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Proteomic Signature of Mesenchymal Stromal Cell-Derived Small Extracellular Vesicles

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Small extracellular vesicles (EVs) are 50–200 nm vesicles secreted by most cells. They are considered as mediators of intercellular communication, and EVs from specific cell types, in particular mesenchymal stem/stromal cells (MSCs), offer powerful therapeutic potential, and can provide a novel therapeutic strategy. They appear promising and safe (as EVs are non-self-replicating), and eventually MSC-derived EVs (MSC-EVs) may be developed to standardized, off-the-shelf allogeneic regenerative and immunomodulatory therapeutics. Promising pre-clinical data have been achieved using MSCs from different sources as EV-producing cells. Similarly, a variety EV isolation and characterization methods have been applied. Interestingly, MSC-EVs obtained from different sources and prepared with different methods show in vitro and in vivo therapeutic effects, indicating that isolated EVs share a common potential. Here, well-characterized and controlled, publicly available proteome profiles of MSC-EVs are compared to identify a common MSC-EV protein signature that might be coupled to the MSC-EVs' common therapeutic potential. This protein signature may be helpful in developing MSC-EV quality control platforms required to confirm the identity and test for the purity of potential therapeutic MSC-EVs.

1. Introduction

1.1. The Promise of Extracellular Vesicles as Therapeutic Agent

Extracellular vesicles (EVs) are nano-sized vesicles secreted by a wide variety of cells. Within the past decade, EVs have emerged as important mediators of intercellular communication, involved in the transmission of biological signals between cells to regulate a diverse range of biological processes, including a role as mediators of cellular communication in health and disease.[1,2] EVs from specific cell types, in particular mesenchymal stem/stromal cells (MSC), have positive effects on regeneration in many tissues and modulate immune responses, offering powerful therapeutic potential.[3,4] Indeed, several studies compared the therapeutic effects of MSCs and their EVs and did not discover significant differences.[5,6] Apparently, MSCs exert their regenerative and immune modulatory actions mainly though paracrine effects rather than by direct cellular mechanisms.[7,8] As EVs are not self-replicating and, due to their small size can be sterilized by filtration, MSC-EVs appear a promising novel class of therapeutics. It is considered that MSC-EVs can be evolved to a standardized, off-the-shelf product for allogeneic regenerative and immunomodulatory therapies. Preclinical data indicate that MSC-EVs obtain a huge regenerative potential in many human diseases, including kidney disease, and a first in man application of MSC-EVs underscores that MSC-EVs mediate clinical impacts, concretely, a steroid refractory Graft-versus-Host-disease patient was successfully treated with MSC-EVs.[8–12]

1.2. Current Bottlenecks in Clinical Translation of Therapeutics EVs

With the demonstration that MSC-EV-therapy is feasible and safe and the successful application of MSC-EV therapy in a case of Graft-versus-Host disease,[10] the field is now approaching the point to tackle the remaining bottlenecks to exploit the potential of (MSC-)EV-based therapies.[13] There are, however, significant bottlenecks for therapeutic translation. EVs are not classified as Advanced Therapy Medicinal Product (ATMP), in Europe they are not regulated centrally. Therefore, no internationally applicable regulatory framework exists, which regulates their production, quality assurance, and clinical application in an internationally accepted manner.[14,15] Furthermore, there is great diversity in existing protocols for MSC culture, EV isolation and purification, lack of standards in EV quantification and characterization, and lack of quality control (QC) and release criteria.

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1.3. Heterogeneity of Therapeutic MSC-EV

The great heterogeneity of the origin of MSCs, their culture conditions, the way MSC-EV-containing supernatants are harvested, enriched, and characterized forms an important hurdle between the promising pre-clinical data and an eventual broad successful clinical application in the future, as it suggests that MSC-EV products of different research groups vary tremendously from each other[16] and overall standardization has to be implemented first. Concerning MSC standardization, guidelines for minimal criteria have been provided in a position paper by the International Society for Cellular Therapy (ISCT).[17] Similarly, the International Society for Extracellular Vesicles (ISEV) has formulated more general guidelines for the isolation and characterization of EVs.[18] Along with the use of proper controls to allow broad interpretation, the above guidelines are not restrictive, and allow much room for variation, for example in the source of MSCs and the isolation and characterization of EVs.

