, Cambridge, UK). After the incubation with appropriate peroxidase-conjugated secondary antibodies (sc-2004, Santa Cruz Biotechnology, Dallas, TX, USA and 62-6520, Thermo Fisher Scientific, Waltham, MA, USA), bands were revealed using ECL (Cyanagen, Bologna, Italy) and images were acquired by ChemiDoc imaging system (Bio-Rad, Milan, Italy). Stain free detection was used as loading control and normalization of signal intensity to total protein loading was performed by Image Lab Software (Bio-Rad, Milan, Italy).

2.4. Data analysis and statistics

The lists of quantified proteins deriving from proteomics were manually checked prior to all subsequent analyses (PCA, clustering and statistics). In details, we filtered targets with valid values in 2 out of the 3 samples in at least 1 group. PCA and clustering were performed by Clustvis (https://biit.cs.ut.ee/clustvis) [31]. Statistical analyses were performed by Prism 5 (GraphPad Software, La Jolla, CA, USA) and Excel. For the comparisons between CM and EV differential *p*-values were FDR-corrected using the Benjamini-Hochberg (FDR < 0.01). Processes/pathway analysis was performed by DAVID [32] version 6.8 (https://david.ncifcrf.gov/home.jsp) (default settings). For ASC/DF comparison we applied less stringent statistical criteria (*t*-test with p value < 0.05). Processes/pathway analysis was performed by STRING [33] with default settings. Venn diagrams were obtained by Venny (http://bioinfogp.cnb.csic.es/tools/venny).

3. Results

Differential proteomics was applied to two different preparations deriving from ASC and DF that are being investigated as potential novel therapeutic tools: CM, complete with soluble factors and vesicular fraction, and EV. The first was prepared by concentrating cell conditioned medium through 3 kDa MWCO centrifuge filters. This procedure allows the retention of extracellular vesicles [25,34] and freely dissolved molecules above 3 kDa. The second type of sample consists in the well-established EV preparation by ultracentrifugation [21]. CM (accounting for both EV and soluble proteins) presented a 55-fold higher protein content than EV per number of donor cells (CM: 48.2 ± 23.3 μg per 10^6 donor cells; EV: 0.9 ± 0.4 μg per 10^6 donor cells). Here, we analyzed 20 μg of total proteins for each sample. It follows that CM samples will present a largely reduced amount of vesicular proteins compared to EV preparations. Nanoparticle Tracking Analysis showed that EV samples deriving from ASC and DF share a comparable size distribution with the 50% of events falling inside the dimensional range of 145.5 ± 16.9 and 151.8 ± 22.4 nm respectively (Supplementary Fig. S2A, B and C). Moreover, also the vesicular yield between the two cell types is similar (~2.8 × 10^8 particles/10^6 cells for ASC and 3.8 × 10^8 particles/10^6 cells for DF, Supplementary Fig. S2D).

The analysis of selected proteins by immunoblotting, performed on 4 CM and 4 EV samples, validated the nLC-MS/MS data (Supplementary Fig. S3).

3.1. CM (all samples) versus EV (all samples)

Of the 1977 factors quantified by differential proteomics, 1147 (Supplementary Table S2) were present in at least 2 samples of at least 1 group and therefore considered for the further analyses. Unsupervised hierarchical clustering analysis distinctly clustered the whole secretome from EV component alone (Fig. 1A). Principal component analysis confirmed the clear distinction between CM and EV, with more than 56% of variance explained by factors 1 and 2 (Fig. 1B). We identified 68 and 202 proteins significantly more abundant and/or exclusively quantified in CM and EV samples, respectively (Supplementary Table S3, column A and B). In order to identify enriched pathways and processes in the whole secretome and EV component alone, the lists of significantly more abundant and/or exclusive proteins in CM and EV samples were analyzed using DAVID. A number of significantly enriched pathways/processes were identified in each kind of sample (Supplementary Tables S4 and S5 for CM and EV, respectively). The analyses of specific proteins more abundant in CM identified strong enrichment of endoplasmic reticulum, Golgi apparatus and lysosome proteins (Fig. 2A), suggesting that these organelles are involved in the release of factors that are not associated to EV. Differently, EV resulted enriched in GTPase activity-related factors which probably regulate the vesicles traffic. Moreover, an EV enrichment in translation and ribosomal proteins was evident, too (Fig. 2B).

