Accurate Quantitation of EV-associated Insulin in Pancreatic Extracellular Vesicles (EVs)

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1 Abstract

Type 1 diabetes (T1D) is characterized by the immune cell-mediated destruction of insulin-producing beta cells in the pancreas. Insulin is also a major autoantigen against which autoantibodies are developed in T1D. Previous studies have shown the presence of preproinsulin and insulin on the surface of EVs, while insulin was also reported to be present inside of EVs as cargo[1,2] . Accurate measurement of the EV-associated insulin both on the surface and inside EVs is challenging but may lead to valuable insights into the possible role of EVs in the development of autoreactive T-cells and autoantibodies in Type 1 diabetes[3]. To understand the molecular composition of pancreatic EVs, we first measured common tetraspanin markers on human islet derived EV samples and identified CD63 as the dominant tetraspanin protein on islet EVs. Further characterization of selected samples with ultrasensitive electrochemiluminescence (ECL) intact-EV immunoassays demonstrated the presence of several pancreatic cell surface markers on islet EVs.

To measure EV-associated insulin, we first purified EVs from human pancreatic islet conditioned culture medium (CCM) using tangential flow filtration (TFF) followed by size exclusion chromatography (SEC). Islet CCM were found to contain insulin in the range of hundreds of ng/mL. TFF removed about 85% of this and SEC further removed most of the remainder, with the EV-containing fractions retaining about 0.5% of total insulin in the CCM. To further determine the location of insulin, we performed a protease protection assay which showed that approximately 40% of the insulin in the EV-containing SEC fractions was protected from digestion, possibly within EV cargo. To determine whether this insulin was associated with tetraspanin positive EVs, we immunoprecipitated (IP) the EVs using either CD63, CD81, CD9, or isotype control antibodies, then lysed and assayed the immunoprecipitates. We observed the highest amount of insulin in the CD63-IP (8.5 pg per mL of CCM); however the tetraspanin IP isolated only ~1% of the total insulin measured in the EV-containing SEC fractions. Further studies are ongoing to determine the amount of EV cargo insulin in IP samples, ascertain whether there are other protease-protected forms of secreted insulin and investigate whether T1D pathogenesis alters the level of EV-associated insulin.

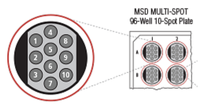
2 Methods

I. MSD® ECL-based assays measure the concentrations of soluble proteins and intact EVs in complex biological samples

Electrochemiluminescence (ECL) Technology

MSD's ECL detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

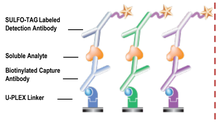
- **High sensitivity:** Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- **Broad dynamic range:** High and low abundance analytes can be measured without multiple sample dilutions.
- **Low background:** Electronic stimulation of detection label is decoupled from the output signal (light), resulting in extremely low background.



U-PLEX® Multiplexed Immunoassays

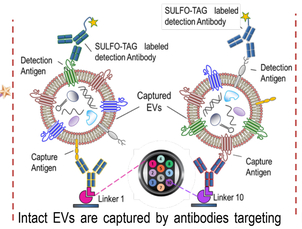
Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. Multiplexing conserves valuable samples by allowing up to ten determinations per well.

Assay Format "A" for soluble analytes: e.g. insulin, glucagon



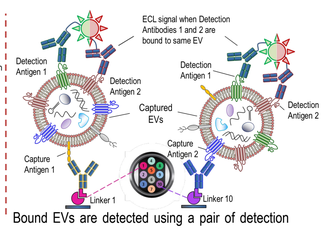
Soluble analytes are captured and detected using sandwich immunoassay format with SULFO-TAG ECL labels

Assay Format "B" for intact EVs: e.g. CD63⁺ or CD9⁺ EVs



Intact EVs are captured by antibodies targeting distinct surface antigens, e.g. CD63, CD9 etc. Bound EVs are detected using SULFO-TAG labeled detection antibodies that recognize common detection antigen(s) on the EVs; usually, this is a cocktail of antibodies targeting common EV proteins: CD81, CD9, and CD63.

