Advancing EV Research: Novel Technique for EV's Surfaceome Profiling.

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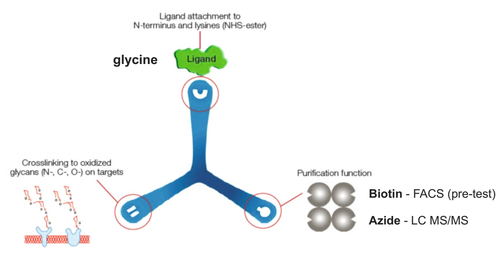
INTRODUCTION:

The extracellular vesicle (EV) surfaceome plays a crucial role in vesicle targeting, uptake, and functional interactions. However, its characterization remains challenging due to EV heterogeneity and small size, limiting conventional proteomics approaches.
In this study, we employed a chemoproteomic approach originally designed for the identification of cell surface proteins in living cells, and adapted it for the untargeted profiling of EV surfaceome. This methodology uses selective enrichment of N-, C-, and O-glycosylated proteins, which are ubiquitously present on the plasma membrane of living cells and, by extension, on the EV surface.

METHOD:

Surfaceome-TriCEPS™ technology for capturing the surface proteins of living cells (Evs) *(a, b, c)*

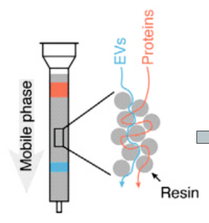


Human hepatic stellate cells (HSCs)

CTRL (Vehicle)

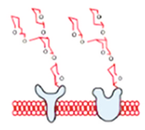
TGF-ß (1ng/ml)

Dichotomic size-exclusion chromatography (SEC)



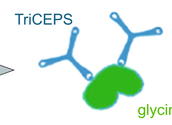
Sepharose CL-6B resin (pore size 24 nm)

Mild preriodate oxidation of surface glycoproteins



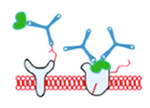
generates aldehyde from surface glycans

Control ligand



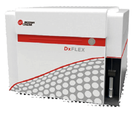
TriCEPs pre-quenched with glycine

EVs surface labeling

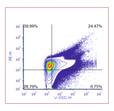


Pre-test Flow TriCEPS

FACS (DxFLEX)



Data evaluation



LC MS/MS analysis

Data evaluation

RESULTS:

Identification of EV-specific markers and HSC activation markers

Fig. 1

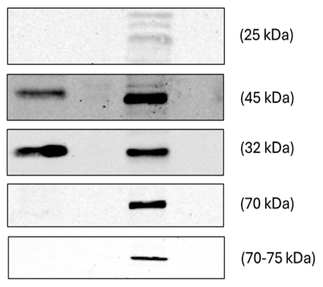


Fig.1. Western blot analysis of EV protein markers (MISEV2018[a]) isolated from cell culture supernatants of hepatic stellate cells using dichotomic SEC[c] method. Cells were cultivated for 24h in serum-free medium. Medium was collected (40 ml), concentrated to 1 ml (Amicon 30kDa MWCO ultrafiltration device) and loaded to 20 ml Sepharose CL-6B resin column. Eluate 1 (Evs), Eluate 2 (proteins), cell lysate and cell culture medium were immunostained for CD9, CD63, CD81,TSG101, Syntenin, HSP70, albumin and calnexin.

Fig. 2

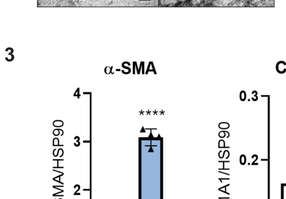


Fig.2. Representative transmition electron microscopy (TEM) images of the Eluate 1 and Eluate 2. Evs were fixed with 2,5% formaldehyd + 2 % glutaraldehyd O/N at RT, absorbed on formvar-carbon coated TEM grid and sained with 2% uranyl acetate.

