Exosomes: A source of novel disease biomarkers in bladder cancer

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Dedication

This thesis is dedicated to my family, especially my fiancé Sean and parents who have believed in me and supported me wholeheartedly throughout all my studies.

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Summary

Exosomes are nanometre-sized vesicles secreted by most cells into the extracellular milieu. They have been proposed as a good source of disease-related markers as they are available non-invasively (for example from urine) and express a repertoire of proteins enriched in cancer antigens and stress proteins. The major aim of this thesis was to perform the first ever proteomics study on bladder cancer exosomes.

Initially, exosomes were isolated from urine specimens but hypervariable yields and poor sample quality made proteomics analysis challenging. As an alternative approach, exosomes were isolated from HT1376 bladder cancer cells. Exosomes were purified by ultracentrifugation on a sucrose cushion, and preparations verified as high quality by immunoblotting, flow cytometry and electron microscopy.

For global proteomics analysis, the sample was solubilised using SDS and DTT and subjected to LC-MALDI-TOF/TOF MS. We identified 353 proteins with high confidence and 63 of these have not been previously identified in other proteomics studies on human exosomes.

Overrepresentation analysis demonstrated that the proteome was consistent with that of other exosomes with significant overlap with exosomes of carcinoma origin. Comparisons with the Gene Ontology database also highlighted strong associations with carcinoma of the bladder and other sites. A GeneGo generated protein interaction network highlighted c-Myc as a major node of protein interaction within this dataset.

Several MS-identified proteins were confirmed as genuinely exosomally expressed using a combination of immunoblotting, flotation on continuous sucrose gradients, and flow cytometry. Expression was also verified in exosomes from a variety of sources, including urine.

In conclusion we report the first proteomics dataset on exosomes derived from bladder cancer cells. We identified 353 exosomal proteins with high quality MS data and highly pure exosomes. The data will aid our understanding of exosome biogenesis and function and may inform the development of urine exosome-based clinical tools in bladder cancer.

Publications and presentations

Publications

Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD, Clayton A. (2010) **Proteomic analysis of bladder cancer exosomes.** *Molecular and Cellular Proteomics.* 9(6):1324-38

Mitchell PJ*, Welton J*, Staffurth J, Court J, Mason MD, Tabi Z, Clayton A. (2009). **Can urinary exosomes act as treatment response markers in prostate cancer?** *J Transl Med* 7:4 (*equal contribution to manuscript)

Presentations and conferences attended

Welton JL, Brewis IA, Brennan P, Staffurth J, Mason MD, Clayton A (2009). **Bladder cancer exosome proteomics for biomarker discovery** (Oral Presentation). *24th Annual Postgraduate Research Day, Graduate School of Biomedical and Life Sciences.* School of Medicine, Cardiff University, Cardiff, UK.

Welton JL, Brewis IA, Brennan P, Staffurth J, Mason MD, Clayton A (2009). **Proteomic analysis of bladder cancer exosomes** (Oral Presentation). *Velindre NHS Trust R&D conference.* Village Hotel, Cardiff. UK

Welton JL, Brewis IA, Brennan P, Staffurth J, Mason MD, Clayton A (2009). **Proteomic analysis of bladder cancer exosomes** (Poster Presentation). *EMBO conference "Cancer Proteomics 2009: Mechanistic Insights, Technological Advances, and Molecular Medicine".* University College Dublin, Ireland

Welton JL, Brewis IA, Staffurth J, Clayton A (2008).**Differential proteomic analysis of exosomes in association with cancer: method development** (Poster Presentation). *5th Joint BSPR/EBI Proteomics Meeting.* Hinxton, Cambridge, UK

Welton JL, Brewis IA, Tabi Z, Staffurth J, Mason MD, Clayton A (2007). **Differential proteomic analysis of exosomes isolated from human urine** (Poster Presentation) *22nd Annual Postgraduate Research Day, Graduate School of Biomedical and Life Sciences*. School of Medicine, Cardiff University, Cardiff, UK.

Welton JL, Brewis IA, Tabi Z, Staffurth J, Mason MD, Clayton A (2007). **Developing methods for differential protein analysis in cancer** (Poster Presentation). *Velindre NHS Trust R&D conference*. Village Hotel, Cardiff, UK.

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Abbreviations

All abbreviations are listed and defined unless they are a standard abbreviation accepted for publication in Molecular & Cellular Proteomics without definition.

1DE	One dimensional electrophoresis
2DE	Two dimensional electrophoresis
Ab	Antibody
ADT	Androgen deprivation therapy
ANOVA	Analysis of variance
APC	Antigen presenting cell
AQP1	Aquaporin-1
ATCC	American tissue culture collection
BCA	Bicinchoninic acid
BCa	Bladder cancer
BPH	Benign prostate hyperplasia
BTA	Bladder tumour antigen
CBS	Central biotechnology services
CEA	Carcinoembryonic antigen
CHCA	α-cyano-4-hydroxycinnamic acid
CK18	Cytokeratin 18
CM	Cell-conditioned media
CRUK	Cancer Research UK
CTL	Cytotoxic lymphocyte
D_2O	Deuterium oxide
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell

DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
dpi	Dots per inch
EBV	Epstein Barr virus
ECACC	European collection of cell cultures
EMEM	Eagle's minimal essential medium
ER	Endoplasmic reticulum
esRNA	Exosomal shuttle ribonucleic acid
ExoQA	Exosome quality assurance
F	Female
FBS	Foetal bovine serum
FBS^{exo-}	Exosome depleted foetal bovine serum
FC	Flow cytometry
FDA	Food and Drug Administration
g	G-force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GPI	Glycosyl phosphatidylinositol
HA	Hyaluronic acid
HAase	Hyaluronidase
HBSS	Hanks' balanced salt solution
HD	Healthy donor
hCFHrp	Human complement factor H-related protein
Her2/neu	Human epidermal growth factor receptor 2
HFF	Human foreskin fibroblasts
hnRNP K	Heterogeneous nuclear ribonucleoprotein K

HSP	Heat shock protein
I/R	Renal ischemia-reperfusion
INT/mm^2	Intensity per millimetre squared
IRMA	Immunoradiometric assay
IVP	Intravenous pyelography
kVh	Kilovolt hours
LAMP	Lysosomal-associated membrane protein
LBPA	Lysobiophosphatidic acid
LGL	Large granular lymphocyte
LREC	Local research ethics committee
M	Male
Mcm	Minichromosome maintenance
MFG-E8	Milk fat globule-EGF-factor VIII
MIIC	MHC class II-enriched compartments
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MP	Microparticle
MS/MS	Tandem mass spectrometry
MUC1	Mucin 1
MudPIT	Multidimensional protein identification technology
MV	Microvesicle
MVB	Multivesicular body/bodies
NEAA	Non-essential amino acids
NHS	*N*-Hydroxysuccinimide
NL	Non linear
NMP	Nuclear matrix protein

OMIM	Online mendelian inheritance of man
ORA	Overrepresentation analysis
PCa	Prostate cancer
PET	Polyethylene terephtalate
PI3K	Phosphoinositide 3-kinase
PMF	Peptide mass fingerprinting
PS	Phosphatidylserine
RP	Reverse phase
S/N	Supernatant
SA: V	Surface area to volume ratio
SCX	Strong cation exchange
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
TCC	Transitional cell carcinoma
TEAB	Triethylammonium bicarbonate
TEM	Transmission electron microscopy
TGFβ1	Transforming growth factor-beta 1
THP	Tamm-Horsfall protein
TURBT	Transurethral resection of bladder tumour
U/ml	Units per millilitre
UBC	Urinary bladder carcinoma antigen
uPA	Urokinase-type plasminogen activator
UTI	Urinary tract infection
v/v	Volume per volume
w/v	Weight per volume

Chapter 1:

Introduction

1.1 *Bladder cancer*

Bladder cancer (BCa) is the 5th most common cancer in the UK and is ranked 9th in the world [1]. Every year in the UK alone around 10,000 people (17 per 100,000 of the population) are diagnosed with BCa. Of these 90% are diagnosed with transitional (urothelial) cell carcinomas (TCC) with the remaining 10% being squamous cell carcinomas (SCC) or adenocarcinomas. TCC is the second most common cancer of the genitourinary tract, prostate cancer being the first, and hence represents a significant clinical problem. Yet it is a research area that remains under-investigated.

The urinary bladder is a muscular organ that acts as a reservoir for urine, produced by the kidneys, prior to its excretion through the urethra. The area where an individual is most likely to develop carcinoma of the bladder is in a triangular region of the bladder known as the trigone (Figure 1.1). It is here where the two ureters enter the bladder and the urethra exits [2].

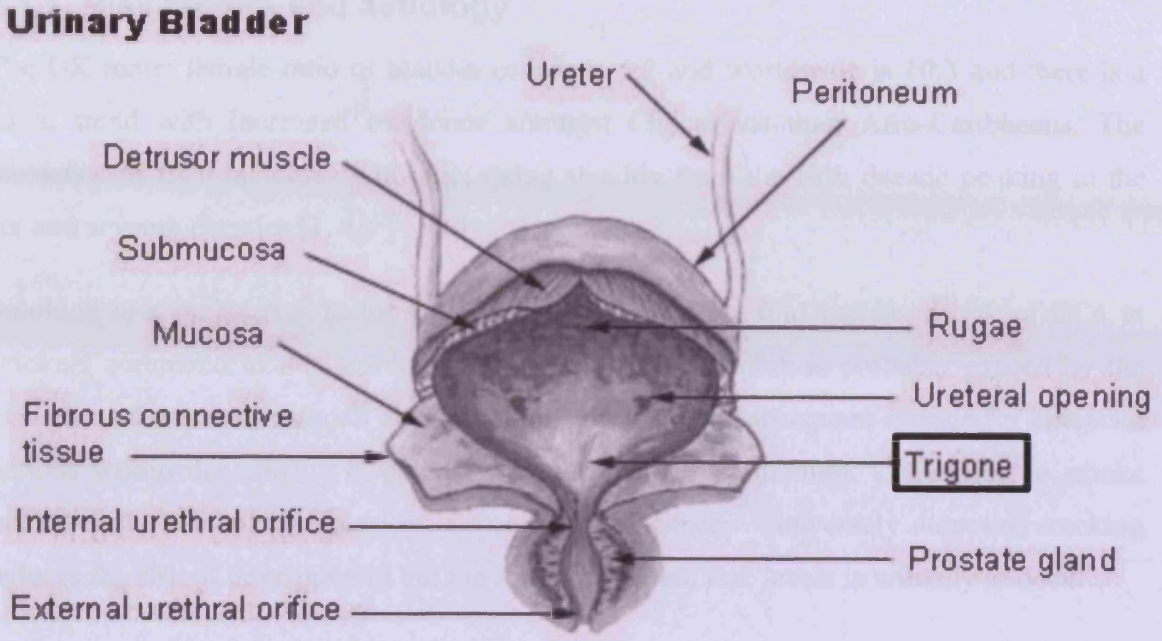


Figure 1.1: Anatomy of the bladder

The trigone region of the bladder highlighted is the area in which a carcinoma is most likely to develop (from SEER Training Modules, NIH, US, 9th March 2010 [3])

1.1.1 Risk factors and aetiology

The UK male: female ratio of bladder cancer is 5:2 and worldwide is 10:3 and there is a racial trend with increased incidence amongst Caucasians than Afro-Caribbeans. The incidence of BCa increases with age; rising steadily from the fifth decade peaking in the six and seventh decades [1, 4].

Smoking is a major risk factor in BCa with a two to six-fold increased risk of BCa in smokers compared to non-smokers [5, 6]. This increased risk is probably caused by the excretion of carcinogens such as 4-aminobiphenyl and its subsequent storage for extended periods within the urine – in contact with the bladder urothelium. Continuing to smoke increases the rate of progression of non-invasive cancer. Conversely stopping smoking reduces the risk of development but the return to normal risk levels is unlikely to occur.

In addition TCC is associated with exposure to industrial chemicals such as aniline dyes, 2-naphthylamine, 4-aminobiphenyl, 4-nitrobiphthol, benzidine, 2-amino-1-naphtol and acrolein [7]. Industries have taken measures to reduce the risk of developing TCC associated with these chemicals.

BCa is also linked to chronic bladder infection particularly in the Middle East where it is associated with chronic infection caused by schistosomiasis (a waterborne parasitic flatworm). In these instances the disease is usually SCC and the mean age of onset is usually earlier than that of TCC [1, 4, 7].