Assay format "C", ultrasensitive assays for intact EVs: e.g. IA-2⁺ or ENTPD3⁺ EVs



Bound EVs are detected using a pair of detection probes in two positions (both positions include mixture of CD63, CD81, and CD9 in the current format) targeting two distinct detection antigens. The ultrasensitive assay format only generates signal when both detection antibodies bind to the same EV. MSD's ultrasensitive technology allows even rare EV populations to be detectable by this assay format.

Description of samples measured

- EndoC-bh5 cells were purchased from *Human Cell Design* and cultured in house in a defined, serum-free medium. CCM was collected after 72 hours of culture.
- Islets from the University of Florida (SAMN36845032, HP-24172-01, SAMN39523303 and HP-23241-01) were obtained from Integrated Islet Distribution Program (IIDP) or Prodo Laboratories, Inc. and cultured at the *University of Florida* in the same medium as the EndoC-bh5 cells. CCM was collected after one week of culture.
- Prodo Bulk Islet is the CCM from a high density islet culture directly obtained from Prodo Laboratories, Inc. and purified by TFF at MSD. This medium contains 5% pooled human serum.

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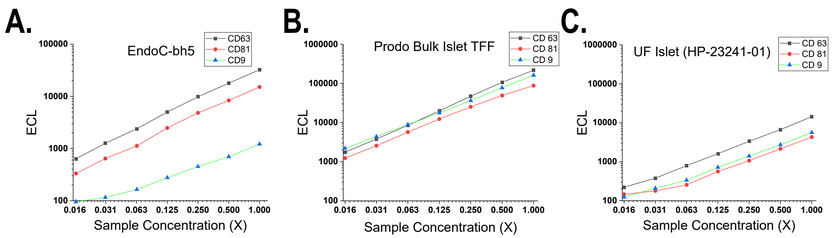
3 Screening of Putative Surface Markers for Pancreatic EVs

I. MSD's ECL-based EV assays measure intact EVs in pancreatic islet and EndoC-bh5 samples



To understand the relative levels of EVs presenting common tetraspanin proteins in pancreatic samples, CCM from three cultures of pancreatic islet origin **(A.-C.)** were serially diluted and assayed by ECL sandwich assays using *assay format B*. The signal ratios between each of the tetraspanin capture antibodies is generally consistent across the whole dilution range for each sample and the signal is linear with dilution. **D.** The signal-to-background is shown as a ratio of signal on each tetraspanin capture spot to the signal on an isotype control capture spot using the undiluted sample. CD63+ EVs were found to be the most abundant among all the tetraspanin+ EVs in each of the islet derived samples. The ratios of CD63 to CD81 or CD9 are given in the table. This pattern is different than what we have observed in most other cell types (data not shown) where CD9+ is usually the most abundant population.

D. EV Signals in Pancreatic and Non-pancreatic Samples

Sample	Signal to Background			TSPAN Ratios	
	CD63	CD81	CD9	CD63/CD81	CD63/CD9
EndoC-bh5	312.0	228.1	35.4	1.4	8.8
Prodo Bulk Islet	1505.1	610.1	1124.6	2.5	1.3
UF Islet (SAMN36845032)	53.2	27.7	30.7	1.9	1.7
UF Islet (HP-24172-01)	126.8	60.6	56.5	2.1	2.2
UF Islet (SAMN39523303)	541.4	132.2	144.5	4.1	3.7
UF islet (HP-23241-01)	170.9	51.0	66.8	3.3	2.6
Islet Media Control	1.4	0.8	1.0		
DPBS	1.2	0.9	1.0		

II. MSD's ECL-based ultrasensitive EV assays detect EVs with putative pancreatic cell-surface markers in pancreatic islet cultures and a cell model