1.4. Unique Characteristics of MSC-EV: A Protein Signature?

Despite the variability in the MSC-EV production processes and the different therapeutic areas, MSC-EV literature shows great consensus regarding the clinical effects of investigates MSC-EV samples. Hence, it can be speculated that there are common activities in all reported MSC-EV samples, which might be reflected by a common protein signature, found in all samples. We hypothesized that, in line with the surface markers defined for MSCs,[17] a specific set of proteins could be identified to define MSC-EV preparations as a unique EV entity derived from MSCs. Such a signature might be valuable to improve the characterization of MSC-EV samples and help to standardize the MSC-EV QC platforms. Being interested in translating MSC-EVs into the clinics, we explored the published proteomics datasets of MSC-EVs for such putative protein signatures. By comparison proteome profiles of EVs from other cell sources, we could indeed define a protein signature common to most published MSC-EVs samples. Furthermore, we identified proteins whose presence is related to the tissue source the MSCs were raised from. Last but not least we stress the identified signature by analyzing non-human MSC-EV samples.

Significance Statement

We compared public available protein profiles of MSC-EVs with proteome profiles of non-MSC-EV samples. Although there is a huge diversity in MSC sources and the methods of how MSC-EV were prepared, an MSC-EV specific protein signature has been identified. In addition, we identified 22 proteins which were exclusively found in profiles of BM-derived MSC-EVs but not in MSC-EV samples of non-BM-MSCs. The collection of the identified proteins should provide a helpful tool to improve the standardization of MSC-EV quality control analyses and might help to unravel potential mode of actions of corresponding EVs.

2. Experimental Section

2.1. Literature Search and Selection

To identify publically available proteomics datasets of MSC-EVs, PubMed was searched using the following search string: (msc OR (mesenchymal AND (stem OR stromal))) AND (exosom* OR microvesic* OR vesicle*) AND proteom*. This search (executed May 2018) yielded 59 matches. These hits were curated individually to identify studies including a proteomic analysis of MSC-EV, leaving 18 papers for further follow-up. From these 18 publications, datasets from ten were found suitable for inclusion in our analysis. Reasons for not including datasets and accompanying papers at this stage were the use of porcine (2x) or rat (2x) MSC, insufficient description of MSC characterization (2x), a repeated analysis of a published dataset (1x), and use of a protein-array instead of an LC-MS/MS approach (1x).

2.2. Data Analysis

Proteomic data for each selected publication were retrieved (from table or supplementary data files), IDs were converted to Gene Names and duplicates were removed. Next, all ID lists were combined in a single file and occurrences for each ID were counted. The same approach was used for the analysis of non-MSC EV datasets and proteomic datasets of MSC and other cells. Gene Ontology (GO) enrichment analysis for functional pathways was performed using GOrilla,[19] and protein–protein interactions were analyzed using STRING.[20] For the STRING analysis, the parameter "required confidence" was set to "low."

3. Results

3.1. MSC-EV Proteomics Dataset Selection

Aiming at inclusion of as many MSC-EV proteomics datasets as possible, our search string, *(msc OR (mesenchymal AND (stem OR stromal))) AND (exosom* OR microvesic* OR vesicle*) AND proteom**, was very broad, with the risk of many false positives. Indeed, manual curation of the 59 PubMed hits revealed that 41 did not contain novel proteomics data on MSC-EV, leaving 18 publications for further in-depth inspection. Only publications containing a complete LC-MS/MS based dataset on well-characterized human MSC-derived vesicles were included, and data from manuscripts describing porcine (2x) or rat (2x) MSC, with insufficient description of MSC characterization (2x), a repeated analysis of a published dataset (1x) and use of a protein-array instead of an LC-MS/MS approach (1x) were excluded.[21–26] Finally, from the initially selected 18 publications, datasets from 10 were found suitable for inclusion in our analysis.[26–39]

Between the ten selected articles and appending datasets there is diversity regarding the source, including bone marrow, embryonic stem cells, adipose tissue, and umbilical cord (**Table 1**). Also EV harvesting times, varying from 24 h to seven days, and isolation protocols vary considerably between the different groups, and include filtration, centrifugation,

Table 1. MCS characteristics. Differentiation: Black: Positive; Cross: negative; White: not analyzed. Markers: White: positive marker, not detected; Black: positive or negative marker, detected; Cross: negative marker not detected; ESC: Embryonic Stem Cells; PL: Placenta; BM: bone marrow; Umb: Umbillical cord; AT: Adipose Tissue; AD: Adipogenic; OS: Osteogenic; CH: chondrogenic.