1.1.2 Diagnosis, treatment and monitoring

The most common presentation of BCa is gross painless haematuria (85% patients). Other symptoms include urgency, urinary frequency and dysuria (painful urination). All of these symptoms can however be indicative of less serious problems such as urinary tract infections, so cannot be used as definitive diagnostic tools alone, and patients require further investigations. The evaluation of suspected BCa usually includes urine cytology, flexible cystoscopy and imaging of the upper urinary tracts by ultrasound and intravenous pyelography (IVP). Transurethral resection of the bladder tumour (TURBT) is performed

under general anaesthesia using a resectoscope (cystoscopy is an endoscopic procedure in which the inside of the bladder can be visually examined) removing all visible tumour as well as biopsy of the surrounding muscle. This tissue may provide important information about the cancer grade and tumour depth. Local staging may also be furthered by MRI scanning of the tumour.

If bladder cancer is confirmed, careful staging is important as treatment is dependent on the stage of the disease at presentation. Detailed classification for malignant carcinomas of the bladder is given in Table 1.1 and depicted in Figure 1.2. This classification offers a shorthand description of bladder tumours at presentation. For example T1 is representative of tumours that are limited to the lamina propria whereas T4 represents tumours that have invaded local structures such as the vagina, prostate (T4a) or abdominal wall (T4b). Knowing the depth of tumour invasion also indicates the risk of lymph node metastasis. Individuals with cancer limited to the lamina propria (T1) have a 20% risk of developing lymph node metastasis whereas patients with full-thickness muscle invasion (T2b) the risk increases to 60%.

There are three grades of BCa G1, G2, and G3. Low grade G1 is the least aggressive. Here the cells are well differentiated and analogous to normal bladder cells. G3 is known as high grade disease and exhibits the most anaplastic (de-differentiated) cells which have a more embryonic form compared to the normal cells. G3 tumours have the highest growth rate, risk of recurrence and metastatic potential [8]. Carcinomas *in situ* are non-invasive flat lesions of the bladder epithelium and are likely to be high grade malignancies.

Once the stage and grade of the cancer has been determined, the treatment may consist of a combination of surgery, radio-, immuno-, and chemo-therapy. Superficial/papillary tumours (Ta/T1) account for 70% of TCCs and are usually of relatively low malignant potential (G1 or 2). These are removed by TURBT or diathermy where the tumour tissue is destroyed using an electric current. Regular cystoscopic follow-up is also performed due to a tendency to recur. The risk of recurrence and risk of progression to muscle invasive disease (T2-T4) can be predicted from the history, tumour stage, grade and multifocality. If

recurrence is considered likely, adjuvant intravesical chemotherapy with mitomycin C or immunotherapy with BCG (bacillus Calmette-Guérin) may be used [9].

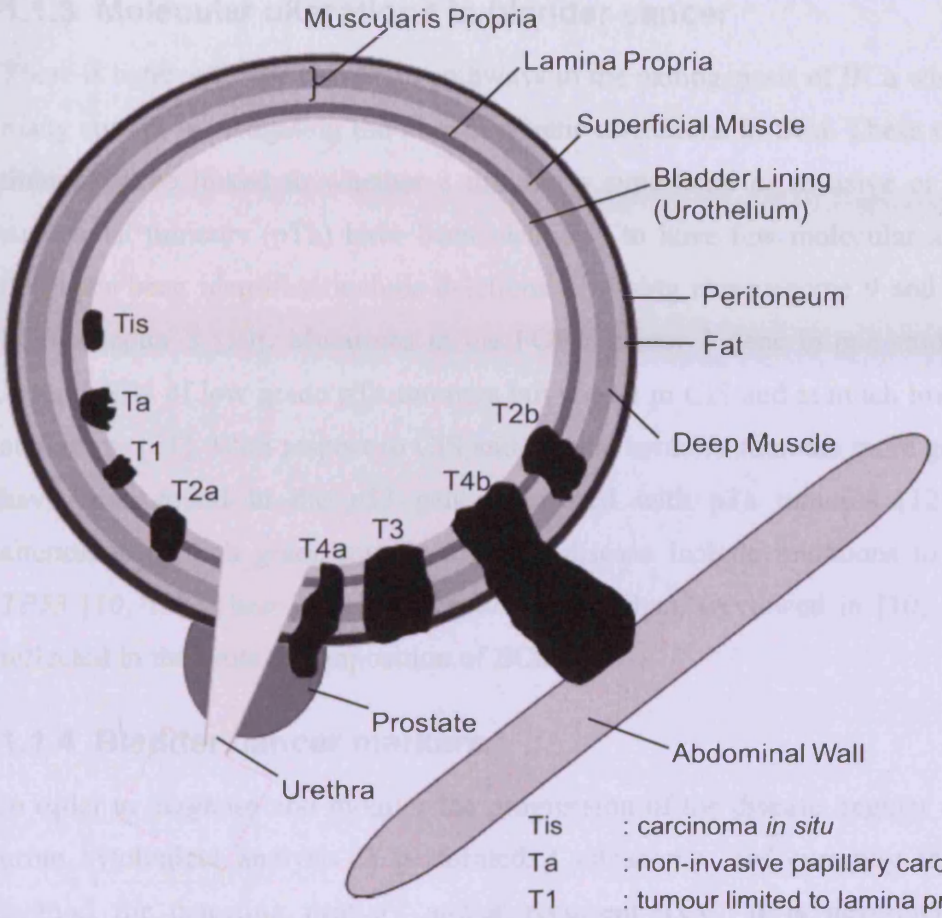
The standard curative treatment options for muscle-invasive tumours (T2-T4) are surgery or complete TURBT followed by external beam radiotherapy. Cystectomy is standard practice in the US and there is increasing evidence that extended lymph node clearance improves outcome. Quality of life following cystectomy is affected by the need to divert urinary flow directly or indirectly into a stoma bag, but modern surgical techniques can overcome much of this with the creation of continent neo-bladders. Radical radiotherapy allows preservation of the organ function of the bladder in the majority of patients. However, patients require life-long cystoscopic follow-up and approximately 15% will require salvage cystectomy for locally recurrent disease. Chemotherapy used pre or post operatively has been shown to improve survival rates by about 5%. Where adenocarcinomas and SCCs are concerned the tumours tend to be insensitive to radiotherapy and therefore the treatment of choice is surgery.

Bladder cancer is a difficult cancer to manage, from its initial presentation to its treatment and monitoring, it involves a lot of careful staging and interpretation of clinical information. Many of the patients are elderly, have significant medical co-morbidities (most are smokers), and require repeated invasive cystoscopic assessment. Hence there is a need for tools that can reduce the amount of invasive clinical work needed to diagnose and monitor these patients.

Table 1.1: TNM bladder cancer staging

Primary Tumour (T)		
Tis		Carcinoma *in situ*
Ta		Noninvasive papillary tumour
T1		Tumour invades the lamina propria, but not beyond
T2		Tumour invades the muscularis propria
	pT2a	Tumour invades superficial muscle (inner half)
	pT2b	Tumour invades deep muscle (outer half)
T3		Tumour invades perivesical tissue
	pT3a	Microscopically
	pT3b	Macroscopically (extravesical mass)
T4		Tumour invades any of the following: prostate, uterus, vagina, pelvis or abdominal wall
	T4a	Tumour invades prostate, uterus, vagina
	T4b	Tumour invades pelvis or abdominal wall
Regional Lymph Nodes (N)		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Metastasis in a single lymph node, 2cm or less in greatest dimension
N2		Metastasis in a single lymph node >2cm but <5cm in greatest dimension, or multiple lymph nodes, none >5cm in greatest dimension
Distance Metastasis (M)		
MX		Distant metastasis cannot be assessed
M0		No distant metastasis
M1		Distant metastasis

From the American Joint Committee on Cancer 2002[4]



Tis : carcinoma *in situ*
Ta : non-invasive papillary carcinoma
T1 : tumour limited to lamina propria
T2 : tumour invades lamina propria
T2a : invades superficial muscle
T2b : invades deep muscle
T3 : tumour invades perivesical tissue
T4a : invading neighbouring structures (prostate, uterus, vagina)
T4b : involvement of rectum, fixed to pelvic wall

Figure 1.2: Tumour staging of bladder cancer in a male patient

(adapted from Bladder Cancer UK, CancerStats, CRUK (2006) and DeVita (2007) [1, 4])

1.1.3 Molecular alterations in bladder cancer

There is believed to be two main pathways in the pathogenesis of BCa which has triggered many studies investigating the known genetic alterations in BCa. These two pathways are thought to be linked to whether a tumour is superficial or invasive or CIS. Low-grade superficial tumours (pTa) have been identified to have few molecular alterations. Those that have been identified include deletions involving chromosome 9 and mutations of the FGF receptor 3 [10]. Mutations in the FGF receptor 3 gene in one study were found in around 80% of low grade pTa tumours but not all in CIS and at much lower levels in pT1 and above [11]. With respect to CIS and muscle invasive tumours more genetic alterations have been found in the *p*53 gene compared with pTa tumours [12]. Other genetic alterations in high grade muscle invasive disease include mutations in *PTEN, RB,* and *TP53* [10, 13]. These genetic alterations and others (reviewed in [10, 13, 14]) may be reflected in the protein composition of BCa cells.

1.1.4 Bladder cancer markers

In order to diagnose and monitor the progression of the disease, regular cystoscopies and urine cytological analysis is performed. Cystoscopies are currently the most efficient method for detecting primary and/or recurrent TCC. It is nevertheless an invasive procedure causing significant discomfort to the patient. Therefore eradicating or reducing the need for cystoscopies is likely to be beneficial not only for the patient but may also reduce the time in clinic and costs. Urine cytology is the standard method for detection or follow up of urothelial carcinomas. The cytological analysis examines exfoliated cells present in voided urine analysing morphological abnormalities (e.g. size, shape, and prominent nucleoli). Urine cytology is sensitive for the detection of high grade tumours, but problems arise with low grade tumours as the cell abnormalities are minor. The detection rate of urine cytology is highly variable (10 to 90%) with the highest rate of detection for high grade carcinomas *in situ*. Flow cytometry has also been used to assess the DNA content of cells to determine the aneuploid population, but this has been found not to be particularly valuable [15]. The variability of urine cytology means that better urinary markers for detection are needed to identify early stage high grade disease before it

becomes invasive, and to detect low grade superficial TCC recurrence. This would enable earlier treatment which is more effective.

1.1.4.1 What makes an ideal tumour marker?

A biomarker is a molecule whose presence, absence, or abnormal physiological status can be used as an indicator of disease or injury. An assay, as a means of measuring/detecting the biomarker needs to be simple, highly sensitive and specific, reproducible and ideally low cost. The marker may be useful for different purposes such as screening, diagnosis and/or disease monitoring.

Screening involves testing seemingly healthy individuals for disease and is normally performed on a specific group in a population. For example a test for prostate specific antigen (PSA) combined with digital rectal examination has been approved by the U.S. food and drug administration (FDA) to screen men over the age of 50 for elevated levels of PSA which may be indicative of prostate cancer. Markers for diagnosis may be most important in situations where biopsy is not feasible for example in certain brain cancers. An assay for monitoring disease should be quantitative for a given marker of interest, and suited to longitudinal analysis. The levels of the marker could be useful for determining whether: the treatment has been successful, there is disease recurrence, or disease progression.

Simple detection methods

For BCa, finding a minimally invasive means of detection is important as the current gold standard for detection is cystoscopy which is an invasive procedure. Ideally therefore, an assay for detecting a marker should be suitable for use with voided urine specimens, because BCa extends into the urine therefore the urine itself has the potential to contain numerous biomarkers for TCC. These markers may be in the soluble fraction, present on excreted tumour cells or within the urine sediment.

Ideally any biomarker assay should be a point-of-care assay (performed in clinic, at patients' bedside, or through a quick response laboratory) giving an immediate result to the

clinician and patient. This would reduce stress for the patient and the need to send sample to the laboratory. This is however not essential and may not always be possible, as other non-invasive tests such as PSA for prostate cancer require samples to be sent to the laboratory [16]. The most important thing is that it is simple to perform and analyse and is accurate. Some currently available tests and those under investigation for both point of care and tests requiring laboratory analyses will be discussed later.