We also performed a pathway analysis on the proteins which were not significantly more abundant nor solely expressed in one of the two sample types (Supplementary Table S3, column C). These factors appeared to be linked to extracellular matrix (ECM) organization,

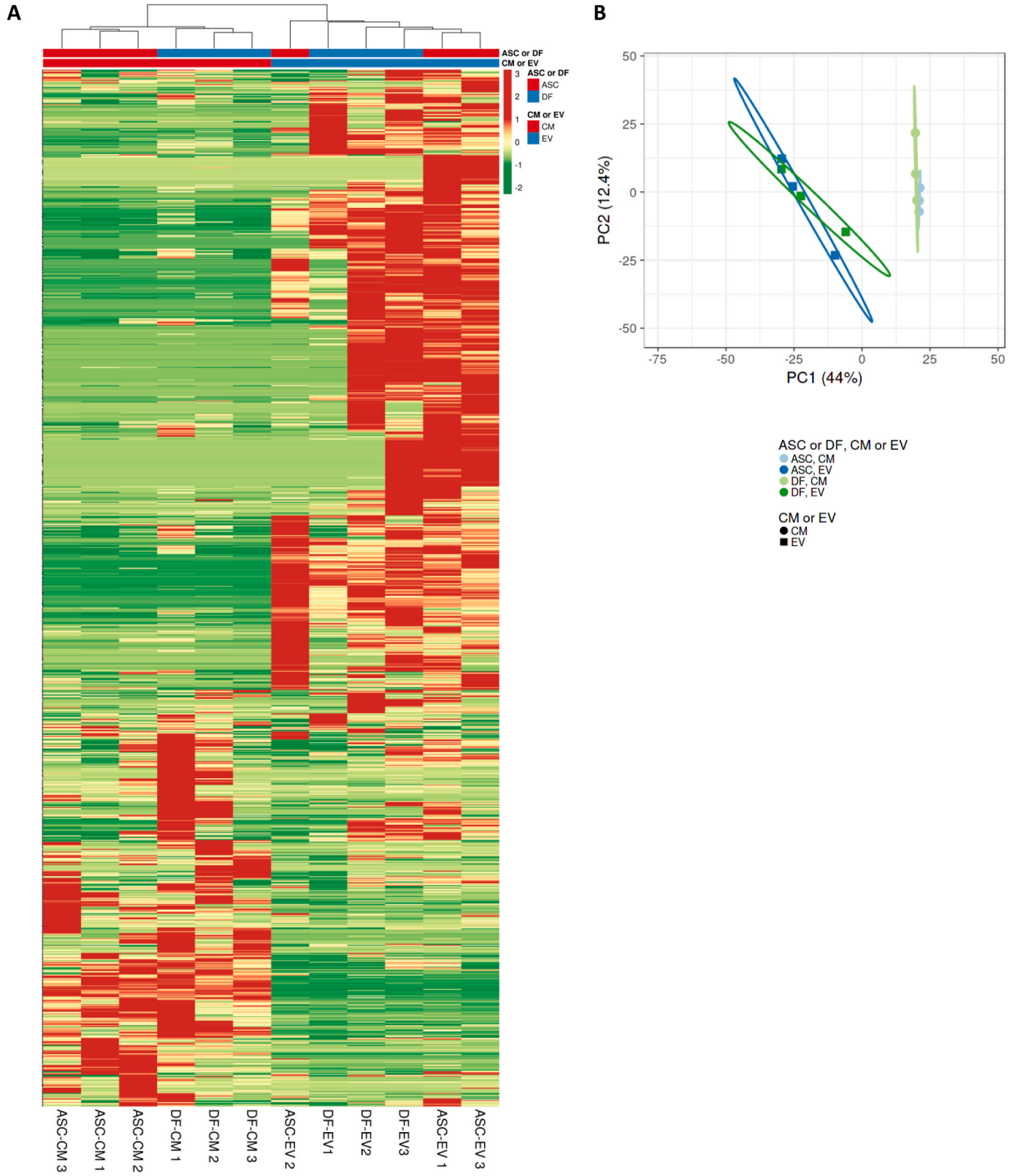

Fig. 1. (A) Unsupervised clustering analyses of CM and EV from ASC (1–3) and DF (1–3). Heatmap clearly shows the clustering of CM and EV samples in 2 groups. (B) PCA plot of the proteomic signatures of CM and EV from ASC and DF. Protein abundance levels of the 1147 identified proteins were used for both analyses.

extracellular vesicles and cell-cell or ECM adhesion (Supplementary Fig. S4, Supplementary Table S6), suggesting that the shared factors account mostly for basic/structural properties.