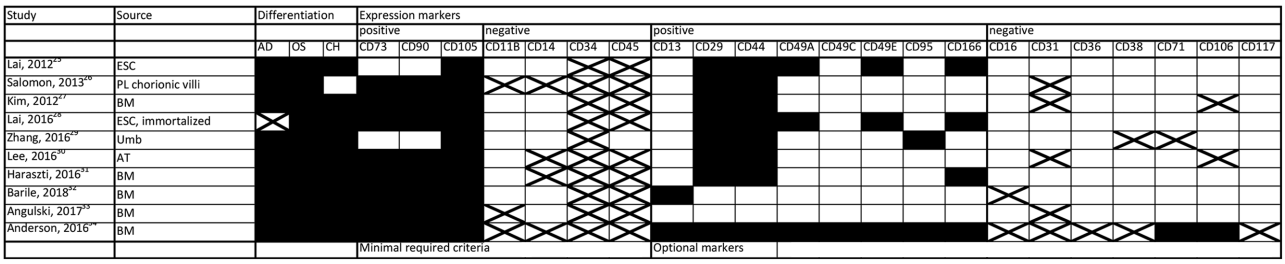

Table 2. EV isolation and characteristics ESC: Embryonic Stem Cells; TFF: Tangential Flow Filtration; HPLC: High pressure liquid chromatography; (seq) UC: (sequential) Ultracentrifugation Umb: Umbilical cord; SG:sucrose gradient; mod: modular size; NTA: Nanoparticle tracking analysis; EM: Electron microscopy; WB: Western Blot.

Study	MSC source	Harvest	Isolation	Size [nm]	NTA	EM	SG	WB	# Proteins
Lai, 2012[25]	ESC	72 h	TFF-HPLC-filter	110-130			Y	CD9, CD63, CD81, CD59	766
Salomon, 2013[26]	PL chorionic villi	48 h	UC (12K-filter-100K)	40-100		Y		CD9, CD63, CD81	394
Kim, 2012[27]	BM	24 h	100 kd filter-seq UC	50-200		Y		CD63, Hsp90, Galectin-1	662
Lai, 2016[28]	ESC, immortalized	72 h	TFF-HPLC-filter	50-150	Y			CD9, CD81, Alix, Tsg101, CD59	987
Zhang, 2016[29]	Umb	36 h	UC: 10K-100K-filter	40-100		Y		CD9, CD81	229
Lee, 2016[30]	AT	48 h	TFF-sucrose cushion 100K-optiprep	50-200		Y		CD63, Hsp90, Galectin-1	2334
Haraszti, 2016[31]	BM	72 h	UC (10k-filter-100k)	50-200	Y	Y		CD9, CD63, CD81, Tsg101	538
Barile, 2018[32]	BM	7 days	UC (filter-10k-100k)	50-200	Y	Y		CD63, Alix, Tsg101	145
Angulski, 2017[33]	BM	24 h	UC (4K-100k)	158 (mod)	Y	Y		n/a	783
Anderson, 2016[34]	BM	40 h	UC (filter-1k-17k-120k)	50-200		Y		n/a	2546

size exclusion chromatography, and combinations thereof (**Table 2**). For EV characterization, techniques include electron microscopy, nanoparticle tracking analysis, and immunoblotting for common EV marker proteins. Commonly, all reports share a characterization of MSCs in agreement the ISCT guidelines (Table 1), a detailed description of the EV isolation method and characterization of the isolated EV population used for proteomic analysis (Table 2).

3.2. Proteomic Content of MSC-EV

To obtain insight into the presence of typical EV-associated proteins and MSC-specific proteins in MSC-EV samples, published MSC-EV proteomic datasets were analyzed. For a global view of common EV-associated proteins in MSC-EV samples identified by LC-MS/MS proteomic approaches, occurrences ("present calls") for all reported protein IDs were cumulated and compared to the top-100 proteins commonly identified in EV samples according to the ExoCarta Exosomal Marker list.[40] Among the ten MSC-EV proteomic datasets, 134 proteins were detected in seven or more datasets and 60 were detected in eight or more datasets. Interestingly, NT5E (CD73) and ENG (CD105), two of the minimally required cell surface antigens for MSC[17] were detected in seven out of ten, and THY1 (CD90), in eight out of ten datasets (**Table 3**). Please note that some datasets contain relatively low amounts of reported IDs (<500), which can be explained by that fact that one datasets only reports quantified IDs,[33] and that for two other studies, the proteomics approach was limited to identifying only the relatively more abundant proteins.[27,30]

For the subsequent comparison with the "common" EV proteins, we considered the 134 proteins that were detected in at least seven datasets. As depicted in Table S1, Supporting Information, 59 proteins from the ExoCarta top-100 EV-associated proteins are represented in at least seven datasets. 75 proteins of the 134 common MSC-EV proteins, including CD73, CD105, and CD90, were not detected in the top-100 EV-associated proteins, leaving them as candidates for MSC-EV specific proteins. Notably, 26 of these proteins were detected in more than seven of the ten datasets being analyzed (Table S1, Supporting Information, shaded).