Accuracy

How accurate an assay is depends on several features including the inherent sensitivity and specificity of the marker being detected, but also the instability of the specimen, poor specimen storage, or shelf life of the assay. Furthermore, the population being tested will also influence not the accuracy of the assay but certainly the accuracy of the biomarker considerably. For example patients with benign genitourinary conditions such as benign prostatic hypertrophy or a urinary tract infection (UTI) may cause an increase in false positives due to the biomarker being present in these benign conditions. Conversely excluding these patients may decrease the number of false positives. Therefore it is important that the biomarker is tested thoroughly taking into account different patient groups allowing the reliability and accuracy of the biomarker to be thoroughly analysed. It is also important to know for what group of people the test is being designed. For example is the test going to be used for screening of the general population, for people presenting with suspected bladder cancer, or being followed up for known bladder cancer. If an biomarker was used that had not been thoroughly tested a patient could be misdiagnosed potentially leading to a patient not receiving treatment or having to go through unnecessary procedures [17].

Therefore the efficiency of the marker, as an indicator of disease, and the assay to detect the marker effectively are statistically analysed using a contingency table. This consists of four compartments representing the following in terms of BCa marker and assay evaluation respectively: 1) True positives – BCa and biomarker present/ positive test; 2) False negatives – BCa and but biomarker not present/ negative test; 3) True negatives – no BCa

and biomarker not present/ negative test; 4) False positives – no BCa but biomarker present/ positive test [17].

This information is used to calculate the sensitivity, false negative rate, specificity and false positive rate. Sensitivity is the percentage of people with BCa and a positive test calculated as true positive/ (true positive + false negative) × 100, whereas false negative rate is the opposite (false negative/ (true positive + false negative) × 100). An ideal tumour marker or tumour marker assay should have a sensitivity approaching 100% and a false negative rate approaching 0%.

Specificity is the percentage of individuals without BCa with a negative test calculated as true negative/ (true negative + false positive) × 100. Conversely the false positive rate is calculated by false positive/ (true negative + false positive) × 100. Again an ideal tumour marker assay should have a specificity approaching 100% and a false positive rate approaching 0%. Therefore to have an accurate tumour marker assay both the sensitivity and specificity have to be high.

Prior to development of a biomarker assay it is paramount the biomarker is tested and found to be highly sensitive and specific. Without assessing the markers capability to be indicative of the disease, any subsequent assay development would be flawed as the true effectiveness of the test would not be determinable.

Marker assay development

Once a putative biomarker has been identified a detection assay has to be developed and tested prior to being clinically approved. Lokeshwar (2005) describes the need for standardising the phases of assay development and describes four phases summarised here [17].

Phase 1: Assay development and evaluation of clinical prevalence (feasibility studies)

Feasibility studies are the first step where an assay has to be optimised and shown to be reproducible. Feasibility studies should record the prevalence and expression of any

markers and also examine the markers association with demographic and clinical characteristics in a representative study cohort.

Phase 2: Evaluation studies for clinical utility

Further optimisation may be performed to improve the assay, but the ultimate goals are to (1) refine hypothesis and (2) define standards for phase 3 studies so the assay can be performed precisely by other investigators.

Phase 3: Confirmation studies

Phase 2 is repeated with sufficient power in a larger defined clinical setting of an independent prospective cohort of patients. The clinical utility of an assay its performance and interpretation is established here.

Phase 4: Validation and technology transfer as application studies

The aims here are to (1) transfer techniques and established methods of assays and other aspects of the technology and (2) to evaluate other investigators abilities to apply the methods and interpret the results. In this phase the assay is incorporated into clinical practice [17].

Phase 1 and 2 are often carried out within a single institute whereas phases 3 and 4 require multi-institutional and international involvement to obtain as much information from as many patients as possible allowing all aspects of the assay to be examined thoroughly.

1.1.4.2 Current and potential bladder cancer tests

Reviewing all of the available literature on current and potential BCa markers is beyond the scope of this work. However a number of the non-invasive clinically approved BCa tests and markers under investigation will be reviewed and many others are detailed in Table 1.3.

BTA stat and TRAK

BTA *stat* (Polymedco Inc., Redmond, Washington, USA) is and immunochromatographic assay for BTA (bladder tumour antigen). The antigen detected by the test was identified to be a human complement factor H-related protein (hCFHrp) [18]. The test detects BTA using five drops of voided urine placed into the test device which is left for 5 min to allow the reaction to take place [19]. The urine comes into contact with latex particles coated with IgG targeted against BTA. When agglutination occurs there is a colour change indicating a positive result.

There is also a laboratory based test, BTA TRAK, which is a quantitative sandwich immunoassay measuring the levels of hCFHrp. As with other tests variability was seen between stages and grades of tumours the sensitivity being highest for high grade tumours. Both BTA *stat* and BTA TRAK show improvements in sensitivity in comparison to urine cytology especially in low tumour grade superficial TCC detection. The sensitivity for G1 was 13% to 55% and G2 and G3 were 36% to 67% and 63% to 90% respectively furthermore the specificity of the tests was >90% [17].

A major drawback of these tests is the increase in false positives in patients with benign genitourinary conditions, particularly haematuria. Patients presenting with these conditions would therefore have to be excluded. However, the tests may aid in the monitoring of patients with a history of BCa. They have the advantage of being clinic-based with an almost immediate result.

ImmunoCyt™

ImmunoCyt™ (Diagnocure, Inc., Saint-Foy, Quebec, Canada) is an immunofluorescence based test for three cellular markers. These markers are tumour associated antigens M344, 19A211 and LDQ10. The M344-antibody identifies a mucin-like high molecular weight determinant on a cytoplasmic protein, while the 19A211-antibody detects a highly glycosylated form of carcinoembryonic antigen (CEA). Lastly the LDQ10-antibody is directed against a mucin [15, 20]. The assay detects these markers in exfoliated cells of the transitional epithelium [21]. This laboratory based test requires 20-40 ml of urine that is

fixed immediately with 50% ethanol followed by the addition of a special fixative solution. The cells are then filtered out and fixed onto slides for immunocytochemical analysis [22].

The combination of urine cytology and ImmunoCyt™ has been shown to give an overall sensitivity of 92% (89, 88, and 94% for grade 1-3 malignancies respectively) in one study. The overall sensitivity for ImmunoCyt™ alone was 89% (89, 86, and 91% for grade 1-3 malignancies respectively) whereas cytology was 50% (11, 55, and 74% grade 1-3 malignancies respectively). The specificity of cytology was however 98% [15].

This test may provide a useful addition to urine cytology in the management of BCa but is insufficient to replace urine cytology. In addition the assay could not be used to determine tumour grade as the distinction between the grades is too small.

NMP22 test

Nuclear matrix protein 22 (NMP22) test kit (Matritech, Inc., Newton, Massachusetts, USA) is an immunoassay detecting elevated levels of a nuclear mitotic apparatus protein which is a component of the nuclear matrix. NMPs make up the non-chromatin structure that confers nuclear shape, organises the chromatin and regulates critical aspects of mitosis [23]. This test is the only one approved for use in diagnosing BCa for patients displaying symptoms or at high risk of developing TCC [22].

The test kit is able to detect complexed and fragmented forms of the protein quantitatively via a microtiter sandwich enzyme-linked immunoassay (ELISA). Simon *et al.,* (2003) offers a simple review of NMP22 test studies showing an overall test sensitivity of 60-70% and specificity of 60-90%. Simon noted that the ranges observed may be influenced by the cut off point (recommended to be 10 U/ml) and the patient cohort used in a study [22].

Overall the NMP22 test has shown potential to be used by clinicians to help them determine a suitable time frame for repeat cystoscopies thus potentially decreasing patient discomfort and anxiety and also costs. This test cannot differentiate between tumour grades and is influenced by other medical conditions but has found a role in a specific cohort of patients.

Mcm5

A potential new test to detect BCa is an immunofluorometric marker assay (analytical sensitivity of 10 pmol/L; equivalent of ~4,000 cells) to measure the level of minichromosome maintenance 5 (Mcm5) protein in exfoliated cells in the urine. This test is currently undergoing phase three clinical trials. Mcm5 protein expression is deregulated in epithelial cells in the first stages of carcinogenesis [24]. The results so far have shown 92% sensitivity (cf. 15% for urine cytology) and a negative predictive value (true negative / (true negative + false negative)) of 97% for patients with low grade superficial cancer. In addition applying a higher cut off point (6,000 cells) to samples from patients with a higher risk for BCa may be more applicable and also improve performance of the test for high grade disease detection.

Improvements in performance are needed to reduce the number of false-negatives. Additional trials are underway to determine whether the Mcm5 assay can replace or reduce the frequency of cystoscopies in the management of patients with or at high risk of developing TCC. Furthermore, the Mcm5 assay showed potential for the detection of different tumour types such as prostate carcinomas so may not be BCa specific [25].

Table 1.2: Bladder tumour markers for detection and surveillance

Test/Marker	Marker Detected	Specimen	Assay Type	Marker Type	Manufacturer	Sensitivity (%)	Specificity (%)
Cytology	Tumour cells	Voided urine, barbotage specimen	Microscopy	Cell morphology	Diagnostic reference laboratories	11-76	>90
Haematuria detection	A: Haemoglobin	A: Voided urine	A: Dipstick	A: Soluble protein	A: Bayer Diagnostics‡	A: 50-90	A: Low
	B: Red blood cells	B: Voided urine	B: Interference-contrast microscopy or red blood cell analyser	B: Red blood cell morphology	B: -	B: ~100	B: ~100
BTA-Stat	hCFHrp (also CFH)	Voided urine	Dipstick immunoassay	Soluble antigen	Polymedco Inc. ¥	36-89 (low for low grade tumours, low tumour volume)	50-70 (low among benign urologic conditions)
BTA-TRAK	hCFHrp (also CFH)	Voided urine	Sandwich ELISA	Soluble antigen	Polymedco Inc. ¥	57-83 (depends on cut-off limit selection)	~50 in benign urologic condition; ~90 in healthy individuals
NMP-22	Nuclear mitotic apparatus protein	Voided urine	Sandwich ELISA (newer version: point-of-care device)	Soluble antigen	Matritech, Inc ¤	47-100 (depends on cut-off limit selection, tumor volume and patient population)	55-80 (depends on presence of benign urologic conditions)
BLCA-4	Nuclear matrix protein	Voided urine	ELISA (using a rabbit polyclonal antibody)	Soluble antigen	-	96.4	100 in healthy individuals; 81 in other urologic conditions

Table 1.2: Bladder tumour markers continued

Test/Marker	Marker Detected	Specimen	Assay Type	Marker Type	Manufacturer	Sensitivity (%)	Specificity (%)
Survivin	A member of inhibitors of apoptosis gene family	Voided urine	Bio-dot test (dot blot assay using a rabbit polyclonal antibody)	Soluble antigen	-	100	87-100
UBC	CK8 and 18 (cytoskeletal proteins)	Voided urine	Sandwich ELISA or point-of-care test	Soluble antigen	IDL Biotech *	36-79 (may be low to detect Ta, T1 tumours)	88-92 (may be low in benign urologic conditions)
Cytokeratin 20	Cytoskeletal protein	Exfoliated cells	RT-PCR or immunocytology	mRNA or cell-associated protein	-	82-87	55-70 (low in benign urologic conditions)
CYFRA 21-1	CyK 19 (a cytoskeletal protein)	Voided urine	Immunoradiometric assay or electrochemiluminescent immunoassay	Soluble antigen	Cis-Bio International ◊; Roche Diagnostics ♩	75-97; ~55 to detect G1 tumours	67-71 (low for urolithiasis, stenosis, BPH, and UTI)
HaA-Haase	Hyaluronic acid and Hyaluronidase	Voided urine	ELISA-like assays using a biotinylated HA-binding protein	2 soluble matrix components	-	88-94	Overall 84; 63-71 in recurrent tumours: 60 % false positives turn true positive in 5 months
Microsatellite DNA test	Microsatellite markers on chromosomes	Exfoliated cells	Genomic DNA PCR	Genomic DNA	-	72-97	>95 in healthy individuals; false positives if BPH, cystitis
Telomerase (TRAP assay)	Enzyme activity	Exfoliated cells	TRAP assay	Cell-associated enzyme	Qbiogene §	70-90; but as low as 7-46 (enzyme unstable in urine)	60-70 (low if UTI, urolithiasis, or inflammation present)

Table 1.2: Bladder tumour markers continued

Test/Marker	Marker Detected	Specimen	Assay Type	Marker Type	Manufacturer	Sensitivity (%)	Specificity (%)
Telomerase (hTERT)	hTERT	Exfoliated cells	RT-PCR (conventional or real-time)	mRNA for hTERT	-	83-95 (but as low as 24)	60-70 (low if UTI, urolithiasis, or inflammation present)
ImmunoCyt	CEA, 2 bladder tumour cell-associated mucins	Exfoliated cells	Immunocytochemistry	Cell-surface antigen	DiagnoCure, Inc. ‡‡	38-90 (low for low grade tumours)	73-80 (low id microhematuria, BPH, cystitis present)
DD23	185-kDa tumour-associated antigen	Exfoliated cells	Immunocytochemistry	Cell-surface antigen	Urocor ¤	73-100	33-67.5
Quanticyt	Mean nuclear shape and DNA content	Exfoliated cells	Computerised analysis of light microscopy images	Nucleus, DNA	-	59-69	70
Mcm5	Mcm5 (minichromosome maintenance 5) protein	Exfoliated cells	Immunofluorometric	Cellular antigen	-	92	73
UroVysion	Alterations in chromosomes 3, 7, 17, and 9p21	Exfoliated cells	Multicoloured, multiprobe FISH	Denatured chromosomal DNA	Vysis ¥¥	68-87; (low for low-grade tumours [36-55])	>90

Table 1.2: Bladder tumour markers continued

BPH = benign prostate hyperplasia; BTA = Bladder tumour antigen; CYFRA = cytokeratin fragment; ELISA = enzyme-linked immunosorbent assay; FISH = fluoresence in situ hybridisation; HA = hyaluronic acid; HAase = hyaluronidase; hTERT = human telomerase reverse transcriptase; NMP = nuclear matrix protein; PCR = polymerase chain reation; RT = reverse transcription; TRAP = telomeric repeat amplification protocol assay; UBC = urinary bladder cancer.