3.2. *ASC-CM vs ASC-EV and DF-CM vs DF-EV*

The direct comparison between CM and EV, restricted to each cell type, allowed the identification of proteins significantly more abundant

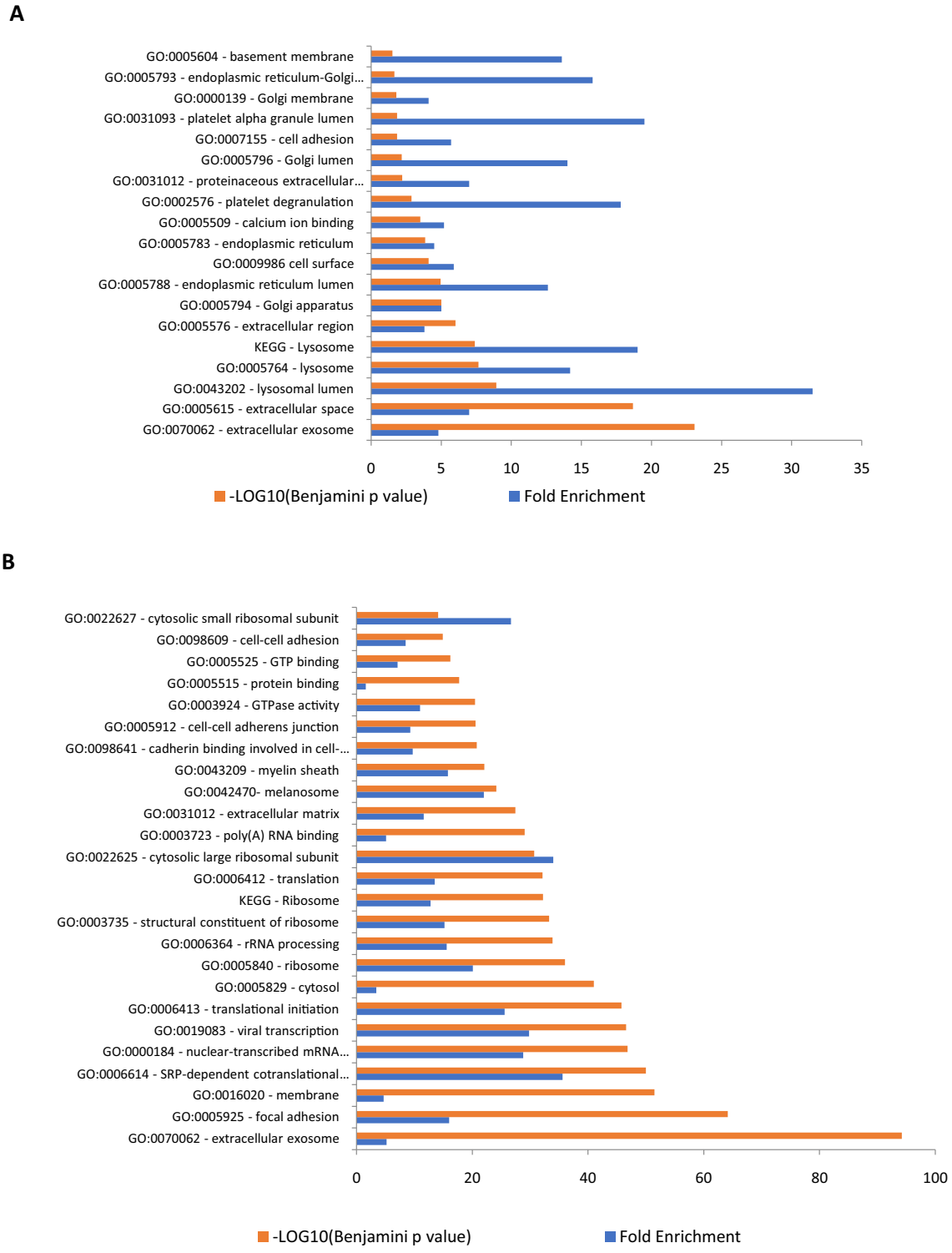

Fig. 2. David Gene Ontology analyses of proteins more abundant or exclusively identified in CM (A) and EV (B). Top 25 processes/pathways were selected on the basis of Benjamini *p* value (−Log Benjamini p value are reported as orange bars). Fold enrichment is also reported as blue bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and/or exclusively quantified in the different samples (Supplementary Table S3, column D-G). The analysis of the pathways/processes enriched in CM and EV divided per cell type confirmed the previous observation reported in Fig. 2 and Supplementary Tables S4 and S5. CM of both ASC and DF is enriched in lysosome-associated proteins (Supplementary Fig. S5A and B for ASC and DF, respectively) while EV contains translation/ribosome associated proteins (Supplementary Fig. S6A and B for ASC and DF, respectively).