- EVs from islet cultures were screened using *Assay format C* with capture antibodies targeting 21 putative pancreatic surface markers and a cocktail of detector antibodies targeting CD63, CD81, and CD9 to maximize sensitivity.
- EV populations expressing ten of the target surface markers were detectable, as defined by ECL signals at least three times higher on the specific capture spot than an isotype control capture spot.
- DPP4, F3, IA-2, SPINT2, and TM4SF4 were observed in all three islet-derived samples while ENTPD3, GAD-65, GLUT2, SSTR1, and SSTR2 were observed in some samples.

Putative Surface Markers in Pancreatic Samples

Marker/Sample	Signal to Background				
	EndoC-bh5	Prodo Bulk Islet (TFF)	UF Islet (HP-24172-01)	Media Control	DPBS
ANXA3	1.8	1.0	1.0	1.2	1.0
CFTR	0.7	0.8	0.8	1.3	1.6
DLK1	1.7	1.0	0.8	0.8	0.8
DPP4	3.5	17.1	3.8	1.1	1.0
DPP6	2.1	1.2	0.7	0.8	1.0
ENTPD3	2.3	9.5	6.7	0.9	1.1
F3	3.5	14.2	3.0	1.1	1.1
FXYD5	1.2	0.9	0.9	1.0	1.0
GAD-65	3.4	0.5	0.6	0.9	0.8
GHSR	0.6	0.6	0.7	1.0	0.9
GLP-1R	1.3	0.8	0.7	0.8	0.9
GLUT2	2.1	15.8	1.1	1.0	0.9
IA-2	191	66.6	104	1.3	1.2
LEPR	1.3	1.1	0.8	0.9	0.8
PLAT	0.8	2.1	1.6	0.7	0.7
SPINT2	39.8	75.1	19.0	1.0	0.8
SSTR1	13.6	1.8	6.3	1.0	0.9
SSTR2	15.7	1.5	1.1	1.0	1.0
TGFBR3	1.0	1.1	0.9	0.8	1.1
TM4SF4	16.6	134	23.3	1.0	1.0
TRPC1	0.9	0.7	0.6	0.8	0.8
IgG1	1.0	1.0	1.0	1.0	1.0

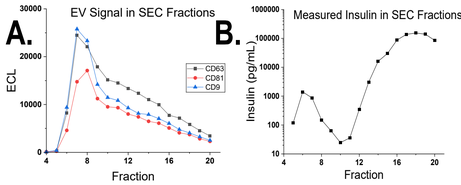
Acknowledgements:

Research reported in this publication was partially supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) under R01 Research Award Number 1R01DK133861. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

4 Measurement of EV Cargo in Pancreatic EVs

I. High levels of insulin are present in islet cultures and insulin co-isolates in EV preparations

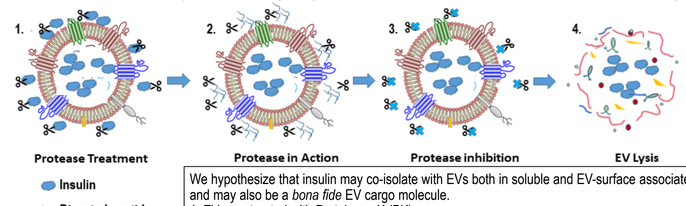
Prodo Bulk Islet CCM was first purified by TFF with a 300 kDa membrane (Pall), followed by size exclusion chromatography (SEC) using an I*zon qEV Original 70 nm* column. **A.** *Assay format B* was used to measure total intact EVs in each SEC fraction to confirm the location of the majority of EVs, in this case, fractions 7-8. The total insulin concentration in each sample and in each SEC fraction was assayed using MSD's V-PLEX® insulin assay (*K151S5D*). 1% Triton X-100 was added to assay diluent to ensure that EVs were lysed and the entirety of soluble and EV associated insulin was measured. **B.** The total insulin concentration in each SEC fraction shows a distinct peak in EV-containing fractions. **C.** A summary table of the total insulin concentration at each stage of the purification process, dilution corrected for volume changes.