3.3. Unique Proteome Signature for MSC-EV

To obtain more insight into common, or maybe exclusive proteins in MSC-EV, 12 proteomics datasets representing EVs (compliant with Minimal Information for Studies on Extracellular Vesicles [MISEV] criteria) from a variety of non-MSC cells, including endothelial cells, epithelial cells, and cancer cells,[41–46] were grouped and analyzed in a similar manner, and proteins detected in at least nine datasets were compared with the ExoCarta top 100 EV proteins (Table S1, Supporting Information). The datasets for these proteins contained on average much more IDs than the MSC-EV datasets (1331 vs 938), and

Table 3. MSC surface markers identified in MSC-EV proteomics datasets. White: positive marker, not detected; black: positive or negative marker, detected; cross: negative marker not detected ESC: Embryonic Stem Cells; PL: Placenta; BM: bone marrow; Umb: Umbilical cord; AT: Adipose Tissue; AD: Adipogenic; OS: Osteogenic; CH: chondrogenic.

Study	Source	Expression markers																						
		positive			negative				positive								negative							
		CD73	CD90	CD105	CD11B	CD14	CD34	CD45	CD13	CD29	CD44	CD49A	CD49C	CD49E	CD95	CD166	CD16	CD31	CD36	CD38	CD71	CD106	CD117
Lai, 2012[25]	ESC	■	■	■	⨯	⨯	⨯	⨯	■	■	■	□	■	■	□	□	⨯	⨯	⨯	⨯	■	⨯	⨯
Salomon, 2013[26]	PL chorionic villi	□	■	□	⨯	⨯	⨯	⨯	□	■	■	■	□	□	□	□	⨯	⨯	⨯	⨯	⨯	⨯	⨯
Kim, 2012[27]	BM	■	■	■	⨯	⨯	⨯	⨯	■	■	■	□	■	■	□	■	⨯	⨯	⨯	⨯	■	⨯	⨯
Lai, 2016[28]	ESC, immortalized	□	■	■	⨯	⨯	⨯	⨯	■	■	□	□	□	□	□	□	⨯	⨯	⨯	⨯	⨯	⨯	⨯
Zhang, 2016[29]	Umb	□	□	□	⨯	⨯	⨯	⨯	□	□	□	□	□	□	□	□	⨯	⨯	⨯	⨯	⨯	⨯	⨯
Lee, 2016[30]	AT	■	■	■	⨯	⨯	⨯	⨯	■	□	□	■	■	□	□	■	⨯	⨯	⨯	⨯	⨯	⨯	■
Haraszti, 2016[31]	BM	■	■	■	⨯	⨯	⨯	□	■	■	□	□	■	■	■	■	⨯	⨯	■	⨯	■	⨯	⨯
Barile, 2018[32]	BM	■	□	□	⨯	⨯	⨯	⨯	■	□	■	□	□	□	□	□	⨯	⨯	⨯	⨯	⨯	⨯	⨯
Angulski, 2017[33]	BM	■	■	■	⨯	⨯	⨯	⨯	■	■	■	□	■	■	■	■	⨯	⨯	⨯	⨯	■	⨯	⨯
Anderson, 2016[34]	BM	■	■	■	⨯	⨯	⨯	□	■	■	■	■	■	■	■	■	⨯	⨯	■	⨯	■	⨯	⨯
		Minimal required criteria							Optional markers															

correspondingly, the number of proteins detected in at least nine datasets (equivalent to at least seven datasets for the MSC-EV datasets) is 358, with 132 proteins being detected in at least ten different datasets. Notably, none of the proteins were detected in all 12 datasets.

A direct comparison of the combined MSC-EV- and non-MSC-EV datasets and the ExoCarta top-100 list was performed to identify unique MSC-EV proteins. Additional stringency was added by selecting only those proteins identified in at least 8 of the 10 MSC-EV datasets, and at least in 9 of the 12 non-MSC-EV datasets. **Table 4** summarizes the proteins which were detected in at least 8 of the 10 MSC-EV datasets but not nine or more non-MSC-EV datasets. Identified MSC-EV specific proteins include THY1 (CD90), one of the commonly used MSC markers. Incidentally, THY1 was one of three surface antigens found to distinguish MSC-EVs from EVs produced by K562 leukemic cell line.[47]

Collagens I and VI are well represented in MSC-EV samples compared to other EV samples, which reflects the GO-term enrichment analysis.[19] This analysis showed "GO:0030020: *extracellular matrix structural constituent conferring tensile strength*" as the most significantly enriched functional pathway (p-value 1.26×10^{-11}) (**Figure 1**a), with most proteins closely interacting as demonstrated by the EMBL string[20] analysis (Figure 1b).