3.3. *ASC (all samples) versus DF (all samples)*

While a clear-cut difference between CM and EV was evident, a

distinction based on cell source was not always obvious. From here on, we applied less stringent statistical criteria (*t*-test with p value < 0.05) comparing ASC versus DF samples. Out of 1147 identified proteins, we recognized 18 proteins that are differentially secreted (both freely dissolved and/or conveyed into vesicles) by ASC (n = 9) and DF (n = 9) (Fig. 3). The differential expression of all 18 factors was confirmed also comparing the products from ASC and DF harvested from a single donor (Supplementary Table S7). Among the proteins enriched in ASC secretome, 3 protease inhibitors (inter-alpha-trypsin inhibitors ITIH1, 2 and 3), the antiviral mediator LGALS3BP (Supplementary Fig. S3), the tenascin TNBX (recognized twice with the protein IDs A0A3B3ISX9 and A0A140TA33, reported just once in Fig. 3) and the MSC key regulator ABI3BP [35]are listed. On the other hand, DF secretion is significantly richer in the fibroblast marker TNC [36] and the matrix remodeling factors CTHRC1 [37] (Supplementary Fig. S3) and FLNA [38]. By STRING analysis (Fig. 3), the first 3 biological processes for ASC and DF specific factors are hyaluronan metabolic process, negative regulation of endopeptidase activity and extracellular matrix organization for the first cell type, while tissue development, epithelium development and morphogenesis of an epithelium for the latter. All other enriched biological processes are listed in Supplementary Table S8.

3.4. ASC-CM versus DF-CM

Out of the 1016 proteins identified by nLC-MS/MS, 821 (Supplementary Table S9) were selected since represented in at least 2 samples of 1 group. Statistical analysis and manual check for cell population exclusive factors reveal a total of 26 molecules enriched in ASC-CM (Fig. 4). Of these, 7 were already identified in the previous analysis. The remaining proteins include the negative regulator of cell proliferation RARRES1 [39],the neurotrophic factors NENF and CRLF1, the regulator of cell metabolism GSN [40], the catabolic enzymes PAPPA2 and IDUA [41], the inhibitor of blood coagulation TFPI [42] and PRELP, a collagen-binding proteoglycan required for osteoblast differentiation [43]. Conversely, 28 proteins (Fig. 4) are enriched and/or exclusively found in DF-CM, 4 of which were already depicted in the wider comparison between ASC and DF released factors. The list includes also the pro-inflammatory mediator CIRBP [44], the pleiotropic factor KITLG, the stem cell regulators DPT [45]and CNN3 [46], and the extracellular matrix components EDIL3, FBN2, COL5A1 and OLFML3. Among the biological pathways identified by STRING (Fig. 4), the top 3 for ASC-CM are glycosaminoglycan metabolic process, mucopolysaccharide metabolic process and glycosaminoglycan catabolic process, while for DF-CM cell adhesion, positive regulation of cellular process and positive regulation of biological process are listed. The remaining biological processes are shown in Supplementary Table S10.

3.5. ASC-EV versus DF-EV

For this comparison, 1372 proteins out of the 1785 identified by nLC-MS/MS (Supplementary Table S11) were considered, 38 of which resulted differentially expressed by ASC and DF (Fig. 5). Of the 15 factors significantly enriched and/or exclusively found in ASC-EV, 12 were identified here for the first time, since the above reported analyses did not indicate them as relevant proteins neither in ASC whole secretome nor in ASC-CM. Among these, IGFBP3 [47], an important MSC homing

