Viewpoint: Redefining tissue cross-talk via shotgun proteomic analyses of plasma extracellular vesicles

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Abstract

Protein signalling between tissues, or tissue cross-talk is becoming recognised as a fundamental biological process that is incompletely understood. Shotgun proteomic analyses of tissues and plasma to explore this concept are regularly challenged by high dynamic range of protein abundance, which limits the identification of lower abundance proteins. In this viewpoint article, we highlight how a focus on proteins contained within extracellular vesicles (EVs) not only partially addresses this issue, it can reveal a underappreciated complexity of the circulating proteome in various physiological and pathological contexts. Furthermore, we highlight how quantitative proteomics can inform EV mediated cross talk and stress the importance of high coverage, sensitive proteomic analyses of EVs to identify

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both the optimal methods to isolate EV subtypes of interest and proteins that characterise them.

Proteins in inter-tissue cross-talk

Fundamental to the maintenance of homeostasis is the complex and coordinated signalling between endocrine glands and other tissues. Proteins or peptides such as insulin, glucagon and oxytocin, and their receptors, are therefore the foundation of endocrinology and inter-tissue cross talk. While the majority of focus in the field has been on protein (and steroid) secretions of widely recognised endocrine glands, such as the pancreas and pituitary, landmark findings have also implicated other tissues in endocrine –like roles. Indeed, the identification of adipokines, such as Leptin[1] and Adiponectin[2], myokines such as IL6[3] and Myonectin[4] and hepatokines such as Follistatin[5] and FGF21[6], widen interest in tissue protein secretion and fully implicate inter tissue protein signalling as a fundamental biological process that warrants further investigation.

Proteomic approaches to investigating tissue cross talk

The intricate, but vast coordinated signalling between tissues is clearly highly complex. However, a deeper understanding of these processes, particularly in the contexts of so-called 'lifestyle diseases' such as obesity and metabolic syndrome[7], has great potential in the discovery of novel therapies. Illustrating the challenge facing research in this area, a deep, integrated 'omics' analysis of the human tissue proteome reveals that a larger

proportion of tissue-enriched proteins are secreted or membrane spanning than are intracellular[8]. The magnitude of the human secretome is clearly vast, suggesting a broad range of tissue cross talk remains largely unexplored. Unbiased, hypothesis free and high coverage proteomic experiments lend themselves in this regard and in an attempt to characterise the secretome of various tissues. Accordingly, several groups have applied mass spectrometry analyses to reductionist, *in vitro* models of tissues such as skeletal muscle[9], Liver[10] and adipose[11]. However, *in vitro* models, particularly considering secretion as a coordinated process, can lack physiological relevance. Direct analyses of blood samples, especially when sampled across tissues via careful cannulation[12] likely offers the most insightful view of tissue protein secretion. That said, this approach also comes with considerable technical challenges. Proteins exist in plasma across a massive dynamic range of a minimum of 10 orders of magnitude[13] with albumin constituting roughly half the total protein mass[14]. Small proteins involved in tissue cross talk, such as cytokines, generally circulate in the pg/ml range. Despite rapid advances in mass spectrometry technology in recent years, accurate quantitation of such proteins at the very bottom of this dynamic range, in an unbiased, untargeted manner, has largely been impossible to date.

Proteomic analysis of extracellular vesicles – a convenient side step of the dynamic range issue.