C.

Samples/Condition	Insulin (pg/mL)	Percent retained
Prodo Islet CCM	231,500±10,500	100%
TFF Retentate (1X)	35,100±2,700	15.2%
SEC EV Fractions	1,103±123	0.48%

II. Protease protection assay shows majority of insulin in SEC EV isolates is not EV Cargo



We hypothesize that insulin may co-isolate with EVs both in soluble and EV-surface associated form, and may also be a *bona fide* EV cargo molecule.
1. EVs are treated with Proteinase K (PK)
2. PK digestion removes the surface bound and soluble insulin
3. PK is inactivated without exposing the cargo insulin
4. EVs are lysed and cargo is measured using ECL immunoassay

Protease protection assay was conducted as shown above. EV-containing SEC fractions were treated with PK or a buffer control, then inhibited with AEBSF, a protease inhibitor, followed by lysis using 1% Triton X-100. Separate optimization experiments were conducted to determine the appropriate concentrations of PK and AEBSF to ensure complete digestion of relevant amounts of insulin in the presence of relevant amounts of total protein and to ensure complete inhibition of the protease (data not shown). Samples were measured with the V-PLEX insulin assay. Approximately 1,103 pg/mL of total insulin was detected in EV-containing SEC fractions, while only 409 pg/mL (37%) was detected after PK treatment. This insulin may be *bona-fide* EV cargo though it could possibly be protected in some other structure or aggregate. We conclude that 63% of insulin in EV-containing SEC fractions is not present in EV cargo, though some may be associated with the EV surface.

Insulin Content in PK Samples

Samples/Condition	Insulin (pg/mL)
SEC Purified EV (No PK)	1,103±123
SEC Purified EV (with PK)	409±5
Insulin Control (No PK)	263±7
Insulin Control (with PK)	Not detected
DPBS (No PK)	Not detected
DPBS (PK)	Not detected

II. Immunoprecipitation demonstrates that ~1% of insulin in SEC EV isolates is associated with tetraspanin-positive EVs

To further investigate the distribution of insulin in the SEC fractions, we performed Immunoprecipitation (IP) using CD63, CD9, CD81, or isotype control antibodies on magnetic beads. Intact EV assay (format B) was used on the supernatant after the IP reactions to confirm nearly complete depletion of each tetraspanin-positive EV population from the sample (data not shown). Among the IP conditions, the highest amount of insulin was detected in CD63-IP, though this was less than 1% of the insulin contained in the SEC EV fractions. This suggests that the majority of the insulin that was protected in the protease protection assays is not contained within tetraspanin-positive EVs. It may be contained within tetraspanin-negative EVs or other structures that protect it from digestion.

Insulin Content after IP

Sample	Insulin (pg/mL)	Percent Retained
SEC Fractions 7-8	1,103±123	100%
CD63 IP	8.5 ±0.5	0.77%
CD81 IP	3	0.27%
CD9 IP	1±0.2	0.09%
IgG IP	Not detected	0%

5 Conclusion and Future Directions

- CD63+ EVs were the most abundant tetraspanin positive population in all the islet-derived EV samples that we measured.
- EV populations expressing several putative surface markers were observed in islet culture samples including IA-2, a Type 1 Diabetes (T1D) autoantigen, and TM4SF4.
- ~0.5% of the total insulin in the islet culture co-isolated with EVs using SEC; however, less than 1% of this is associated with tetraspanin+ EVs.
- Further studies are ongoing to determine the surface and cargo distribution of insulin in IP samples, the nature of the protected insulin within the SEC fractions, as well as whether T1D pathogenesis alters the level of EV-associated insulin.

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6 References

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