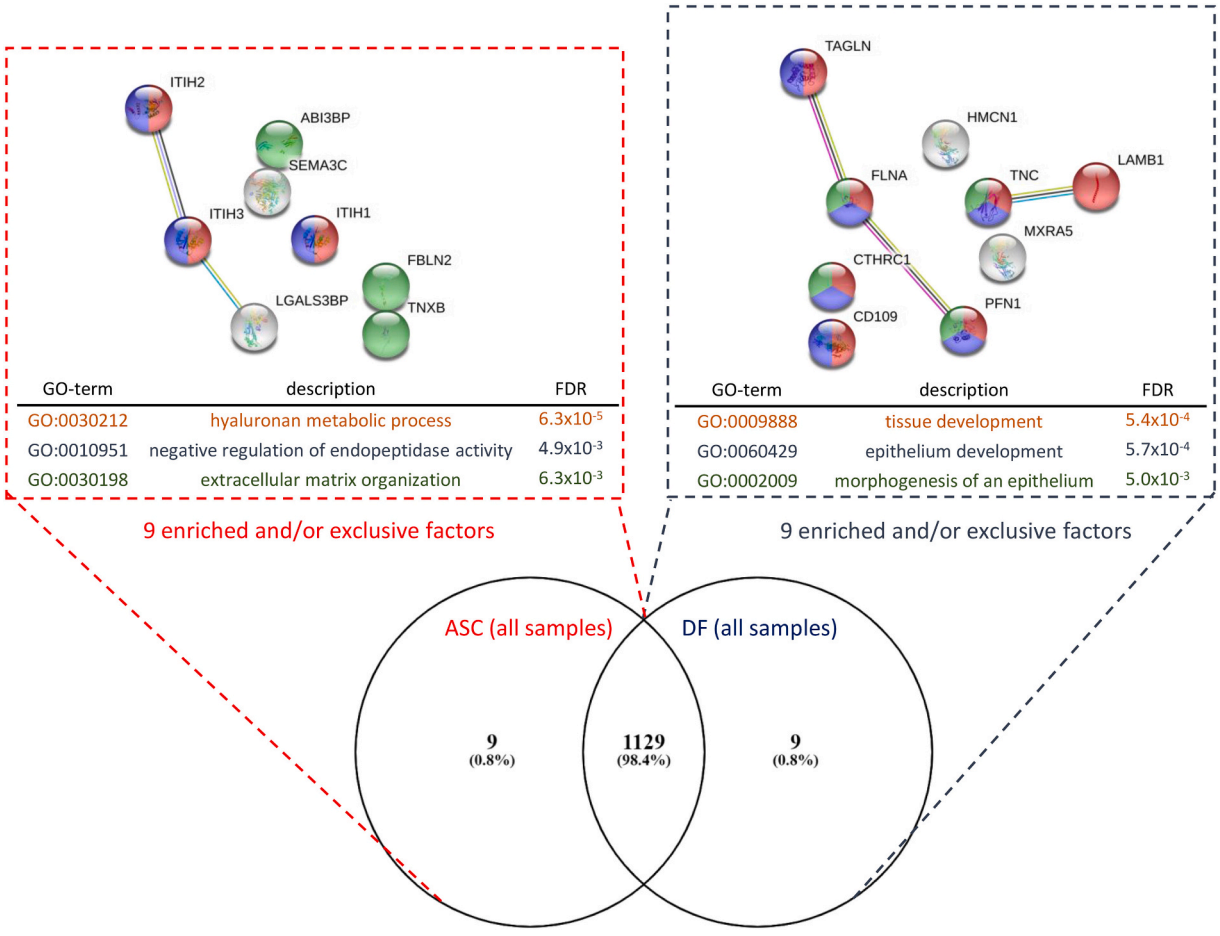

Fig. 3. STRING analysis uncovering protein-protein interactions and top 3 biological processes associated to the factors solely or preponderantly secreted by ASC (left panel) and DF (right panel) whole secretome.