By and large, the simplest and quickest iteration of mass spectrometry based proteomics on plasma, so called single shot LC MS-MS, generally leads to the identification of no more than a few hundred proteins. While this type of analysis can be improved via depletion of abundant plasma proteins via immunoaffinity columns or beads, poor antibody specificity or affinity of proteins to albumin itself can result in a poorly representative sample[14]. Extensive fractionation, at the protein or peptide level is another option, but this can also magnify

analysis time and limit throughput[15]. An alternative is to focus more closely on the constituents of blood and carry out fractionation prior to commencing the lysis, reduction, alkylation and digestion of proteins typical in proteomic sample processing. Generally, cells are removed from blood via centrifugation and the resultant plasma is assumed to contain protein, lipid and metabolites representative of the site of blood collection at that time. However, extracellular vesicles (EVs) of varying size and cellular origin are also present, and are increasingly recognised as important carriers of nucleic acid, lipid, metabolites and pertinently, proteins. Importantly, EVs can be isolated from plasma with relative ease, largely depleting the plasma sample of high abundance proteins. A focus on the proteins circulating in EVs, at least from an analytical perspective, represents a convenient sidestep of the problems associated with obstructive, high dynamic range and facilitates a deeper look into the circulating 'secretome'. Vesiclepedia, an online compendium[16] of EV protein identifications across 54 studies, lists 306 proteins identified via a range of analytical and isolation methods. When compared with the 8548 proteins identified in EVs across all cell types and tissues (Figure1), this, in many ways demonstrates the technical difficulty encountered in analysing EVs derived from plasma. That said, several groups, including our own, have recently made use of the rapid advancements in mass spectrometry technology to gain deeper insight into the circulating EV proteome in various physiological and pathological contexts[17, 18, 19].

Rethink on the circulating secretome?

While there are, undoubtedly some gaps in our knowledge of classical protein secretion, it is largely a well-characterised process involving the recognition of N terminal amino acid signal sequences by the signal recognition particle (SRP), subsequent processing in the endoplasmic reticulum and golgi apparatus and eventual release into the extracellular environment[20]. Such is the apparent uniformity of signal or leader sequences in secreted

proteins, it is possible to predict, via algorithms, their presence in proteins with known amino acid sequences[21]. In the context of identifying the secretome of a particular tissue therefore, one avenue of discovery is to identify tissue expression of mRNA or proteins in response to various physiological or pathological stimuli and screen, via bioinformatic means, for the presence of a signal peptide to inform which proteins are likely secreted. This approach has been adopted with success in skeletal muscle in the search for myokines or proteins secreted in response to contraction and exercise[22]. While this approach clearly has some merit, our recent, in depth analysis of the circulating EV proteome in humans[19] emphasises why a sole focus on proteins involved in classical secretion may underestimate the extent of the circulating proteome. In isolated small vesicles and exosomes of a size range of 50-350nm, we identified over 5000 proteins at a false discovery rate of 1%[19]. Analysis of the amino acid sequence of each of these proteins via the SignalP server[21] predicted the presence of a signal peptide in just 16% of all protein identifications[19]. While the method of EV isolation, centrifugation, possibly led to some contamination with non EV proteins in these samples, the identification of such a high number of apparently non-secreted proteins in circulation suggests EVs provide a way in which cells can interchange proteins independently of classical secretion. So not only are secreted proteins of interest in understanding tissue cross talk, a new avenue, that being EV mediated protein exchange, may also be highly relevant. Indeed, the identification of thousands of proteins in EVs, in the human circulation, via in depth, high coverage UHPLC-MS/MS [17, 19], supports the existence of complex tissue cross talk as a fundamental biological process.

Using quantitative proteomics to determine specific EV protein tissue uptake

Of course, identifying vast numbers of proteins within EVs in plasma does not provide specific evidence of tissue cross talk *per se*. The field is therefore challenged to identify techniques by which tissue specific release and uptake of proteins in EVs can be

characterised. With regard to the latter, quantitative proteomic approaches can make a contribution. For example, stable isotope labelling with amino acids in cell culture, or SILAC[23], has, in some ways been superseded by multiplexed isobaric[24] or non-labelled[25] quantitative approaches to mass spectrometry based proteomics. However, metabolic labelling with stable isotope amino acids provides a unique scenario whereby labelled cells *in vitro* or labelled rodents *in vivo*[19, 26] release EVs containing proteins that are distinguishable in mass spectrometry analyses, owing to their heavier mass (Figure 2B). Quantitative proteomic analyses of cells or tissues exposed to labelled EVs can therefore indicate not only the extent of EV derived protein uptake, but also the adhesion molecules potentially mediating this transfer[19]. This represents another example by which, unbiased, shotgun proteomics can inform EV mediated tissue cross talk.