‡ *Bayer Diagnostics, Leverkusen, Germany*
¥ *Polymedco Inc. (formerly Bard/Bion Diagnostics, Redmond, WA)*
Matritech, Inc., Newton, MA ¤
**IDL Biotech, Borläbger, Sweden*
◊ *Cis-Bio International, Gif-sur-Yvette, France*
ᵻ*Roche Diagnostics, Burgess Hill, UK*
§*Qbiogene, Geron Corp., Menlo Park, CA*
‡‡ *DiagnoCure, Quebec City, Quebec, Canada*
¤¤ *Urocor, Oklahoma City, OK*
¥¥ *Vysis/Abbott Labs, Abbott Park, IL*

from Lokeshwar *et al.*, (2005) [17]

1.1.5 The future for bladder cancer markers

Overall the marker assays currently available for use by clinicians all have their uses but no single marker has excellent sensitivity and specificity for all TCCs in order to replace cystoscopies or even urine cytology. Many of the assays such as ImmunoCyt may nevertheless complement urine cytology and thus reduce the interpreter subjectivity seen with urine cytology. Other assays such as BTA-TRAK and the NMP-22 test could be used to help the clinician determine a suitable time period between cystoscopies.

Unfortunately many of the tests listed in Table 1.3 are also affected by one or more benign conditions and/or other cancers. As described earlier it is very important to thoroughly test an assay with samples from individuals with varying benign conditions and stages and grades of cancer. However, if it is established that a test is affected by conditions such as haematuria, increasing the number of false positives, how useful can that assay actually be? Especially when taking into account that 85% of people diagnosed with bladder cancer present with haematuria, thus limiting such an assay to those patients not presenting with haematuria. Only 15% of bladder cancers would therefore be identified. However, it might be useful in the follow-up phase.

Any new potential markers need to be thoroughly examined assessing various aspects such as its detectability, stability, and quantification of the effects of benign conditions. Furthermore, is the assay specific to a particular stage or grade of tumour or does it detect them all equally? Where quantitative assays are concerned it is important to establish a suitable cut off point giving the assay its best sensitivity and specificity. This also needs to be maintained throughout multicentre trials. Once all the data has been collected and analysed it may then be possible to determine an assays best possible application. For example a test such as the HAase ELISA may be able to identify high grade disease specifically and potentially before it becomes invasive [26]. Others may be useful in identifying recurring disease such as the UBC test which showed higher low grade tumour sensitivity than other available tests although sensitivity was only 66% [27]. It is apparent that many years of work and data analysis is required to determine a markers true potential.

In recent years it has become clear that the molecular heterogeneity of bladder tumours makes it unlikely that one molecule will enable an accurate diagnosis. There is therefore a need for a panel of markers which are likely to be more useful in the diagnosis and monitoring of the TCC [28-30]. One way of identifying molecular markers may be through molecular profiling. However, the molecular heterogeneity of cancers may also pose problems here as subpopulations of cells may be present within the tumour that are specific to a particular stage in the tumours evolution [30]. Alternatively, some studies have demonstrated the benefit of using several of the currently available urine based BCa tests. For example Sánchez-Carbayo (2001) examined the use of UBC, CYFRA 21-1 and NMP-22 together as a means to individualise intervals between cystoscopies [31].

It may be beneficial to perform molecular profiling on many different tumours allowing the identification of genes and proteins that are common to the different grades and stages of disease. This may help identify a panel of markers that may be useful in diagnosing and monitoring BCa. These markers could also be important in the pursuit for new therapeutic targets and understanding the molecular pathways of the disease. Markers identified may also allow for improvements in patient stratification to maximise therapeutic interventions which is the holy grail of individualised medicine.

In order to identify a potential marker panel it may be necessary to utilise novel urine biomarker sources because of the complexity of the urinary proteome and genome. Looking at the urinary proteome specifically it is likely that this will contain abundant proteins such as Tamm-Horsfall protein (THP) that may mask other less abundant proteins of interest in BCa. Chattergee (2005) compared the proteome of lyophilised urine samples from BCa patients and patients presenting with microhaematuria under suspicion of bladder cancer (their control). They identified just three proteins (Reg-1, CK2, and CD5) elevated in cancer [32]. This was probably due to high levels of abundant proteins masking the less abundant proteins that may be relevant in cancer. Other contributing factors which may impact upon the complexity of the urine include proteinuria and haematuria [33]. Proteinuria would lead to a large number of abundant non disease specific proteins whereas haematuria would alter the cellular composition of the compartment and introduce blood

proteins to the sample, such as highly abundant albumin and immunoglobulins. The volume of fluid poses problems when analysing urine as potential biomarkers may be very dilute. Also in relation to dilution is the hydration state of the individual this may affect the solute concentration and potential biomarker concentration. Also the presence of salt within the urine may create difficulties in analysing it, particularly with respect to proteomics where salt is a major interfering substance. Proteases present may also affect the susceptibility of the sample to spontaneous degradation. Furthermore the biological content of the urine will be derived from multiple cell types (from the kidney and downstream of the renal tract) [34-36] and thus urine represents the genitourinary tract as a whole and is not a means of selecting just bladder derived proteins.

Therefore it may be prudent to examine a sub-proteome of the urine which is likely to contain fewer if none of the most abundant proteins in urine, such as THP, thus reducing the sample variability. This may reveal relatively low abundant proteins which may be of greater clinical interest. Smalley *et al.,* (2008) undertook a proteomics study examining one such subset: urinary microparticles and identified eight proteins that had altered levels in bladder cancer compared to healthy donors [33]. However, the method used here means the sample analysed although a subset of the urinary proteome it is still nonetheless a highly complex mixture of microvesicles, exosomes and other urinary constituents that can be pelleted by high speed ultracentrifugation. As a consequence, the true source of the markers is unknown. If there was a single source for all of the markers of interest, such as excreted tumour cells, they would be more likely to be consistently present and possibly related functionally to one another. This study does nevertheless offer insight into the potential advantages of analysing a subset of the urinary proteome for biomarker identification.

Of the urinary constituents, nanometre sized vesicles termed exosomes may offer themselves as a particularly good source of potential biomarkers. Exosomes are secreted into the extracellular space by most if not all cell types. Many tumour cells have been shown to secrete particularly high amounts of exosomes and these are known to be enriched in tumour associated antigens and membrane proteins in particular [37-42].

With respect to BCa, cancerous urothelial cells due to their proximity to the urinary space are likely to secrete exosomes into the urine. These exosomes can be purified from the urine [34] and might provide a complex panel of BCa associated proteins that could represent disease status.

1.2 *Exosome biology and purification*

Exosomes are nanometre sized vesicles (30-100 nm in diameter) naturally secreted by living cells into the extracellular milieu both *in vivo* and *in vitro*. They were first described in the 1980's as a means of purging cellular mass and transferrin receptor from reticulocytes during their maturation into erythrocytes [43]. They have since been shown to be released by numerous cells types including B-cells [44-47], T-cells [48, 49], mast cells [50-53], neuronal cells [54, 55], hepatocytes [56], fibroblasts [44], numerous cancer cells [40, 57, 58] and others. Furthermore they have been isolated from biological fluids including urine [34, 59, 60], saliva [61], breast milk [62], blood [63-65] and malignant effusions [66-68]. Exosomes have been isolated from various mammalian species (humans and rodents), reptiles and birds [69-71]. Furthermore the cellular compartment that gives rise to exosomes is present in yeast and plants [71, 72]. Exosomes may therefore be a fundamental feature of eukaryotic cells.

1.2.1 Exosome formation

Exosomes are formed in the endocytic tract within multivesicular body (MVB) compartments. The pre-exosomes contained within these compartments are released into the extracellular fluid space when the outer membrane of the MVB fuses with the plasma membrane. This endosomal origin of exosomes has been shown by several electron microscopy studies demonstrating the fusion of MVBs with the cell membrane releasing the exosomes (Figure 1.3) [47, 73, 74]. However, more recently an additional mechanism for formation and release of exosomes from T cells (specifically Jurkat) has been presented. This mechanism involves the outward budding of plasma membrane domains enriched in exosomal and endosomal proteins [75]. It has also been proposed that this

manner of exosome formation may be hijacked by HIV for the formation of infectious particles [76, 77].

For endocytic tract exosome formation, proteins are segregated in the outer membrane of the MVB prior to being internalised by membrane invagination. It has long been thought that the sorting of proteins within the MVB involves a group of proteins known as the endosomal sorting complex required for transport (ESCRT). This sorting normally involves the mono-ubiquitinylation of proteins as a means of tagging them for recruitment by the ESCRT machinery [78, 79]. A proportion of cellular proteins within the endosomal tract may ultimately be delivered through MVBs to the lysosome for degradation. Alternatively, this compartment can also result in vesicular secretion.

Endosomal sorting of MHC class I into MVBs has been shown to involve the ESCRT machinery as follows (Figure 1.4). Once the MHC class I protein is ubiquitinated it appears to be recruited by the ESCRT machinery into the endosomal pathway at the MVB limiting membrane. The recruitment is in association with ESCRT-0/clathrin lattices. ESCRT-I then binds to the ubiquitinated cargo protein which activates ESCRT-II. ESCRT-II initiates the oligomerisation of more than four small coiled-coil proteins forming the ESCRT-III complex which is a large endosomal associated structure. In this final complex the ubiquitin tag is removed prior to sorting into the MVB. The ESCRT-III complex also appears to be involved in the concentration of the MVB cargo [78, 80, 81]. The ESCRT components found in secreted vesicles may help distinguish these vesicles from the plasma membrane and plasma membrane derived microvesicles. Nevertheless, not all proteins found in exosomes are ubiquitinated. Furthermore the selective knockdown of certain ESCRT constituents impacts upon the lysosomal/degradation route but may not adversely affect exosome formation. Therefore, the ESCRT pathway does not offer a full explanation for exosome formation.

An alternative pathway for the sorting of cargo in to MVBs has been demonstrated relating to the lipid membrane constituents of the MVB. In this sorting mechanism raft based microdomains within the MVB are thought to help segregate the proteins for lysosomal

degradation from those to be incorporated into exosomes. These microdomains within the limiting membrane of MVBs contain large concentrations of sphingolipids. Ceramide is produced from the sphingolipids by the action of neutral sphingomyelinase. This ceramide is able to induce spontaneous domain budding in the MVB (Figure 1.5). Interestingly exosomes have been found to be enriched in ceramide but upon the inhibition of neutral sphingomyelinases the release of exosomes was reduced [82]. It is thus likely that these sphingolipid rich microdomains and the formation of ceramide from these sphingolipids may be responsible for exosome formation within the MVB.