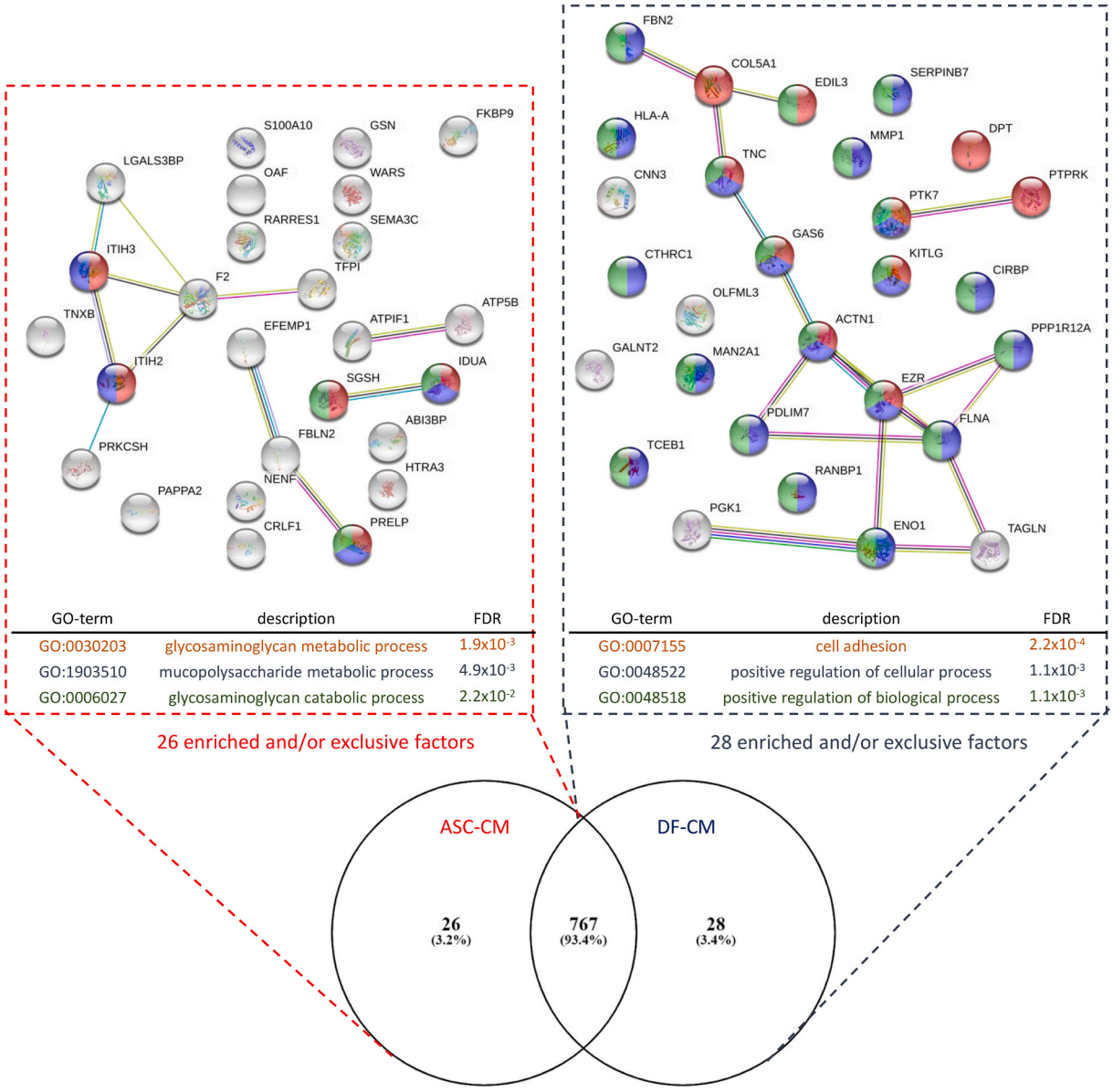

Fig. 4. STRING analysis uncovering protein-protein interactions and top 3 biological processes discriminating between ASC- and DF-CM. The analysis was based on 26 and 28 factors more abundant or exclusively identified in ASC- (left panel) and DF-CM (right panel).

molecule, MFAP5 [48], an anti-inflammatory mediator, and SFRP4 [49], a Wnt inhibitory factor, stand out. DF-EV count 23 proteins statistically overexpressed in comparison to ASC counterpart, 1 already identified in the whole secretome analysis. The others comprise exosome-related factors such as CLTC and TMED2, the regulator of fibroblast proliferation and survival WNT5A [50], the pro-osteogenic factor ENPP1 (Supplementary Fig. S3), the TGFβ controller LTBP1 [51], and MARCKSL1 [52], an important player in regeneration. STRING analysis (Fig. 5) reveals positive regulation of macrophage cytokine production, type B pancreatic cell proliferation and positive regulation of I-kappaB kinase/NF-kappaB signaling as enriched biological pathways for ASC-EV, whereas DF-EV top 3 pathways are non-canonical Wnt signaling pathway, Wnt signaling pathway, planar cell polarity pathway and receptor-mediated endocytosis. The rest of enriched biological pathways are shown in Supplementary Table S12.

4. Discussion

Due to the extreme complexity of secretome composition, a great multidisciplinary scientific effort is needed in the perspective of a future clinical translation.

In this context, the present work aims at disclosing the protein content of the two most common secretome formulas, i.e. CM and EV, from different cell sources, i.e. ASC and DF.

Here, we showed that (i) by differential proteomics, CM samples, accounting mostly for soluble elements, can be recognized from ultracentrifuge-isolated EV (ii) protein enrichment in CM and EV is not linked to cell source, (iii) CM proteome distinguished ASC from DF and (iv) biologically active factors expressed specifically by ASC or DF can be recognized by proteomic analysis.

The processes enriched in CM indicate the preferential secretory pathways for the freely-released proteins. We did not distinguish clear differences between ASC and DF, suggesting that the secretory routes are shared among the two cell types. In details, CM was enriched in endoplasmic reticulum, Golgi and lysosome proteins. As follows, the majority of CM proteins are likely released mainly via the classical secretion pathway (endoplasmic reticulum/Golgi-derived vesicles) or by lysosomial secretion, one of the non-classical secretion pathway.