Searching for optimal EV isolation methods benefits optimal proteomic analyses

The EV field is a rapidly growing one, as researchers recognise the importance of cell to cell communication via vesicles in both physiological and pathological contexts[27]. However, one frequent observation is the lack of consistency in isolation methodologies or validated controls identifying EV subsets[28]. This has led to confusion in nomenclature as to what represents, for example, an exosome or microparticle, which may have differing functional properties[29]. One approach to subjugate this issue is to isolate EV subtypes via a range of centrifugation, density gradient, or immunoaffinity methods and carry out unbiased proteomic analyses on each, with a view to identifying consistent EV markers for each subtype. While there have been some excellent contributions in this area, using EV samples from a range of cell lines[30, 31], proteomic coverage in these studies varies widely. Since the absence of a specific protein in EV subsets is as informative as its presence, researchers should take care in interpreting proteomic data when sample preparation, instrument performance, or both

might be limiting the observable proteome. For example, current consensus in the field suggests that for isolation of ~100nm exosomes, centrifugation should be at greater than 100,000g for over an hour. However, we have recently shown, in high coverage UHPLC-MS/MS analyses of EVs isolated from human plasma, that exosome and small vesicle markers such as TSG101, SDCBP, CD9 and PDCD6IP (ALIX) are reproducibly detected in samples isolated at just 20,000g in a standard laboratory micro-centrifuge (Figure 2A, Table 1)[19]. These data suggest that, while EV isolation methods such as ultracentrifugation through a density gradient[32], size exclusion chromatography[33], or immmuno-isolation[30] will undoubtedly provide a more pure enrichment of small vesicles, adopting high quality proteomic methodologies allows the researcher to carry out exosome and small vesicle research in simple and conveniently derived EV samples. Furthermore, experiments attempting to identify reproducible protein markers of different cell EV subtypes or proteins that might be mediating their biological action in various physiological and pathological scenarios can greatly benefit highly optimised, high coverage proteomic analyses. Encouragingly, recent, new acquisition methods demonstrate large improvements in the coverage and speed of single shot quantitative proteomics[34], suggesting that such analyses of EV subtypes can make telling contributions to the growing research field of EV mediated tissue cross talk.

In conclusion, proteomic analyses of plasma continue to broaden the size of the known circulating proteome in various physiological and pathological states. Recent observations of a large number of proteins in circulation in EVs reminds the field that protein exchange between tissues, or tissue cross talk, might well be occurring independently of classical protein secretion. Furthermore, as the depth of coverage and accuracy of quantitative proteomic analyses continues to improve, it is likely that these approaches can greatly inform critical aspects of EV biology and help delineate specific tissue cross talk mediated by EVs.

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Figure Legends

Figure 1: Representation of proteins identified in EVs isolated from plasma in the context of the entire Vesiclepedia database[16]. Data were extracted from the Vesiclepedia database and visualised in the Funrich program[35]