In addition, Rab GTPases have been shown to be involved in exosome secretion. Rab27a and Rab27b have been demonstrated to have different functions in MVB docking at the plasma membrane. The silencing of two known Rab27 effectors has been shown to inhibit exosome release [83]. Another study using oligodendrocytes suggests that Rab35 may also be involved in exosome release [84]. It is clear that exosome formation is still poorly understood and requires more research to enable us to fully understand the processes involved.

During exosome formation cytosolic proteins are incorporated into their lumen and thus extracellular domains of the transmembrane proteins (Figure 1.5) are oriented toward the extracellular environment following release into the extracellular milieu. This membrane protein orientation has been demonstrated by labelling whole mounts of exosomes for the extracellular domains of membrane proteins such as MHC class II molecules for immuno-electron microscopy [47, 85]. Clayton *et al.,* (2001) also showed that beads coated with antibody specific for MHC class II molecules can bind exosomes in cell-culture supernatants. Furthermore, these exosome-bead complexes also stained for an array of other membrane proteins such as CD63, ICAM-1 and CD59 [86]. These observations are consistent with the proposed membrane orientation and 'inward budding' model exosome biogenesis.

Electron microscopy studies have demonstrated the relative enrichment of membrane proteins and cholesterol in the exosome membrane. The exosome fusion electron

micrograph shown in Figure 1.3 illustrates that cholesterol and MHC class I labelling is almost entirely restricted to the exosomes [87], whereas the plasma membrane and the limiting membrane of the MVB show very little labelling. The recruitment and enrichment of proteins and lipid constituents are fundamental properties of exosomes. It is this specific enrichment of proteins in the exosome membrane, compared to the plasma membrane, which may be of particular importance in facilitating the identification of novel biomarker proteins.

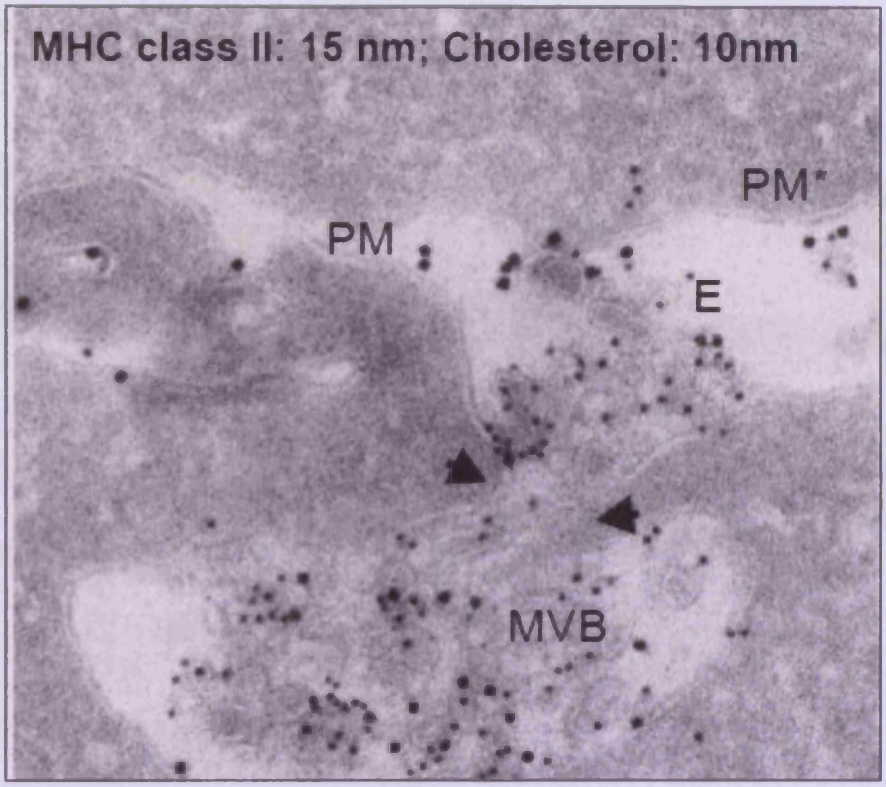


Figure 1.3: Fusion of MVB with the plasma membrane releasing exosomes rich in cholesterol and MHC class II into the extracellular fluid space

Cryosections of B-cells (RN cells) demonstrating the fusion profile (indicated by arrows) between a multivesicular body (MVB) and plasma membrane (PM), releasing exosomes (E) into the extracellular fluid space. The sections were double-labelled for cholesterol (10 nm) and MHC class II (15 nm) showing the staining to be almost entirely on the vesicles and not on the plasma membrane or MVB limiting membrane. Image taken from Wubbolts *et al.,* (2003) [87].

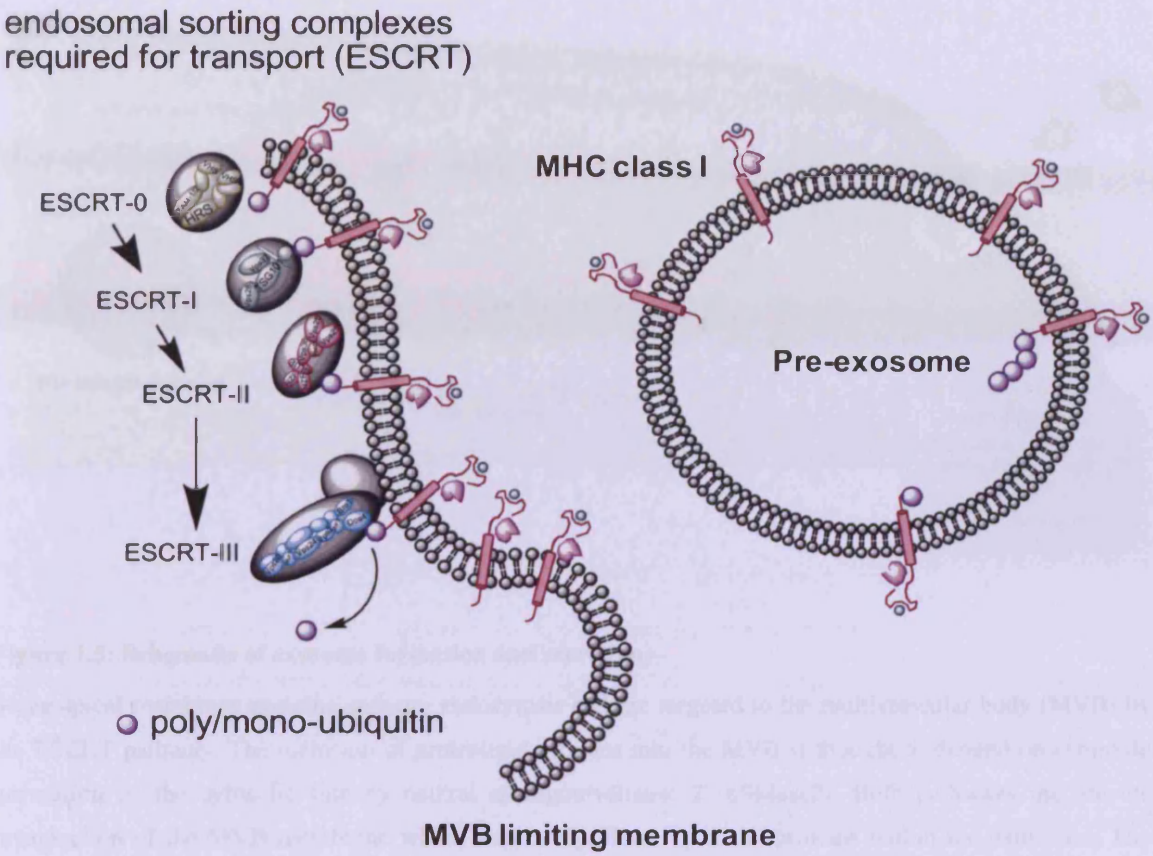


Figure 1.4: The involvement of the ESCRT complex in MHC class I MVB incorporation

Ubiquitinated MHC Class I associated with ESCRT-0/clathrin lattices in the MVB limiting membrane. ESCRT-I then binds to the ubiquitinated Class I, this activates ESCRT-II. ESCRT-II initiates the oligomerisation of more than four small coiled-coil proteins forming the ESCRT-III complex. In this final complex the ubiquitin tag is removed prior to sorting into the MVB.

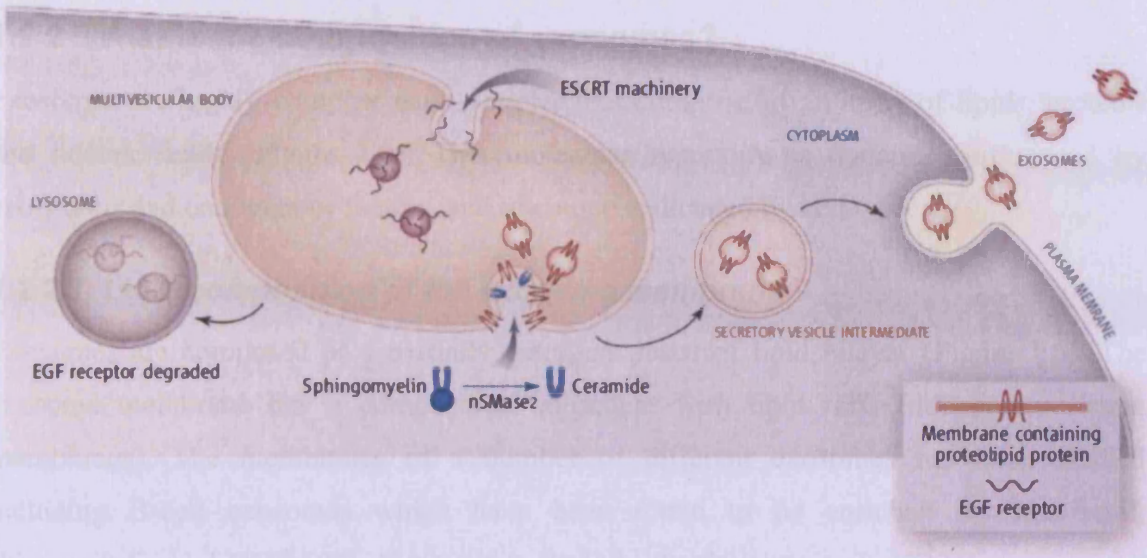


Figure 1.5: Schematic of exosome formation and secretion

Some apical membrane proteins undergo endocytosis and are targeted to the multivesicular body (MVB) by the ESCRT pathway. The inclusion of proteolipid proteins into the MVB is thought to depend on ceramide generation on the cytosolic side by neutral sphingomyelinase 2 (nSMase2). Both pathways include the invagination of the MVB membrane which also encapsulates cytosolic proteins within the exosomes. The extracellular domain of the membrane proteins are returned to the outer surface of the membrane. After many vesicles have accumulated, the MVB fuses with the apical membrane releasing the exosomes in to the extracellular fluid space. From Marsh and van Meer, (2008) [88]. This scheme suggests the potential for multiple types of nano-vesicle perhaps within the same MVB.

1.2.2 What is the composition of exosomes?

Exosomes are highly complex nano-vesicles that comprise of an array of lipids, proteins and nucleic acids (Figure 1.6). This molecular repertoire is dynamic, influenced by exogenous and endogenous factors, and of course is dictated by cell type.

1.2.2.1 Lipid composition of the exosome membrane

Exosomes are composed of a partially detergent resistant lipid bilayer (Figure 1.6). The exosome membrane has a composition consistent with lipid rafts (detergent resistant membranes). The membranes of a number of different exosomes has been studied including B-cell exosomes which have been found to be enriched in cholesterol, shingomyelin and GM3 [87]. However, a study by Laulagnier *et al.,* (2004) on mast cell and dendritic cell (DC) derived exosomes did not show the same enrichment of cholesterol. Laulagnier did nonetheless observe the loss of phospholipid asymmetry in exosomes with increase in flip-flop of lipids between the two leaflets compared with the parent cell [89].

A preliminary study by the same group proposed that the lipid lysobiophosphatidic acid (LBPA) may be essential for exosome biogenesis, within the MVB, but that the LBPA is not passed on to the exosomes. It was also proposed by Wubbolts *et al.,* (2003) that the recruitment of proteins into the limiting membrane of MVBs may involve their incorporation in to tetraspanin-containing detergent resistant membrane domains [87].