3.4. Robustness of the Signature

The protein signature identified based on selected publications has to be validated using external controls. For this, we made a comparison with additional human- and non-human (rat, pig) MSC-EV datasets that were not included in our initial analysis, allowing us to assess the robustness of the identified MSC-EV proteomic signature. Besides human MSC, also MSC, and their EVs from other species have been used for therapeutic approaches in various disease models. As such, proteomic profiles of EV from well-characterized porcine MSC[22,23] and rat MSC,[12,21] used in experimental kidney diseases and intracerebral hemorrhage, respectively, have been determined. As listed in Table S1, Supporting Information, 12 out of 15 proteins defined in the MSC-EV proteomic signature could be identified in rat adipose tissue-derived MSC-EV samples, and one proteomics analysis of porcine MSC-EVs identified all 15 MSC-EV specific proteins, with a second analysis only missing two of these 15 proteins. Also, a recently published proteomics analysis of EV from poorly characterized BM-derived MSC-EVs identified most of the signature proteins, only missing HBB.[24] With porcine models as the goal standard model for cardiovascular research,[48] it is important to note that, based on the two included porcine datasets, the MSC-EV proteome signature is highly conserved in pigs, underscoring the use of such models for translational research. Besides the identification of most hallmark proteins in all MSC-EV datasets, the presence of these proteins in non-MSC-EV samples was investigated to identify the uniqueness of these proteins for MSC-EV samples. As listed in Table 4c, most proteins could be detected in 0 (COL6A2, COL6A3), 1 (COL1A2, ACTN1, ANPEP, COL6A1, FLNB, FLNC, VAT1, VIM), or 2 (COL1A1, THY1) out of 12 of these datasets, with LRP1 (4x) and HBB (6x) as the most commonly detected proteins.

Apart from full proteomic characterization of MSC-EV samples, commercially available antibody-arrays to detect the presence of selected proteins had also been employed.[25,26] As detection of proteins is not unbiased, and depends on the selection of antibodies on the array, it may not be surprising that MSC-EV signature proteins are hardly detected in such experiments, as summarized in Table 4b.

3.5. Different MSC Sources are Reflected in MSC-EV Protein Content

It has been reported that MSC from different sources (BM, AT, UC, or hESC) display different functional properties being reflected in different molecular profiles.[49–51] Within the ten datasets used to establish the MSC-EV specific proteome signature, a sub-analysis comparing BM-derived ($n = 5$, 2868 proteins) versus non-BM-derived (UC, hESC, AT; $n = 5$, 3529) EV was performed. Between groups, 1468 proteins were detected in both groups, and 3433 proteins were detected in up to three datasets of each group. Interestingly, the BM-MSC-EV group contained 22 proteins represented in four out of the five datasets analyzed (**Figure 2**a). In contrast, in the other group not a single protein was represented in four or more of the corresponding datasets. GO-enrichment analysis (Figure 2b) reveals that BM-MSC-EV specific proteins are involved in cell adhesion (GO:0050839) and mediate integrin- and receptor-interactions (GO:0044877, GO:005102, GO:0005178).

Table 4. a: MSC-EV hallmark proteins. b: Identification of MSC-EV hallmark proteins in other MSC-EV proteomics analyses. c: MSC-EV proteins in other EV.

a		b						c	
ID	detected	Rat[20]	Pig[21]	Pig[22]	MSC char.[23]	Ab Array[24]	Ab Array[25]	ID	Detected
COL1A1	9	**Col1a1**	**COL1A1**	**COL1A1**	**COL1A1**	n/a	n/a	COL1A1	2
COL1A2	9	**Col1a2**	**COL1A2**	**COL1A2**	**COL1A2**	n/a	n/a	COL1A2	1
LRP1	9	**Lrp1**	**LRP1**	**LRP1**	**LRP1**	**LRP1**	n/a	LRP1	4
ACTN1	8	**Actn1**	*ACTN1*	**ACTN1**	**ACTN1**	n/a	n/a	ACTN1	1
ALDOC	8	*Aldoc*	**ALDOC**	**ALDOC**	**ALDOC**	**ALDOC**	n/a	ALDOC	1
ANPEP	8	**Anpep**	**ANPEP**	**ANPEP**	**ANPEP**	**ANPEP**	n/a	ANPEP	1
COL6A1	8	**Col6a1**	**COL6A1**	**COL6A1**	**COL6A1**	n/a	n/a	COL6A1	1
COL6A2	8	**Col6a2**	*COL6A2*	**COL6A2**	**COL6A2**	n/a	n/a	COL6A2	0
COL6A3	8	**Col6a3**	**COL6A3**	**COL6A3**	**COL6A3**	n/a	n/a	COL6A3	0
FLNB	8	*Flnb*	**FLNB**	**FLNB**	**FLNB**	n/a	n/a	FLNB	1
FLNC	8	**Flnc**	**FLNC**	**FLNC**	**FLNC**	n/a	n/a	FLNC	1
HBB	8	**Hbb**	**HBB**	**HBB**	*HBB*	n/a	n/a	HBB	6
THY1	8	*Thy1*	**THY1**	**THY1**	**THY1**	**THY1**	n/a	THY1	2
VAT1	8	**Vat1**	**VAT1**	**VAT1**	**VAT1**	n/a	n/a	VAT1	1
VIM	8	**Vim**	**VIM**		**VIM**	n/a	n/a	VIM	1

Bold: present, italic: not detected.

