Identification of anchor proteins for the BioDrone™ platform

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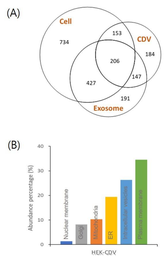
Abstract

BioDrone™, developed at MDimune, is a state-of-the-art drug delivery system based on cell-derived vesicles (CDVs) produced by serial extrusion from diverse cell sources. CDVs exhibit physicochemical similarity to extracellular vesicles (EVs) but a tremendous advantage in production scalability over EVs. Furthermore, with genetic engineering of source cells, CDVs can be equipped with additional biological features such as targeting and cargo loading capabilities. In this study, we identified stable anchor proteins exclusively for HEK-CDVs. First, we analyzed the HEK-CDV proteome and selected a set of CDV-specific membrane proteins that are highly abundant in CDVs compared to cells or EVs. Then, stable cell lines were established to overexpress these anchors fused with GFP proteins and other tags. After CDV production, the expression of anchor fusion proteins was quantified via GFP ELISA, and their distribution in a single-particle resolution was examined by nanoflow cytometry. Among candidates, four HEK-CDV anchors showed a stable and robust presence in the CDVs, with 30 to 150 molecules per CDV particle and 42 to 66 % of the GFP (+) population among total particles. Moreover, their proper topology was confirmed via protease cleavage assay, while their abundance in the CDVs was also confirmed from a large-scale extrusion. Together, we present a set of HEK-CDV anchors for the versatile engineering of the BioDrone™ platform. Currently, we are developing various BioDrone™ therapeutics utilizing these anchors.

Proteome Analysis of HEK-CDVs



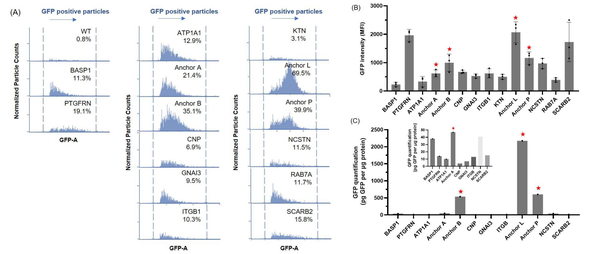
(C)

Protein Name	Gene name	CDV Ratio	CDV Abundance	Exosome Ratio	Exosome Abundance	Subcellular
Anchor B	Anchor B	19.18	578,831,342	11.81	356,519,809	Vesicle, PM
Integrin beta-1	ITGB1	14.28	407,439,475	10.68	304,557,675	ER, PM
Ras-related protein Rab-7a	RAB7A	11.42	387,167,648	1.74	59,032,236	Not defined
Anchor A	Anchor A	14.48	368,836,860	5.93	150,996,699	PM
Nicastrin	NCSTN	8.28	333,029,841	0.95	38,070,235	PM
Lysosome membrane protein 2	SCARB2	21.41	325,954,252	0.53	8,133,431	Vesicle, PM
Anchor L	Anchor L	41.69	625,753,223	1.22	18,317,643	Vesicle, PM
Anchor P	Anchor P	36.38	618,379,380	0.92	15,638,189	Vesicle, PM
Kinectin	KTN1	7.87	502,081,461	2.81	179,452,910	ER
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	20.93	2,844,002,240	8.32	1,130,592,812	PM
Guanine nucleotide-binding protein G(i) subunit alpha-3	GNAI3	27.72	780,309,103	13.88	390,615,313	PM
2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP	10.89	508,835,681	6.71	313,356,859	PM

HEK-CDV proteome analysis revealed several anchor candidate proteins which were exclusively abundant in HEK-CDVs compared to cells and EVs.

Figure 1. Identification of anchor candidates from HEK-CDVs via proteome analysis. (A) Comparative proteome analyses of CDVs vs. cells and exosomes. (B) Percentage of diverse subcellular origin of membrane proteins in HEK-CDVs. (C) A list of proteins for HEK-CDV anchor candidate.

Identification and Characterization of BioDrone Anchor

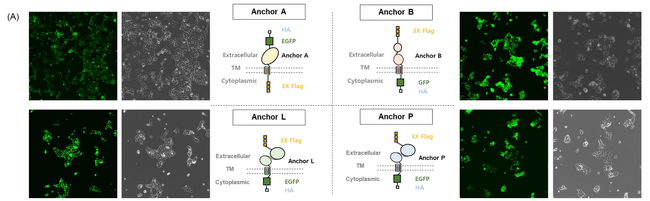


We analyzed the amount of anchor fusion proteins in the CDVs and the distribution at a single particle level to validate anchor protein expression on HEK-CDVs. The stars indicate the finally selected anchors, based on the anchor GFP fusion protein level, the ratio of GFP+ particles, and the number of GFP molecules on CDVs.

Figure 3. Characterization of anchor proteins in HEK-CDVs
(A) Representative histogram from nanoFCM showing GFP positive particles. The mean intensity (B) and quantification (C) of GFP fusion proteins on HEK-CDVs are shown. Anchors with lower GFP expression are shown in the inset.