Figure 1

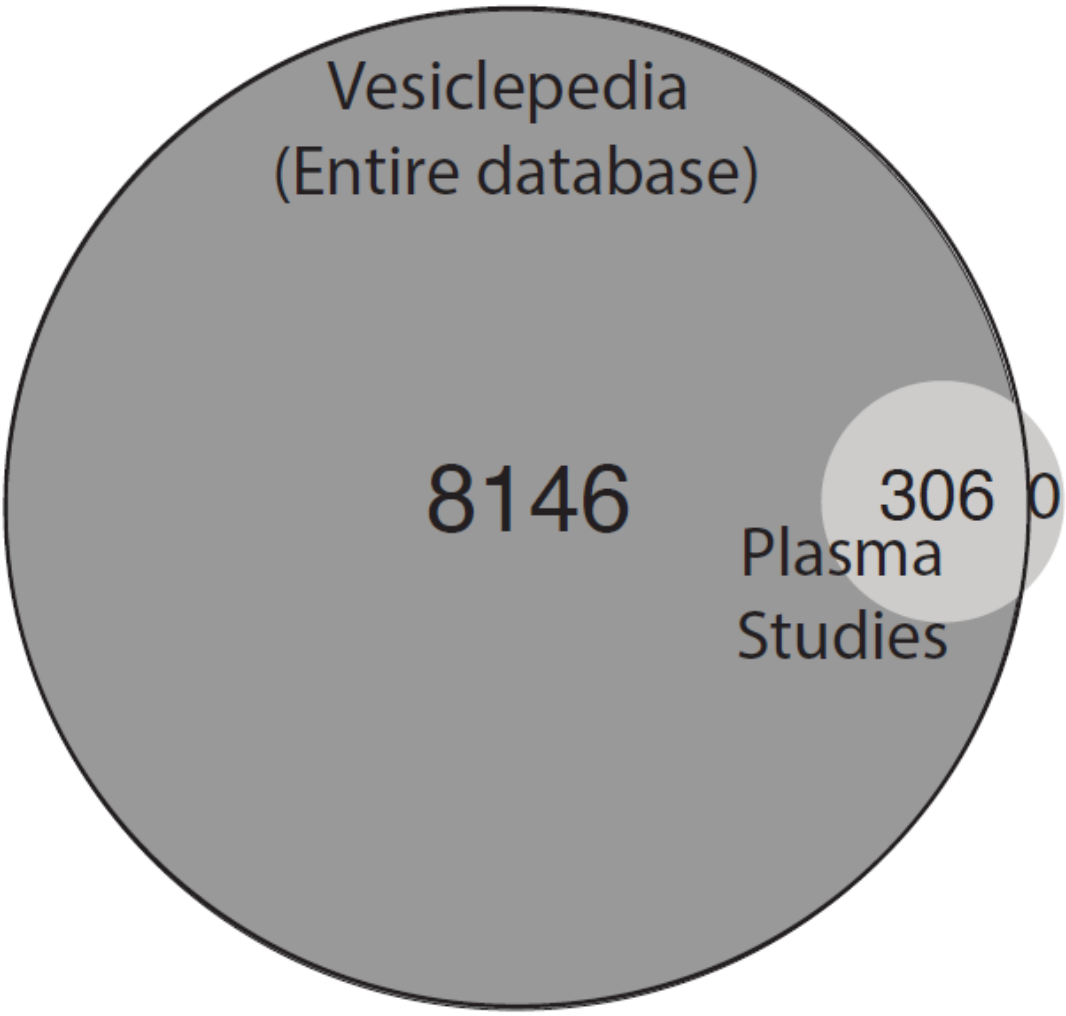

Figure 2: Proteomic approaches to the determination of tissue cross talk via extracellular vesicles (EVs). (A) Plasma is derived from participants using conventional means and EVs isolated via high-speed centrifugation interspersed by washing in phosphate buffered saline. The EV pellets are then lysed, reduced, alkylated and the protein contents digested via proteases 'in solution'. Optimised, single shot analyses on an Orbitrap mass spectrometer can facilitate great insight into protein release into circulation via EVs. (B) To examine specific interchange of proteins between cells and tissues, SILAC labelled cells and mice generate EVs containing 'heavy' proteins distinguishable in LC-MS/MS analyses. Pulse

chasing of these EVs into different, unlabelled cells or mouse recipients, via LC-MS/MS screening of recipient cells and tissues provides insight into specific protein delivery via EVs.