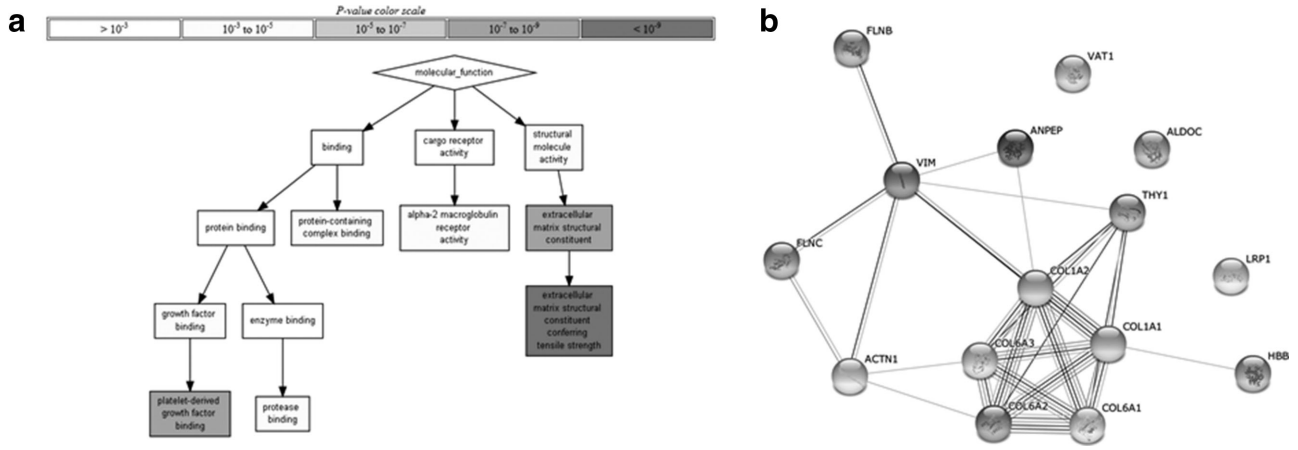

Figure 1. Enriched signaling and structural interactions. MSC-EV hallmark proteins were analyzed for a) enriched GO-terms and b) protein–protein interactions.

4. Discussion

Here, we compared public available protein profiles of MSC-EVs with proteome profiles of non-MSC-EV samples. Although there is a huge diversity in MSC sources and the methods of how MSC-EV were prepared, an MSC-EV specific protein signature has been identified. In addition, we identified 22 proteins which were exclusively found in profiles of BM-derived MSC-EVs but not in MSC-EV samples of non-BM-MSCs. The collection of the identified proteins should provide a helpful tool to improve the standardization of MSC-EV quality control analyses and might help to unravel potential mode of actions of corresponding EVs.

4.1. MSC and MSC-EV Standardization

With the recent developments in basic and translation MSC-EV research it is becoming evident that the broad diversity in experimental designs, and thus the apparent lack of harmonization, is hampering the overall comparability of reported pre-clinical MSC-EV data. To promote harmonization and the usage of more uniform MSCs, the ISCT formulated the "minimal criteria for defining multipotent mesenchymal stromal cells" in 2006.[17] Since then, these recommendations have been widely implemented, and accordingly, generally standardized MSC characterization is reported by most researchers (reviewed by Mushahary et al.[52]). Also for MSC-EV preparations, it remains