Exosome membranes have also been found to contain phosphatidylserine (PS) that is distributed equally between inner and outer leaflets. This is something unlike the plasma membrane of normal cells where PS is principally located in the inner leaflet. PS becomes exposed on the cell surface early during apoptosis where it is recognised by macrophages and mediates phagocytosis of apoptotic cells [90]. Exosomal surface PS has been demonstrated by flow cytometry of Annexin-V stained exosome-bead complexes [63, 91]. Another protein with PS binding properties identified in exosomes is soluble protein milk fat globule-epidermal growth factor-factor VIII (MFG-E8). MFG-E8 (lactadherin) associates with exosomes through binding to the PS exposed on the exosome surface

through its C1C2 domain. MFG-E8 is also able to bind through its EGF-like domain to αvβ5 and αvβ3 integrins on the surface of dendritic cells (DCs) and macrophages [85]. This integrin binding facilitates the uptake of MFG-E8-PS bound entities such as apoptotic cells by macrophages. In terms of exosomal function and MFG-E8, Zeelenberg *et al.*, (2005) demonstrated *in vivo* that dendritic cells could uptake and cross-present antigens that were coupled to the C1C2 domains of lactadherin (i.e. exosomally expressed). This form of antigen acquisition was superior to the uptake of soluble antigen in eliciting anti-cancer immunity [92].

Tim-1 and Tim-4 (T-cell immunoglobulin- and mucin-domain-containing molecule) also bind PS and may assist in the uptake of exosomes [90]. The PS exposed on the surface of exosomes therefore may be a key mechanism for exosomal uptake by cells such as DCs, macrophages and others [85, 90, 92, 93]. Overall, the role of lipids in exosomes is an understudied aspect of these vesicles and certainly warrants further study.

1.2.2.2 Protein composition of exosomes

The protein composition of exosomes has been studied more extensively and has been shown not to fully represent the proteome of the parent cell. Exosomes may be viewed as depleted of proteins of the endoplasmic reticulum (ER), Golgi apparatus, mitochondrial and nuclear origin, but enriched in some membrane proteins.

A large number of proteins common to many exosomes have been identified including membrane adhesion proteins such as integrins [40, 62, 87], which are cell specific. For example αM on DCs, β2 on DCs, mast cells B cells and T cells, and α4β1 on reticulocytes [94]. Other classes of proteins identified include tetraspanins (CD9, -63, -81, -82) [34, 64, 85, 95-97]; heat-shock proteins – HSP90 [34, 56, 57], HSC70 [62, 87, 98]; proteins involved in membrane transport and fusion – annexins [34, 56, 57, 95], Rab protein family [34, 61, 95, 99]; cytoskeletal components – actin [40, 53, 99, 100], cofilin [34, 62, 95, 101], tubulin [97, 99, 100]; antigen presentation – MHC class I [48, 58, 74, 100, 102], MHC class II [59, 61, 62], ICAM-1/CD54 [46, 103, 104]; lysosomal markers – LAMP1 [46, 59, 101], LAMP2 [46, 59, 64] and metabolic enzymes – enolase-1 [62, 87, 96]. A

schematic representation of an exosome is depicted in Figure 1.6 showing the proposed structure of an exosome.

Exosomes may also contain cell type specific proteins and hence may serve some functions akin to the parent cell. For example cell specific proteins such as A33, cadherin-17, CEA, epithelial cell surface antigen (EpCAM), and mucin 13 were identified in colon tumour cell line LIM1215 [96]. Some proteins that are associated with cancers can be found in abundance in cancer exosomes.

Exosomal proteins have been identified by a number of means including western blotting [36, 46-48, 51, 58, 74], flow cytometric analysis of exosome coated beads [46, 48, 86, 99], and using proteomics technologies such as two-dimensional electrophoresis (2DE) coupled with mass spectrometry (MS) [40, 105] or 1DE liquid chromatography tandem MS (LC-MS/MS) [96, 101, 106]. Proteomics analysis of exosomes using various technologies has expanded our knowledge of proteins expressed by exosomes greatly in the last decade and will be discussed in detail later.

Some of these exosome studies have also helped confirm that exosomes are not just membrane fragments because they lack some abundant cell surface receptors. For example DC derived exosomes do not express Fc receptor [99] and B-cell derived exosomes lack transferrin receptor [47, 86]. Furthermore, some of the exosomal proteins such as annexin II, RAB5/RAB7 and TSG101 can be found in the endocytic pathway further supporting the endosomal origin of exosomes [99, 107]. TSG101 in particular has specifically been identified to be important in the transport of proteins in to the endocytic tract and has also been shown to be enriched in exosomes [107, 108].

Overall the protein composition of exosomes is not fixed it is dynamic and heterogeneous, changing as the cell responds to its environment perhaps changing their extracellular function. Dai *et al.*, (2005) showed that exosomes derived from heat shocked CEA positive tumour cells contained increased levels of HSP70. These exosomes were also more efficient in priming cytotoxic T lymphocytes (CTL) than unstressed cells of the same

origin [109]. This is a classical example whereby change in cell status is reflected through molecular changes in the exosomes produced, and this has bearing on exosome function.

In terms of the phenotype plasticity of exosomes, cancer cells may be of particular interest as they can be considered as stressed cells because they may be deprived of oxygen and nutrition and are also under attack from the immune system. This endogenous stress may therefore be reflected in the exosomes. The presence of stress associated proteins such as NKG2D ligands have been identified in exosomes derived from cancer cells. These exosomes were able to function in the suppression of NKG2D expression in peripheral blood leukocytes. As a consequence these exosomes were able to aid the tumour in evading the immune system [110]. Therefore the function and composition of cancer exosomes may be different from non-cancer sources making them a potentially good source of cancer biomarkers.

1.2.2.3 RNA content of exosomes

The presence of messenger RNA (mRNA) and microRNAs (miRNA) in exosomes was first discovered recently by Valadi *et al.,* (2007). Valadi revealed the presence of 1,300 mRNA and 120 miRNAs in exosomes derived from mouse and human mast cells many of which were not present in the cytoplasm of the parent cell. This indicated a mechanism for specifically selecting these RNA species for inclusion into exosomes. The authors also coined the term exosome shuttle RNA (esRNA) for these observations. They further demonstrated that the mRNA was transferable from one cell to another and that the mRNA can be functional in its new location leading to the translation of the acquired RNA. This was demonstrated by new mouse proteins being transiently expressed in recipient cells [111].

Exosomal miRNA have since been identified in circulating tumour-exosomes from ovarian cancer patients and lung adenocarcinoma patients, isolated using magnetic beads coated with anti-EpCAM antibody. These studies identified differences in the miRNA profiles of patients and control samples. The profiles of the cancer patient miRNA were also established to be very similar to the tumour tissue profiles [112, 113]. Other identified

sources of exosomal miRNAs include circulating placental derived exosomes, human saliva, and EBV-infected B-cell exosomes [114-116].

Recently it has been demonstrated that exosomal regulatory miRNAs are functional in their target cells. Pegtel *et al.*, (2010) showed that exosomes derived from EBV-infected cells were able to transfer miRNA to an uninfected cell and once in the cell the miRNA were able to repress EBV target genes [116]. The incorporation of miRNAs into exosomes and their secretion in to the extracellular environment is proposed to be independent of the ESCRT machinery [117]. It appears that the shuttling of specific RNA into exosomes may play an important role in the functioning of exosomes. mRNA and particularly current miRNA analysis of exosomes may offer additional options for identifying disease relevant molecules.

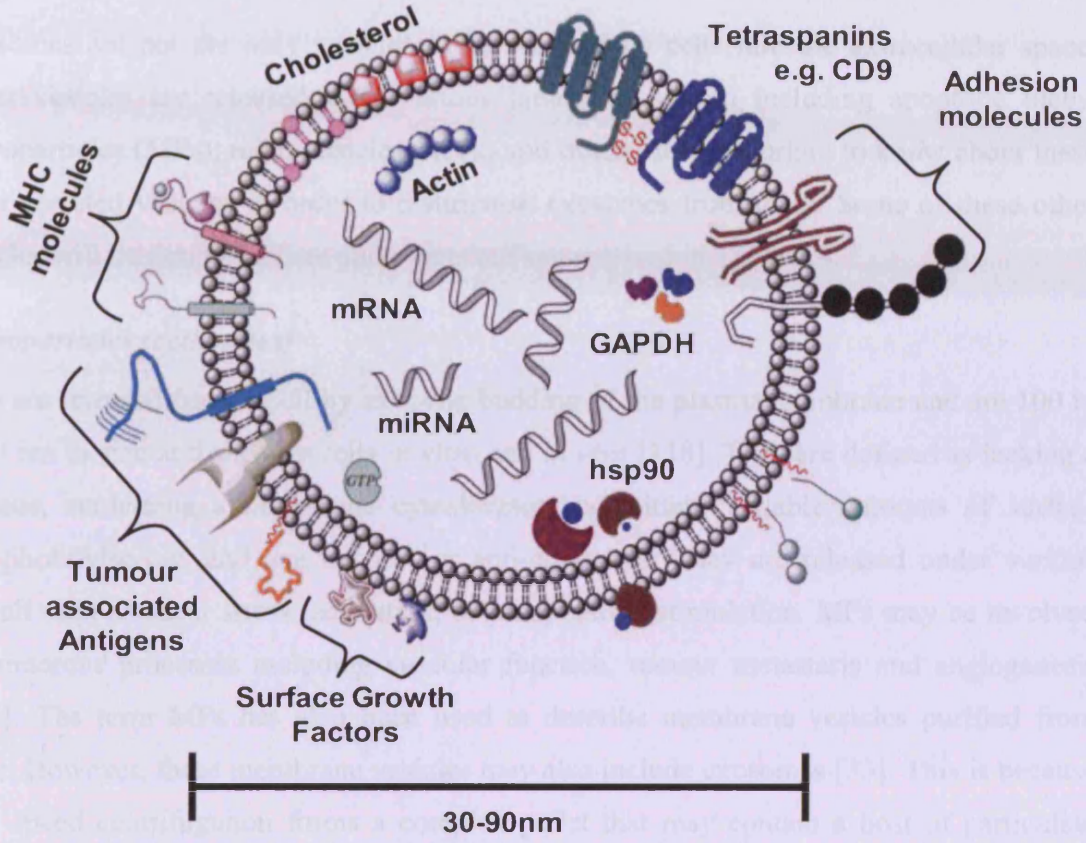


Figure 1.6: Schematic representation of the proposed structure of an exosome

The exosome bounded by a lipid bilayer, which contains cytosol-like intraluminal components from the parent cell. The extracellular domains of various transmembrane proteins are exposed at the exosome surface. Proteins from the groups denoted are known to be expressed by exosomes; mRNA and miRNA are also known to be contained within the lumen. Exosomes are recognised to be between 30 and 90 nm in diameter (from most cell types and most publications).

1.2.3 Comparison of exosomes with other secreted vesicles

Exosomes are not the only vesicles to be secreted by cells into the extracellular space. Other vesicles are released after various biological stimuli including apoptotic blebs, microparticles (MPs), microvesicles (MVs) and others. It is important to know about these other secreted vesicles in order to distinguish exosomes from them. Some of these other vesicles will be described here and others are summarised in Table 1.4.

Microparticles (ectosomes)

MPs are released from a cell by exocytic budding of the plasma membrane and are 100 to 1000 nm in size and shed by cells *in vitro* and *in vivo* [118]. They are defined as lacking a nucleus, containing a membrane cytoskeleton, containing variable amounts of surface phosphotidylserine, and can be pro- or anti-coagulant. They are released under various stimuli such as shear stress, activation, or proapoptotic stimulation. MPs may be involved in numerous processes including vascular function, tumour metastasis and angiogenesis [119]. The term MPs has also been used to describe membrane vesicles purified from urine. However, these membrane vesicles may also include exosomes [33]. This is because high speed centrifugation forms a complex pellet that may contain a host of particulate material including exosomes.

Microvesicles

The term MVs appears in some instances to cover MPs and exosomes as well as other particles/vesicles [101, 120]. It is unclear whether these are distinct bodies or a mix of different secreted vesicles including MPs and exosomes this is something that needs to be clarified.

Prostasomes

Prostasomes (aposomes or seminosomes) are 50-500 nm vesicles secreted from the apical region of prostatic luminal epithelial cells [121]. Prostasomes are enveloped in a storage vesicle and are released into the seminal fluid by diacytosis or exocytosis [122]. Diacytosis is a mechanism by which the storage vesicles tear a repairable hole in the plasma

membrane to release the prostasomes [123]. Prostasomes have high cholesterol to phospholipid ratio as well as high sphingomyelin and monounsaturated fatty acid content, giving the membrane a highly ordered structure [121].