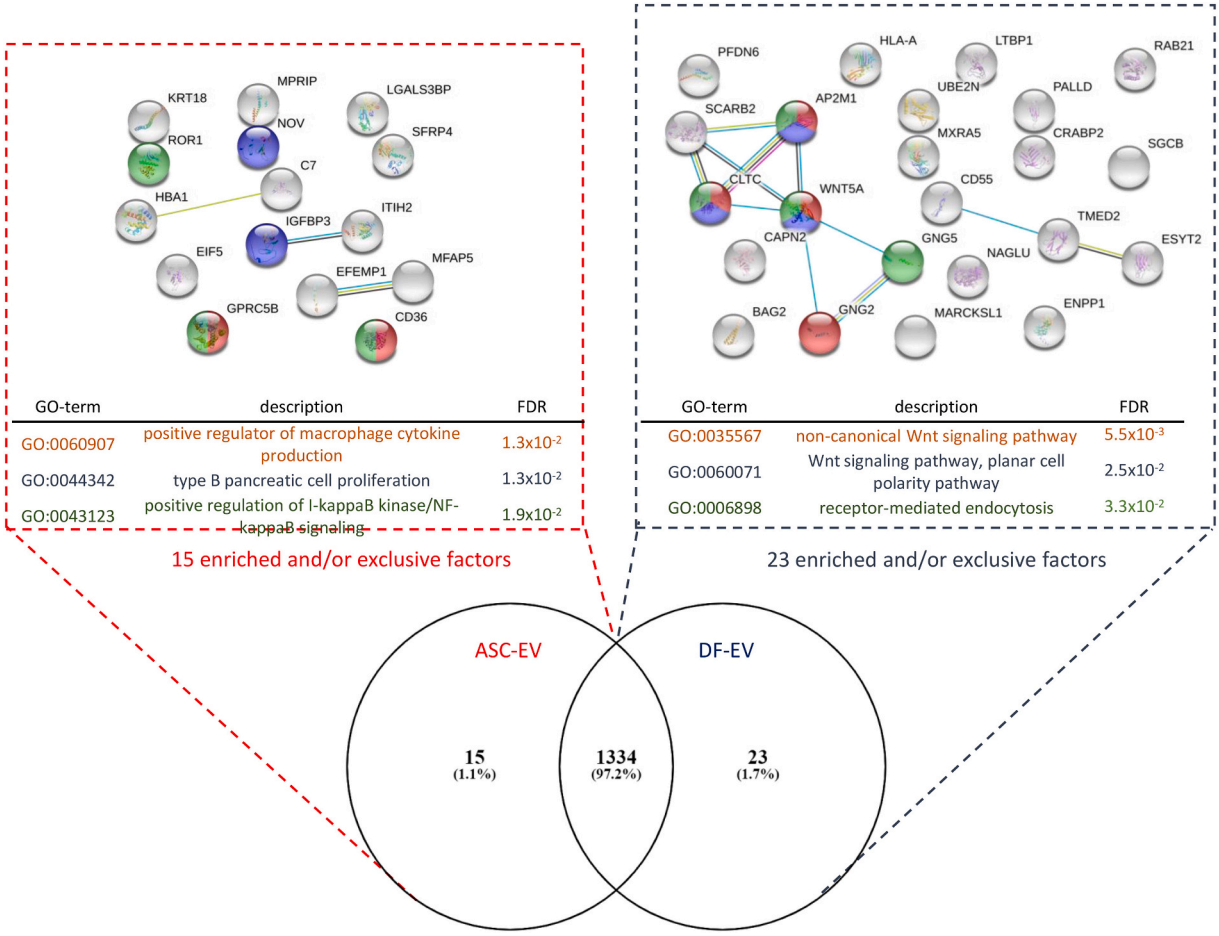

Fig. 5. STRING analysis uncovering protein-protein interactions and top 3 biological processes discriminating between ASC- and DF-EV. The analysis is based on 15 and 23 factors more abundant or exclusively identified in ASC- (left panel) and DF-EV (right panel).

As predictable, the comparison between EV and CM proteome allowed the identification of EV-related proteins. In particular we identified an enrichment in GTPase activity-related proteins such as Rab proteins. These hydrolases are known to regulate the vesicular traffic and exosome formation [53]. These factors have been largely described in exosomes and EV from a plethora of cell types and body fluids ([54], http://www.exocarta.org [55]). Besides GTPase activity-related proteins, also other 48 out of the top 100 proteins reported in Exocarta are significantly more abundant or exclusively quantified in EV compared to CM (Supplementary Table S3, column H). Our analysis also highlights the EV enrichment in proteins related to ribosomes, RNA processing and protein translation. An intriguing theory proposed by Zhu et al. [56] is that the presence of ribosomal proteins together with other translation associated factors (and RNAs) would allow EV to express proteins by their own thus directly influencing the behavior of the recipient cells.

In this study we have also analyzed the processes associated to proteins that were not significantly different in the CM and EV groups. These factors are probably both freely-released into the extracellular space or delivered through EV and resulted involved in the interaction with cells (cell-cell adhesion, integrin-binding) and extracellular matrix (collagen-binding, extracellular space/region/matrix). This observation confirms the ability of these two stromal cell types to strongly interact with the extracellular matrix (production and organization of matrix proteins) and with recipient cells.

Then, we investigated cell-source dependent differences in terms of protein content, focusing on the factors that can mediate biological effects and framing them in the context of the currently available in vitro, preclinical and clinical evidences.

The first trials based on the administration of MSC secretome have recently reached the clinic [16], even though the road towards an exhaustive characterization of this new generation cell-free therapeutic is still a long one. Among the major issues that still need to be addressed, the fine molecular investigation of all the components that constitute MSC secretome stands out. Here we give some evidences to support the potential therapeutic effects of this cell-free product based on the proteins that are enriched in comparison to DF.