Fig. 3

Fig. 3. Western blot analysis of HSC activation markers following TGF-β treatment. Hepatic stellate cells (HSCs) were treated with 1 ng/mL TGF-β or vehicle control (Ctrl) for 24 hours. After treatment, cells were washed with 1× PBS and cultured for an additional 24 hours in serum-free medium. Conditioned medium was collected for extracellular vesicle (EV) isolation, and the cells were lysed for Western blot analysis. Expression of HSC activation markers was assessed, including α-SMA (alpha-smooth muscle actin), Col1A1 (collagen type I alpha 1 chain), and CTHRC1 (collagen triple helix repeat-containing 1). Protein levels were quantified and normalized to the housekeeping protein HSP90.

Pre-test Flow TriCEPS

Fig. 4

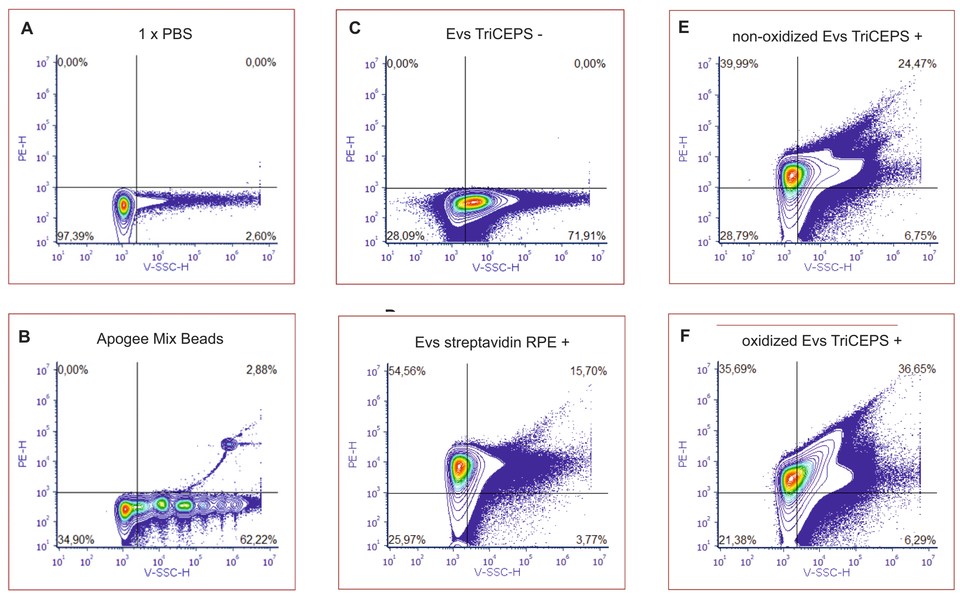


Fig.4. Flow cytometry analysis of Evs labeled with TriCEPS - Biotin and pre-tested for subsequent Evs surfaceome profiling. **The pre-test experiment has been developed to determine the optimal experimental conditions for the Surfaceome -TriCEPS- main experiment (LC MS/MS analysis).** During Flow-TriCEPS pre-test experiment, glycine was conjugated to TriCEPS labeled with biotin. Thus quenched TriCEPS was added onto slightly oxidized and non-oxidized EVs. Samples were then labeled with a streptavidin-RPE and analyzed by flow cytometry (DxFlex, Beckman Coulter). (A) sterile 1xPBS, (B) Apogee Mix calibration beads - silica beads and fluorescent PS beads, (C) pure isolated Evs without TriCEPS (unstained control), (D) Evs labeled with streptavidin RPE, (E) non-oxidized Evs labeled with TriCEPS and Streptavidin RPE, (F) oxidized Evs labeled with TriCEPS and streptavidin RPE.

EV proteins commonly identified in both groups

		number of peptides	
	Marker	**CTRL**	**TGF-β**
Evs	CD63	3	3
	ANXA2 (Annexin 2)	4	13
	CD59 glykoprotein	2	2
	CD44	4	3
	SDCB1 (Syntenin-1)	2	1
	A2MG (Alpha-2-macroglobulin)	6	6
	EF1A1 (Elongation factor 1-alpha 1)	2	4
	ALBU (albumin)	7	11
	CD81	1	0
Cells	G3P (GAPDH)	5	5

Table 1. List of commonly accepted EV markers (as per MISEV2023) identified in the surfaceome of both EV groups.