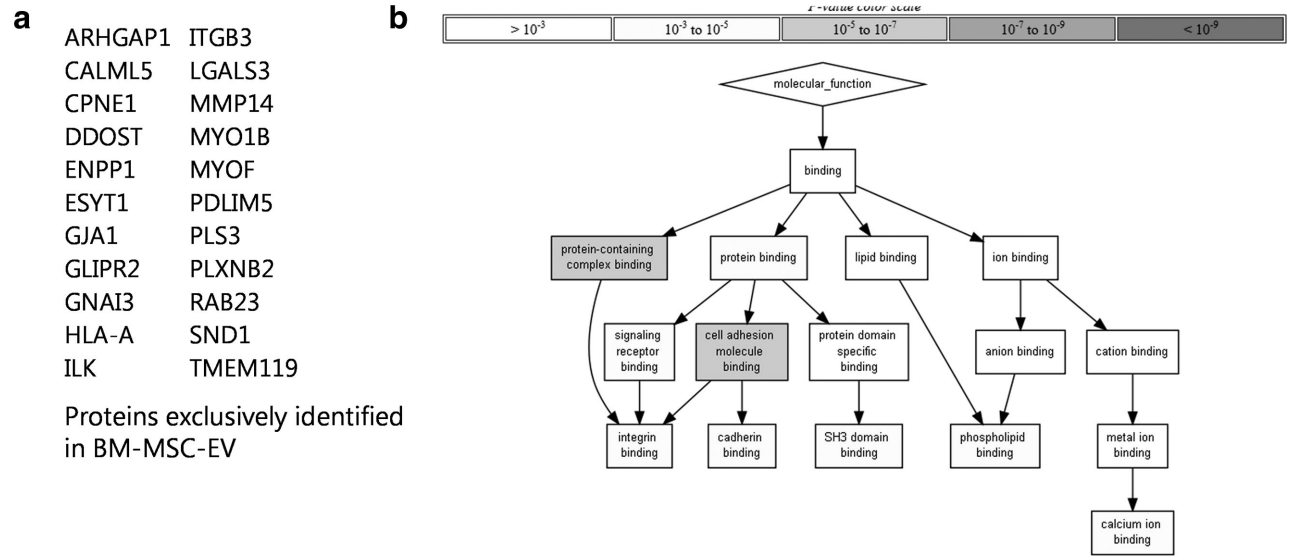

Figure 2. BM-MSC-EV enriched proteins. a) Twenty-two proteins were exclusively detected in BM-MSC-EV, and b) GO-term enrichment analysis indicates that proteins involved in cell adhesion and integrin- and receptor-interactions are overrepresented in these EV samples.

crucial to well characterize the MSCs secreting the EVs to be prepared. Consequently, in our analysis to identify MSC-EV specific proteins, only datasets were considered if convincing MSC characterization data were provided.

Similarly, recommendations (MISEV) for (the description of) the isolation procedure and characterization of EVs have been formulated by the International Society of Extracellular Vesicles (ISEV) in 2014,[53] and are continuously updated.[18] It is the intention of ISEV that EV researcher provide specific information on EV isolation and characterization. However, these recommendations do not favor certain method and are quite liberal. These recommendations are helpful in the rapidly expanding field of EV research in which an increasing plethora of methods and protocol variants are used to prepare EVs from different sources. Although only studies were considered here, which fulfill the recommendations of ISEV, MSC-EVs were prepared with different methods (tangential flow filtration, ultracentrifugation, HPLC, and combinations thereof). Still, as a common nominator, all MSC-EV fractions under consideration were reported to exert beneficial effects in respective preclinical models. Thus, the identified MSC-EV specific protein signature correlates very well with the functional properties of respective MSC-EV fractions. We consider that this signature will not only help to improve the quality assurance of novel MSC-EV preparations in the future, but may also help to unravel the MSC-EVs' mode of action.

4.2. Robustness and Uniqueness of the MSC-EV Protein Signature

For the identification of the MSC-EV proteome, LC-MS/MS proteomics data obtained from EV isolated using different methods and from different MSCs were used. During the process of dataset selection, the ten publications with their accompanying dataset we used for the initial analysis were the only papers that complied with our restrictions. It is intriguing that despite the broad variety in isolation methods and MSC sources, a rather similar population of EVs appears to be isolated. As first control, the most identified proteins in these EV were compared to the ExoCarta top-100 EV proteins, demonstrating that 59 of 134 (44.0%) proteins identified in at least seven out of ten datasets are common EV proteins. Compared to the EV from non-MSC, where 73 out of 358 (20.4%) proteins detected in at least 9 out of 12 datasets, this is a strong indication that EV isolations were relatively properly performed, demonstrating the value of adherence to the MISEV guidelines.[18] Given the higher number of identifications in the non-MSC EV datasets, with 1.4 more identification on average, identifying 73% of the top 100 EV proteins is a similarly solid illustration of the value of the MISEV guidelines.

On the same hand, with an average of 1.4 times more identifications in the non-MSC-EV datasets, and the fact that the bulk of the common EV proteins are identified in most of these analyses, and all of the top 100 proteins are covered in the total amount of identification, it appears safe to assume that these combined datasets represent a common EV population, source cell-independent proteome. Interestingly, most MSC-EV hallmark proteins could be detected in just 0, 1, or 2 out of 12 of these datasets. As these datasets comprise on average 1.4 times the number of protein identifications compared to the MSC-EV datasets, we can safely conclude that our approach has led to the identification of a protein MSC-EV signature that can be considered both uniform and to a certain extend (with exception of LRP1 and HBB) unique.