Confusing nomenclature

These descriptions of some of the different secreted vesicles demonstrate the confusion in the terminology for secreted vesicles. There is a need for clearer definitions to reduce the ambiguity in the literature. A consensus on the definition of secreted vesicles would allow researchers to characterise their samples correctly reducing the number of misidentifications and renaming. Furthermore it is my belief that published works should demonstrate that their sample is what it is claimed to be, based on a given definition. Part of the problem here is the lack of a formal definition for these vesicles (including exosomes) and also the heterogeneity within these particulate populations.

1.2.4 Defining exosomes

I would define an exosome as is a secreted vesicle of MVB origin less than 100 nm in diameter consisting of a partly detergent resistant lipid bilayer containing exposed phosphatidylserine. Exosomes have a specific floatation density between 1.1 and 1.2 g/ ml. They contain mRNA and miRNA specifically shuttled to the exosome and are enriched with endosomal and tetraspanin proteins. Furthermore they express some classical MVB markers such as ESCRT components TSG101, VPS, and LAMP1. They are rich in membrane and cytosolic proteins and contain minimal ER, Golgi, nuclear, and mitochondrial proteins. Lastly the extracellular domains of their transmembrane proteins are oriented toward the extracellular environment. Whilst some non-exosomal vesicles may share some of these properties, it is the overall features which best summarise exosomes.

Table 1.4: Characterisation of selected secreted vesicles

	Particle name	Size	Density in sucrose	Lipid composition	Intracellular origin	Comments
Exosomes	Exosomes	30-90nm	1.12-1.2 g/ml	Enriched in cholesterol and diaglycerol; expose phophotidylserine	Endosomes	Marker proteins include tetraspanins (CD9, CD63), Alix and TSG101.
	Dexosome					Exosomes released from dendritic cells; membrane enriched in shingomyelin; contain CD9. CD81, MHC class I and II.
Non-exosomal	Microparticles (MP) and ectosomes	0.1-1 μm				Size and characteristics of MP defined by Scientific and Standardisation committee (SSC) of the ISTH.
	Microvesicles (MV)	0.03-1 μm	nd	Expose phosphotidylserine	Plasma membrane	Term MV covers both exosomes and MPs?
	P2 and P4 particles (prominosomes)	~600 nm (P2); 50-80 nm (P4)	nd	nd	nd	Both particles contain CD133; P4-particlea lack CD63
	Prostasomes	50-500 nm (mean 150 nm)				Shed from the prostate gland in to the seminal fluid; not clear whether they are exosomes/MPs or a mixture; cholesterol/phospholipid ratio very high (~2)
	Apoptotic vesicles	50-500 nm	1.16-1.28 g/ml	nd	nd	contain histones
	OMVs	20-250 nm				Released by Gram-negative bacteria
Exosome-like	Exosome-like vesicles	20-50 nm	1.1 g/ml	Do not contain lipid rafts	Internal compartments?	contain TNFRI
	Argosomes	nd				Exosome-like vesicles isolated from *Drosophila*
	Epididymosomes	nd				Exosome-like vesicles isolated from human sperm; cholesterol/phospholipid ratio high (~2)
	Tolerosomes	~40 nm				Exosome-like vesicles that contain MHC II

Adapted from Simpson *et al.*, (2008) and Thery *et al.*, (2009) [123, 125].

nd: not determined.

1.2.5 What functions do exosomes have?

Originally exosomes were thought to be a means of ridding the cell of unwanted membrane proteins, such as transferrin receptor from maturing red blood cells [73]. However, exosomes are now thought to have a multitude of roles in normal physiology and assorted pathological scenarios. Mechanisms underlying these functions are varied and complex and include combinations of ligands capable of binding different cell-surface receptors on the target cell stimulating a response. Furthermore, exosomes may be taken up by target cells transferring exosome surface molecules, cytosolic contents and/or RNA. Unfortunately little is truly known about the biological function of exosomes. Some *in vivo* work has been performed suggesting some specific roles for exosomes. The vast majority of studies have concentrated on the possible effects of exosomes on immune function [38, 39, 47, 58, 74, 92, 124]. There are too many to discuss in the context of this thesis nevertheless, Thery *et al.,* (2009) offers an excellent review of proposed functions of exosomes with respect the immune system and these are summarised in Figure 1.7 [125]. A few of these exosome immunological studies will be discussed in more detail along with potential non-immune functions of exosomes.

1.2.5.1 Exosomes in immune function

Activating effects of exosomes on the immune system

The first report of exosome involvement in immune function was of the direct presentation of antigens by exosomes to T-cells (Figure 1.7 (1)). Raposo *et al.,* (1996) demonstrated that multivesicular MHC class II-enriched compartments (MIIC) of B cells are exocytic. When the MIICs fused with the plasma membrane they released exosomes into the culture media. The MHC class II molecules on the surface of these exosomes were recognisable by helper T lymphocytes ($CD4^+$ T cells). The interaction of these exosomes with $CD4^+$ T cells stimulated T cell proliferation in a peptide specific, MHC restricted manner [47]. It is thought that *in vivo* the exosomes from antigen presenting cells (APC) like B cells and particularly dendritic cells may function as carriers of MHC class II complexes for amplifying the immune response.

Wolfers *et al.*, (2001) reported that tumour derived exosomes may also serve an immune activating function. Exosomes can act as a source of tumour rejection antigens for dendritic cell-uptake and processing leading to efficient cytotoxic lymphocyte (CTL) cross-presentation (Figure 1.7 (2)). DCs pulsed with tumour-derived exosomes were able to trigger T-cell-mediated anti-tumour responses *in vivo* (in mice with recently implanted tumours). These responses lead to autologous tumour rejection and strong immune responses against tumours of different origins. These "cross protective" exosomes indicate that they may contain tumour-antigens shared with other cancers. Although the role of murine-viruses in the transmission of this apparent 'shared tumour rejection' process remains a possible alternative explanation. These same effects were not observed with tumour lysates or apoptotic bodies, suggesting these effects are mediated preferentially by the exosomes. Exosomes may therefore act as miniature antigen presenting cells in amplification of immunity, or as vehicles for antigenic transfer [58].

Inhibitory effects of exosomes on immune function

There is also evidence that tumour exosomes may in fact exhibit immune evasive functions. Tumour exosomes have been shown to selectively impair lymphocyte responses to interleukin-2 (IL-2). Strong inhibition of IL-2-driven lymphocyte proliferation has been observed in the presence of tumour exosomes. The lymphocyte subsets were also examined individually showing the main anti-proliferative effect was through $CD4^+$ T-cells implicating an influence on regulatory T cells. In fact exosomes can support inducible T regulatory cells (T-reg), defined by FOXp3 expression, and enhance their suppressive functions [39].

The cytokine, transforming growth factor-beta 1 (TGFβ1) found on exosomes appears to be responsible for T-reg activation (Figure 1.7 (3)) [38, 39]. TGFβ1 expression has been shown to be the principle mechanism for the down-regulation of NKG2D expression on $CD8^+$ T-cells and natural killer cells. NKG2D is an activating receptor for natural killer, $CD8^+$ and $\gamma\delta^+$ T cells and its loss in cancer is a key mechanism for immune evasion [38].

This work suggests that NKG2D is a likely physiological target for tumour derived exosomes.

The apparent conflict between immune-activation/suppression in the literature is not easy to explain. One view is that the molecular phenotype of exosomes is absolutely key to their functions. Exosomes produced by well established, bulky tumours are likely to express a markedly different phenotype from exosomes produced by small neoplastic lesions. Also, differences in how exosomes are handled in these studies may be quite different and may therefore give different outcomes. However it is emerging that obtaining immune responses any tumour exosomes require manipulation of the system. For example heat shock or the addition of adjuvants. It is not likely that exosomes from advanced cancers attack immune activation as the cancers progress unhindered by the immune system.

1.2.5.2 Exosomes in non-immune functions

Role of exosomes in sperm maturation

The surfaces of sperm undergo modification in macromolecular structure as they travel along the male reproductive tract. Sperm cells have no cellular machinery for protein manufacture and therefore are unable to facilitate these modifications [126]. The majority of modifications occur in the epididymis where the epididymal epithelium secretes exosomes, coined epididymasomes, into the lumen of the epididymis. Some of the exosome associated proteins are subsequently transferred to the spermatozoa. The transfer of a selection of proteins is thought to aid the fertilising capabilities of sperm during their maturation including the P26/P34H family which are involved in sperm–zona pellucida binding [127-129]. Overall it appears that exosomes may play a considerable role in the maturation of sperm in a number of mammalian species.

Exosome involvement in angiogenesis

Proteins associated with angiogenesis have also been identified on the exosome surface. For example exosomally expressed tetraspanin CO-029/D6.1A has been demonstrated, *in vitro*, to trip an angiogenic switch supporting angiogenic factor transcription in target cells.

The ability of exosomes to move around the body through the circulation means that this angiogenic effect could be systemic thus aiding tumour progression [130].

The most recent work in this area focuses on exosomal tetraspanin 8 (Tspan8) using a rat adenocarcinoma model (AS-Tspan8) to examine the effects of exosomal Tspan8 on angiogenesis. Here D6.1 was shown to inhibit the uptake of Tspan8 exosomes into target endothelial cells. Tspan8 was found in association with CD49d and this complex is thought to aid exosome uptake. The mRNA profiles of AS and AS-Tspan8 exosomes were also examined showing the Tspan8 exosomes to have elevated levels of five mRNAs expected to be relevant in targeted endothelial cells. Of these two were shown to be transiently expressed in the target cell confirming AS-Tspan8 exosomes uptake [131]. It is also thought that exosomal RNA may be involved in intracellular communication, cellular development, protein synthesis, post-translational modification and possibly stem cell differentiation control [116, 132]. It appears that exosomes certainly exert an influence over angiogenesis potentially through both protein and mRNA loading onto endothelial cells.

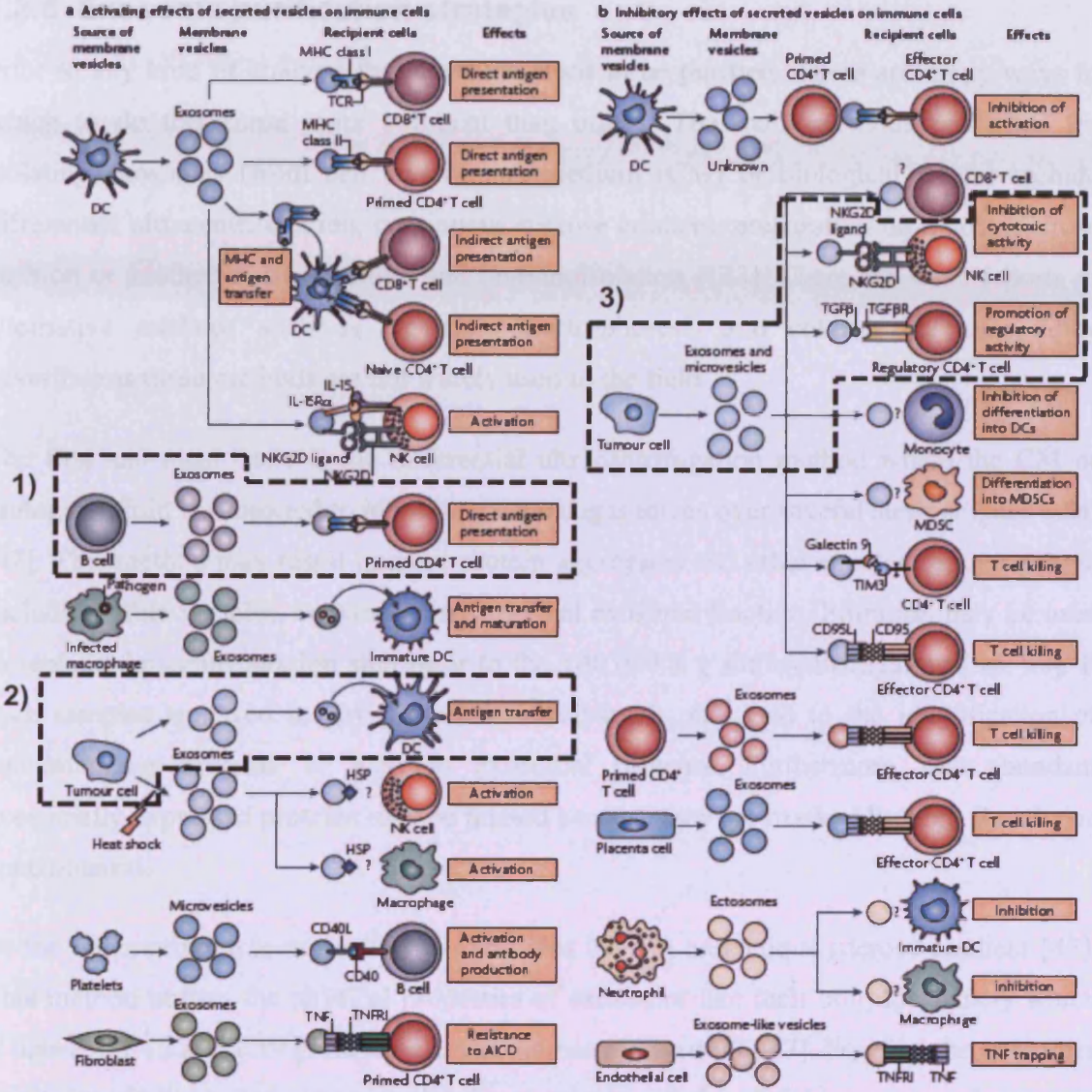


Figure 1.7: Summary of the functions of secreted vesicles on immune cells observed *in vitro*

Vesicles secreted from various cellular sources have numerous activating effects on immune cells (a) as well as inhibitory effects (b) shown in the orange boxes. The three areas highlighted 1-3 are discussed in detail in the text. Taken from Thery *et al.*, (2009) [125].