Selected BioDrone Anchors for HEK-CDVs

We finally determined four BioDrone anchors which are abundantly expressed on the CDV membrane.



(B)

Subcellular origin	Plasma membrane		Lysosome	
Gene name	Anchor A	Anchor B	Anchor L	Anchor P
Percentage of GFP (+) particles (corrected ratio)	42	51	66	52
GFP quantification (GFP ng/ µg protein)	0.40	2.13	0.75	0.88
GFP/CDV* *in GFP positive CDVs	31	152	122	51

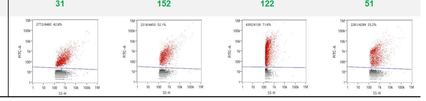
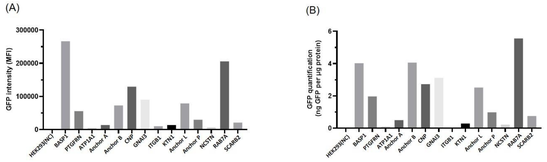


Figure 4. Selected anchors for BioDrone platform. (A) Schematic diagram of selected anchor proteins and GFP fusion strategy in HEK293 cells. (B) Characterization of the selected anchors. The table shows GFP+ particles (red) and GFP protein copy numbers per CDV (green).

Generation of Stable Cell Lines Overexpressing Anchor Candidates

Anchor candidates fused with EGFP were transduced to HEK293 cells using lentiviral particles. Antibiotic selection and cell sorting on transduced cells eliminated non-transduced cells and generated stable cell lines.



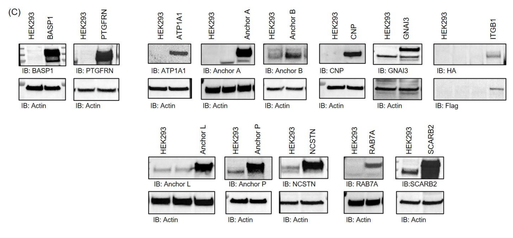


Figure 2. Overexpression of anchor candidates in stable cell lines. Overexpression of anchor fusion proteins was assessed by measuring fluorescence intensities (A) and quantifying GFP expression (B). (C) Immunoblot analyses confirmed their overexpression.

Scale-up of CDV Production Containing BioDrone Anchors

We compared the characteristics of BioDrone anchors in the CDVs produced from different extrusion methods (membrane filter and large-scale extrusion). The anchor expression profiles remained the same in both CDVs.

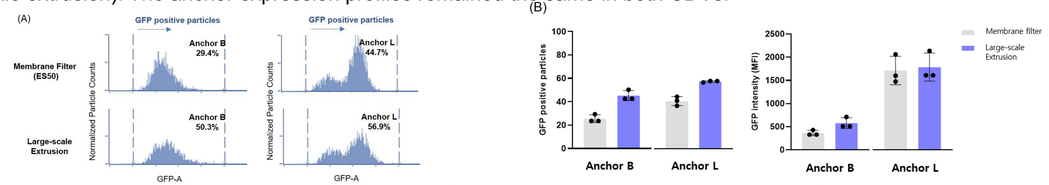
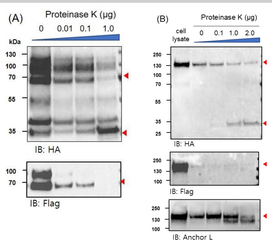


Figure 5. Comparison of BioDrone anchor expression from different extrusion methods (A) Histogram of GFP+ particles. (B) Percentage of GFP+ particles (left panel) and GFP intensity (right panel) of indicated anchors between membrane filter extrusion and large-scale extrusion.

Topology Analysis of Selected BioDrone Anchors



CDVs were treated with proteinase K (PK) to define the topology of selected anchor proteins. Since PK cannot penetrate through the lipid bilayer, only the extravesicular parts of the anchor proteins are susceptible to PK degradation. The CDVs are shown to maintain the original topology of anchor proteins.

Figure 6. Topology analysis of anchor proteins via protease cleavage assay. CDVs containing anchor B (A) or anchor L (B) were treated with various concentrations of PK and analyzed by western blot. HRP conjugated anti-Flag or anti-HA antibodies were used for detection of 3x Flag tag at the N-terminal and HA tag at the C-terminal of the anchor proteins.

Summary & Future Prospects

- CDV engineering requires stable and efficient anchor proteins, which are specific and abundant in CDVs.
- Proteome analysis provided a deep understanding of the subcellular origin of CDVs and helped to find a list of membrane anchor proteins that could be efficient for HEK-CDV engineering.
- We evaluated anchor candidate proteins and chose a set of anchors located in the plasma membrane or intracellular vesicles.
- Anchor proteins will be a basis for the genetic engineering of HEK-CDVs to introduce targeting ligands and therapeutic cargos to BioDrone therapeutics.