Regardless of the analysis type, 2 factors were always found enriched by ASC: lectin galactoside-binding soluble 3-binding protein (LGALS3BP, Supplementary Fig. S3) and inter-alpha-trypsin inhibitor complex component II (ITIH2). LGALS3BP promotes integrin-mediated cell adhesion and stimulates host defense against viruses and tumor cells. It is a ubiquitous multifunctional secreted glycoprotein, often associated to exosomes, expressed by most tissues and detected in a variety of body fluids such as semen, saliva and urine. Originally it was studied as a marker of neoplastic transformation and cancer progression [57], but recently its role as a potent antiviral mediator in a broad range of infections, spanning from HIV to HBV and HCV, has been recognized [58]. In this perspective, MSC antiviral and antimicrobial potential has already been documented [59], and it recently gained popularity following the COVID-19 pandemia. Indeed, MSC administration as cell therapy in the management of COVID-19 pneumonia has already been considered [60,61] and even tested in several clinical trials ([62] and a total of 31 interventional studies listed in https://clinicaltrials.gov/, date of search May 11th 2020). Moreover, also the use of MSC secretome as therapeutic alternative in this context has been hypothesized [63]. ITIH2 is an endopeptidase inhibitor that acts as a binding protein

between hyaluronan and other matrix proteins, it orchestrates hyaluronan metabolic process and it is often found in exosomes. Interestingly, other 2 of the 4 heavy chains ITIH (i.e. ITIH1 and 3) were found significantly enriched comparing ASC and DF secreted factors. To the best of our knowledge, this aspect doesn't seem to entail any clear biological consequence and will require further investigations.

Among the ASC-enriched processes, extracellular matrix (ECM) organization and cartilage associated pathways emerged. In this perspective, these results completely align with what we previously described both in terms of ASC secretome content [13] and its in vitro action [25]. Indeed, we recently demonstrated that the administration of ASC-CM or -EV to chondrocytes, that were induced towards an osteoarthritis (OA)-like phenotype by the inflammatory cytokine TNFα, is able to revert a series of key inflammatory, catabolic and hypertrophic markers (e.g. PGE2 and matrix metalloproteinases) [[25] and Giannasi et al. 2020, unpublished data]. Interestingly, a clinical trial based on the administration of allogeneic Umbilical Cord MSC and/or their CM to 15 participants with grade 3 OA has recently been approved (ClinicalTrials.gov Identifier: NCT04314661).

Moreover, also the regulation of macrophage cytokine production resulted specifically associated to ASC. MSC immunoregulatory properties have been widely characterized and harnessed in the treatment of immune-associated diseases over the years [64,65]. We also demonstrated the beneficial action of ASC-CM in contrasting neuroinflammation in a mouse model of type I diabetes [22].

At last, type b pancreatic cell proliferation pathway appeared among ASC-enriched ones. In both type I and type II diabetes, beta-cell mass and function are compromised, leading to a deficit in insulin secretion and therefore hyperglycemia. In this regard, MSC/ASC administration was successfully tested in a variety of preclinical models of diabetes and several clinical trials confirmed that MSC have the potential to ameliorate diabetes mellitus [66].

In the past decades, several evidences supported the therapeutic potential of DF in terms of regeneration and immune modulation [11]. Recently, these cells started to be considered equal to MSC, as they share both phenotypic and genetic characteristics [12].

The most common therapeutic scenario for DF employment is skin graft applied to a variety of clinical conditions such as acute and chronic wounds, burns, epidermolysis bullosa and ulcers. In this context, our data reveal tissue/epithelium development and cell adhesion among the first pathways enriched in DF-derived secretome in comparison to ASC products.

Beside this straight-forward clinical application of DF, in a previous work [13], starting from proteomics data, we first hypothesized then validated in vitro a pro-osteogenic effect of DF-CM. With these new analyses, DF-EV enrichment in molecules belonging to canonical and non-canonical Wnt signaling pathway, with potential implications in osteogenesis and bone metabolism [67–69], emerged. Moreover, several single factors with pro-osteogenic and/or bone tissue remodeling activity resulted significantly more expressed by DF in the different comparisons, in details: CTHRC1 (Supplementary Fig. S3), that plays a role in osteoblast differentiation and in the coupling between bone resorption and formation [37], DPT, responsible for the induction of progenitors towards an osteoblast phenotype [45], and ENPP1 (Supplementary Fig. S3), involved in bone mineralization and soft tissue calcification [70].

5. Conclusions

In conclusion, with this work we provide evidence of a different protein composition between complete secretome and EV and of the presence in ASC and DF samples of specific bioactive mediators. It's important to underline that all analyses were performed starting from the same amount of proteins for each preparation, therefore all factors that resulted more or uniquely expressed in EV are most probably present in CM in a similar quantity per number of donor cells. Therefore, in the perspective of a clinical translation, CM represents a complete product and the easiest procedure and less manipulation account for a more feasible scale up compared to EV production.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data are available via ProteomeXchange (identifier PXD020219)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.104069.

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