	number of peptides	
Protein	**CTRL**	**TGF-β**
A4 (Amyloid-beta precursor protein)	38	34
AGRIN	14	8
ANT3 (antithrombin III)	5	3
APLP1 (Amyloid beta precursor like protein 1)	4	4
CCN1 (Cellular communication network factor 1)	4	4
CO1A1	31	24
CO1A2	10	7
CO4A1	7	6
CO4A2	10	5
CO5A1	14	11
CO5A2	19	5
CO6A1	32	19
CO6A2	21	8
CO6A3	112	94
CSF1 (Colony stimulating factor 1)	15	9
CSPG2 (Versican core protein VCAN)	13	12
DESP (Desmoplakin)	31	87
DSC1 (Desmocollin 1)	2	4
DSG1 (Desmoglein 1)	9	17
EDIL3 (EGF-like repeat and discoidin I-like domain-containing protein 3)	25	20
FETUA (Fetuin-A, Alpha-2-HS-glycoprotein)	4	4
FINC (Fibronectin 1)	76	65
G3P (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH)	5	5
HSP7C (Heat shock cognate 71 kDa protein)	3	6
IBP7 (Insulin-like growth factor-binding protein 7)	6	5
ITA5 (Integrin alpha-5)	7	6
ITB1 (Integrin beta-1)	23	15
ITIH2 (inter-alpha-trypsin inhibitor heavy chain 2)	7	4
K1C10 (Keratin, type I cytoskeletal 10)	31	38
K2C1 (Keratin, type I cytoskeletal 1)	47	62
LDLR (Low density lipoprotein receptor)	9	4
LG3BP (Galectin-3-binding)	28	27
LRP1 (LDL receptor related protein 1)	11	8
LTBP1 (Latent-transforming growth factor beta-binding protein 1)	7	2
MFGM (Lactadherin, milk fat globule-EGF factor 8)	7	6
NID1 (Nidogen-1)	25	11
NRX3B (Neurexin-3-beta)	3	2
PAI1 (Plasminogen activator inhibitor 1)	9	9
PGS1 (biglycan)	12	15
PGS2 (decorin)	17	9
PLAK (Junction plakoglobin)	12	22
PTX3 (Pentraxin 3)	24	15
SDC4 (Syndecan 4)	4	2
SRCRL (Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D)	11	11
SRGN (Serglycin)	6	5
TARSH (ABI family member 3 binding protein)	2	2
TSP1 (Thrombospondin-1)	25	8
WNT5A (Wnt family member 5A)	8	8

Table 2. Proteins identified in both types of vesicles.

Characterization of surfaceome changes on control EVs and TGF-β EVs

Fig. 5. Volcano plot depicting the comparison between control EVs and TGF-β EVs.

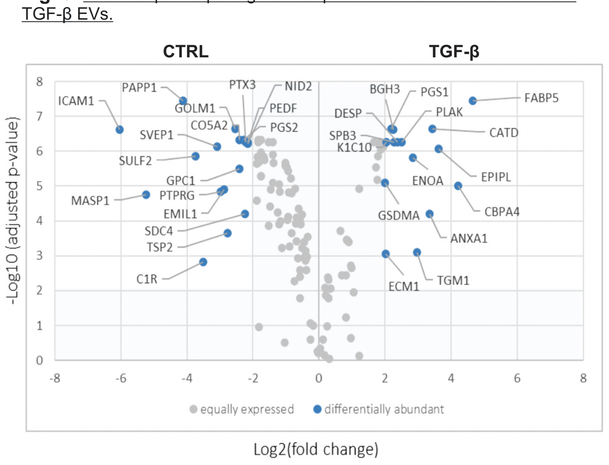


Table 3. Summary of identified differentially abundant proteins along with quantitative information