Figure 2

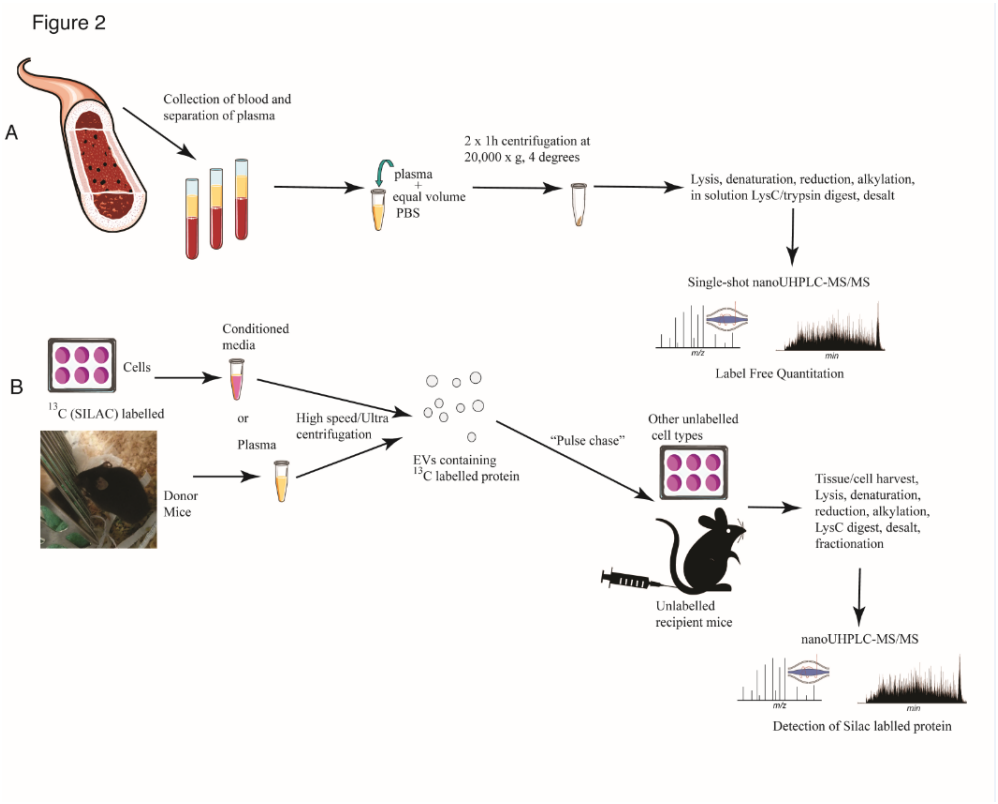

Table 1 Proteins commonly known as exosome markers reproducibly identified in EVs isolated at 20,000g from plasma of three healthy human donors. Listed are the Uniprot identifier for each exosome marker, the IBAQ intensity recorded in the EV sample from each subject, the number of unique peptide sequences associated with that marker and the number of sequencing events, or MS/MS, used to derive this information. For full methodological approach, see[19]. Associated mass spectrometry data are available via the PRIDE partner repository with the dataset identifier PXD006501.

Table 1

			Subject 1	Subject 2	Subject 3
Gene	**TSG101**	IBAQ intensity (log2)	23.1	22.9	25.5

Uniprot ID	Q99816	Unique peptides	2	2	6
		MS/MS count	3	2	8
Gene	**SDCBP**	IBAQ intensity (log2)	25.0	26.6	24.5
Uniprot ID	O00560	Unique peptides	2	1	0
		MS/MS count	6	13	3
Gene	**CD9**	IBAQ intensity (log2)	33.2	32.9	33.9
Uniprot ID	P21926	Unique peptides	6	7	5
		MS/MS count	15	22	19
Gene	**PDCD6IP (ALIX)**	IBAQ intensity (log2)	26.7	25.5	27.6
Uniprot ID	Q8WUM4	Unique peptides	24	12	25
		MS/MS count	27	19	31

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