4.3. Implications for Function or Effect of MSC-EV

The identification of a robust set of proteins specifically present in and on MSC-EV allows speculation on the role of these proteins in the therapeutic function of these vesicles. The most

striking characteristic of the protein signature, based on the String protein–protein interaction and GO-term enrichment analyses, is the significant abundance of collagens I and VI. Collagens are expressed as extracellular proteins and consist of large trimeric fibers. As such, the collagens identified in the proteomes of MSC-EV are presumably attached to the outer surface of these vesicles through their strong binding to collagen receptors. Integrins, the most common collagen-binding proteins, appear very common in MSC-EVs, with components of the collagen-binding pairs $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$, namely ITGB1 (7x), ITGA2 (5x), ITGA11 (5x), and ITGA1 (3x) identified in many MSC-EV proteomics datasets, in contrast to other collagen binding proteins including nectins.[54] Potentially collagen 1 and 6 can directly and indirectly contribute to the therapeutic potential of MSC-EV. Collagen 6, for example, could have a direct antimicrobial effect through the activity of its von Willebrand factor type A-like domains.[55] Also, collagen 6, by influencing the ECM microenvironment, could skew macrophages to the regenerative M2 phenotype,[56] or stimulate fibroblast polarization and migration, as observed in tendon repair, through NG2 binding.[57] Collagen 1 and signaling peptides derived from this protein by hydrolysis have been demonstrated to reduce mucosal damage in ulcerative colitis by down-regulation of inflammatory cytokines.[58] Indirect effects of collagens would depend on the action of MSC-EV components, including proteins such as the proteasome complex[26] or miRNAs.[59] In such instances, the collagens on MSC-EV could enhance the uptake of MSC-EV at the site of injury through promoting directed and prolonged binding of MSC-EV in regions with up-regulated integrin expression, common at sites of injury or inflammation.[60]

Comparing the proteome content of BM-MSC-EV to non-BM-MSC-EV reveals an enrichment for cell- and protein-adhesion proteins in BM-MSC-EV according to the GO-enrichment analysis. This is in parallel to earlier descriptions of different molecular compositions of MSC from different sources[50,51] and their derived EV.[49] Direct analyses comparing the functionality of MSC-EV from MSC derived from different sources have never been described, but some speculation can be made based on comparisons of MSC from different sources. MSC from different sources, even from the same donor, show functional differences.[61,62] Additionally, the secretome of BM-MSCs appears better capable to stimulate angiogenesis and suppress of B-cell activation compared to the secretome of Umb-MSCs.[63,64] Although these findings may be explained by the observed differences in EV protein content, direct comparisons of the activities of isolated EV from MSC from various sources are required to draw any conclusions.

4.4. Limitations

Despite the thorough analysis we performed here, several limitations of our study can be addressed. Firstly, only ten studies on MSC-EV were included, mainly due to the inclusion criteria that were chosen. Unfortunately, in present times, this fact could limit in a high extent the applicability, confidence, or robustness of the results presented here. They are at this point insufficient when included in any prospective regulatory request or proposal for the use of MSC-EV products in humans, which would require additional (targeted) proteomics analysis of specific MSC-EV preparations. Additionally, in none of the ten included publications size exclusion chromatography (SEC) was used as an EV isolation method. Proteomic profiles of MSC-EV isolated by SEC are likely to differ from those of MSC-EV isolated by ultracentrifugation, HPLC, tangential flow filtration, or precipitation, as SEC is likely to better remove overabundant soluble proteins compared to other techniques, resulting in relatively more pure EV preparations. Thus, comparative studies on SEC-isolated MSC-EV, with a strong focus on EV purity and potential contaminations will be required to verify our conclusions on MSC-EV cargo and their potential functional implications.[65,66] Furthermore, besides the mentioned variability in cell source, the harvesting time of the EV-containing conditioned medium shows great variability between studies, ranging from 24 h to 7 days. The optimal harvesting time may vary per EV-producing cell, and depends on a balance between high yield while preventing accumulation of unwanted substances such as apoptotic bodies, as nicely determined by Lee et al., who determined an optimal harvesting time of 48 h for their adipose tissue-derived MSC-EV.[31]

4.5. Recommendations

Based on the robust MSC-EV proteome signature that we were able to obtain from a concise and well-controlled analysis of published proteomics datasets, a standardized approach for the characterization and verification, a quality control, could be envisioned. As the protein signature comprises several membrane- and extracellular proteins, a multiplex bead capture flow cytometry approach,[67] for example, based on COL6A2 or COL6A3 and THY1 (CD90) could be envisioned as a standard, easy to use platform for MSC-EV quality control, either in a research or clinical setting. Alternatively, such proteins could be tested in sandwich immunoassays[68] or antibody arrays[69] adapted specifically for this purpose. Having such tools established would pave the way to a more standardized and controlled identification, characterization, and quality assessment of MSC-EV for therapeutic use, meaning a huge leap forward to the clinical application of such vesicles.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

Sai Kiang Lim is founder of Paracrine Therapeutics and Vesiderm.

Keywords

cell therapy, clinical translation, exosomes, standardization, stem cells

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