Enriched in	Protein	Accession	Protein Description	#peptides
CTRL	C1R	P00736	Complement C1r subcomponent	3
	COA2	P05997	Collagen alpha-2(V) chain	18
	EMIL1	Q9Y6C2	EMILIN-1	5
	GOLM1	Q8NBJ4	Golgi membrane protein 1	9
	GPC1	P35052	Glypican-1	5
	ICAM1	P05362	Intercellular adhesion molecule 1	2
	MASP1	P48740	Mannan-binding lectin serine protease 1	5
	NID2	Q14112	Nidogen-2	6
	PAPP1	Q13219	Pappalysin-1	18
	PEDF	P36955	Pigment epithelium-derived factor	8
	PGS2	P07585	Decorin	16
	PTPRG	P23470	Receptor-type tyrosine-protein phosphatase gamma	3
	PTX3	P26022	Pentraxin-related protein PTX3	18
	SDC4	P31431	Syndecan-4	4
	SULF2	Q8IWU5	Extracellular sulfatase Sulf-2	5
	SVEP1	Q4LDE5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	11
	TSP2	P35442	Thrombospondin-2	2
TGF-β	ANXA1	P04083	Annexin A1	4
	BGH3	Q15582	Transforming growth factor-beta-induced protein ig-h3	9
	CATD	P07339	Cathepsin D	7
	CBPA4	Q9UI42	Carboxypeptidase A4	3
	DESP	P15924	Desmoplakin	74
	ECM1	Q16610	Extracellular matrix protein 1	3
	ENOA	P06733	Alpha-enolase	5
	EPIPL	P58107	Epiplakin	2
	FABP5	Q01469	Fatty acid-binding protein 5	4
	GSDMA	Q96QA5	Gasdermin-A	5
	K1C10	P13645	Keratin, type I cytoskeletal 10	35
	PGS1	P21810	Biglycan	15
	PLAK	P14923	Junction plakoglobin	17
	SPB3	P29508	Serpin B3	5
	TGM1	P22735	Protein-glutamine gamma-glutamyltransferase K	7

Fig. 5. Volcano plot depicting the comparison between control EVs and TGF-β EVs.
Proteins with an enrichment factor of log2(FC) ≥ 2 and -log10(adjusted p-value) ≥ 2, are considered as highly enriched in the corresponding samples (white space in the volcano plot figure). Using this criterion, the abundance of 32 surface proteins was identified as significantly different between CTRL and TGF-β Evs (Table 3).

Pathway analysis of enriched proteins in CTRL and TGF-β vesicles (Reactome)

Fig. 6.

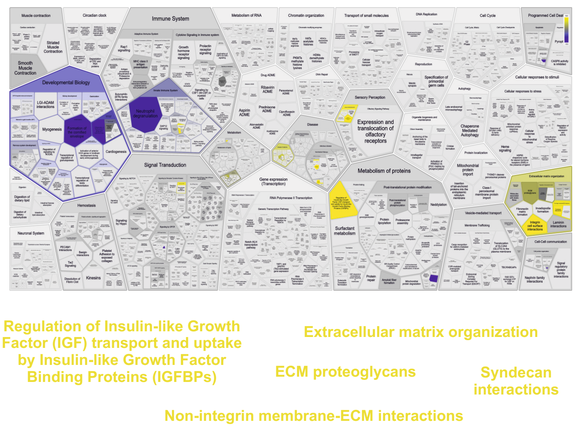


Fig 6. Voronoi map depicting all pathways included in Reactome. Pathways that contain proteins found increased in CTRL vesicles are indicated in yellow and proteins found increased in TGF-β treated vesicles in blue.

CONCLUSION:

- Protein identification and quantitative analysis demonstrated the efficacy of this approach, enabling the **identification of 158 surface-associated proteins on EVs**, with 94 proteins detected based on the presence of at least two peptides per protein.
- The majority of identified proteins have been previously reported in EVs derived from various cell types, and **11 of them are among the top 100 most frequently identified EV**-associated proteins according to Vesiclepedia. These include CD63, ANXA2, CD59, CD44, Syntenin-1, Alpha-2-macroglobulin, Elongation Factor 1-Alpha 1, Albumin, CD81, GAPDH, and CD29.
- Profiling EV surfaceome by c**apturing glycosylated proteins** can play an important role in advancing both fundamental research and clinical applications.
- By refining our ability to map and interpret the EV surfaceome, we can unlock **new diagnostic biomarkers** by capturing specific EV subpopulations in circulating milieu, improve targeted therapies, and deepen our understanding of cell-to-cell communication in health and disease.

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