1.2.6 Exosome purification strategies

Prior to any kind of analysis the exosomes need to be purified. There are many ways in which to do this some more stringent than others. The main methods published for isolating exosomes (from cell conditioned medium (CM) or biological fluids) include differential ultracentrifugation, continuous sucrose gradient, preparation on a 30% sucrose cushion or another dense medium, and immunoisolation [133]. There are some reports of alternative methods such as free-flow electrophoresis and column chromatography nevertheless these methods are not widely used in the field.

The first and most basic is the differential ultracentrifugation method where the CM or biological fluid is subjected to increasing centrifugal forces over several steps (Figure 1.8a) [47]. This method may result in more protein aggregates and other smaller cellular debris, including other vesicles, contaminating the final exosome fraction. Filtration may be used to replace the centrifugation step prior to the 100,000 x *g* ultracentrifugation [95, 99]. If such samples are used in any proteomics analyses it may lead to the identification of contaminating proteins as well as exosomal proteins. Furthermore less abundant exosomally expressed proteins may be missed because they are masked by high abundance contaminants.

At the other extreme is separation of exosomes using a continuous sucrose gradient [47]. This method utilises the physical properties of exosomes like their buoyant density which is between 1.12 and 1.19 g/ ml for most exosomes reported [46, 47]. For this, the exosomes and contaminating proteins are pelleted and re-suspended in 2.5 M sucrose solution and overlaid with a continuous sucrose gradient followed by an overnight high speed centrifugation step and subsequent fraction collection (Figure 1.8b). This method is however very labour intensive and is therefore not widely used for functional studies but may prove useful as an analytical tool.

A compromise between these two methods utilises a sucrose cushion along with differential ultracentrifugation. This method was first utilised by Andre *et al*, (2002) to isolate exosomes from malignant ascites fluid [67]. Our group has subsequently adapted

the method which involves overlaying the source material onto a 30% sucrose deuterium oxide (D_2O) (density of 1.2 g/ ml) cushion allowing the majority of exosomes to be retained within the sucrose D_2O cushion [38, 39, 44, 134]. The sucrose fraction is then collected and the exosomes within are washed and pelleted (Figure 1.8c). The sucrose cushion method in combination with a cross flow ultrafiltration process was the FDA-approved mechanism for clinical DC-exosome preparations [42].

Exosomes can also be isolated using an immuno-magnetic isolation approach in which there is no need for ultracentrifugation. One example utilises magnetic beads coated with a particular antibody against a known exosomal membrane protein. For example when isolating exosomes from antigen-presenting cells an anti-MHC class II antibody may be used [86, 87]. The CM or biological fluid is incubated with the beads for 24 h ensuring bead saturation. The bead exosome complexes are then thoroughly washed leaving just the exosome coated beads which can be subsequently analysed.

Immunoisolation offers some advantages over the ultracentrifugation techniques as it allows the capture of exosomes from samples, CM or biological fluids, which contain many contaminants or substances that may interfere with purification by ultracentrifugation such as foetal bovine serum (FBS) in cell culture medium or albumin and other abundant proteins in plasma [68]. It is also arguably a gentler process that minimises damage to vesicles. However there is one key issue the decision on which antibody to use for exosome capture, as a single antibody may not be suitable for capturing all exosomes. Capture antibodies utilised in various studies include anti-CD63 [64], anti-MHC class II [86, 87, 104], anti-Her2 [135] anti-EpCAM [65, 112], and colon epithelial cell specific anti-A33, essentially selecting a sub-population of vesicles expressing these molecules and do not always reflect the composition or function of the entire exosome population. This technique is also not suited to purifying high numbers of exosomes from samples. Lastly the effect of immunoisolation on the functionality of the exosome is not known therefore exosomes captured by this method may not be appropriate for use in functional immunology and other studies. The exosomes may need to be liberated from the antibody/bead complex and this extra step may cause damage to the vesicle.

The decision on which exosome purification strategy to use will ultimately be based on the sample purity required, the quantity of exosomes needed, the complexity of the sample source, and whether the exosomes need to be functional. Irrespective of the method of isolation used it is imperative that the sample is characterised and that the true nature of the sample is known. There is currently no consensus with respect to the criteria for exosome purity which is something that needs to be addressed. Simpson *et al.,* (2009) support this view especially with respect to exosome samples to be used in proteomics studies, but we would advocate this is equally if not more important for exosome functional studies.

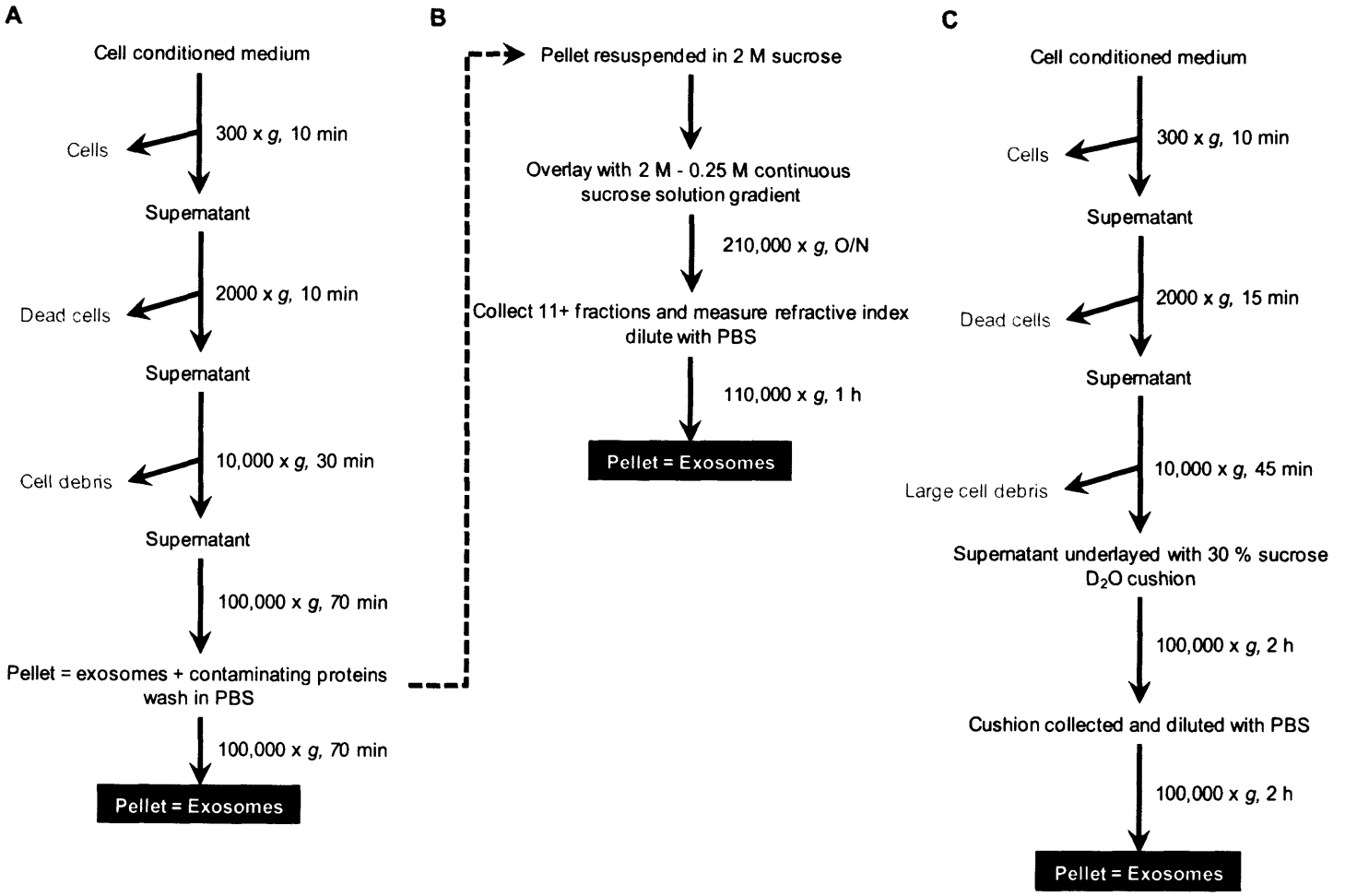


Figure 1.8: Purification of exosomes by different centrifugation methods

Flow charts for the three ultracentrifugation methods of exosome purification. A. Differential ultracentrifugation [133]; B. Linear sucrose gradient (dashed arrow marks the start point) [40]; C. Differential ultracentrifugation with 30% sucrose cushion [42, 67].

1.3 *Proteomics analysis of exosomes*

The use of proteomics technologies to study the proteome of exosomes may offer a gateway to identify potential biomarkers for cancers and other diseases. It may also offer insights into exosome biogenesis and function. Over the past 15 years there has been increasing interest in the exosome proteome and its potential as a biomarker source. Until recent years much exosome proteome analysis has relied on two-dimensional electrophoresis (2DE) and western blotting. The depth of coverage of these techniques is restricted to the more highly abundant proteins thus limiting the data that can be collected [136]. Significant improvements in all aspects of proteomics workflows, including both hardware and software, have occurred over the past several years [137-139]. These developments have lead to a substantial increase in the number of exosomal protein identifications possible, from as few as nine protein identifications in 1999 to as many as 1132 in 2009 (presuming that all of the these protein identifications are genuine) [59, 85]. Furthermore the availability of ExoCarta, a database cataloguing exosome proteome and RNA studies, will enable groups undertaking exosome proteome studies to compare their datasets *in silico* with those generated by others [140].

Exosomes derived from both cell culture and biological fluids have been investigated and some of this work will be discussed in detail. Simpson (2008, 2009) reviews the exosome proteome studies to date and incorporates a summary table of published works [123, 141]. This has been included here for reference (Table 1.5). In addition a summary of the main proteomics workflows used in exosome proteome studies to date can be seen in Figure 1.9. In 2001 Thery *et al.,* reported the first extensive protein map of a particular exosome population in this instance dendritic cell exosomes. Less than 50 proteins were identified of which 21, mainly cytosolic proteins, were newly identified as exosomal. Exosomes were purified by differential centrifugation and the proteins were separated by one-dimensional electrophoresis (1DE), and stained protein bands were excised and trypsin digested. Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (MS) was performed followed by peptide mass fingerprinting (PMF) for

protein identifications. This study confirmed exosomes as uniquely different to apoptotic blebs by their protein constituents and structure (by electron microscopy) [99].

A study of B-cell derived exosomes by Wubbolts *et al.*, (2002) used a different approach to purifying exosomes utilising several of the methods described earlier (section 1.2.6). Initially differential ultracentrifugation was used followed by separation on a sucrose gradient. Exosome fractions were then incubated with anti-MHC class II magnetic beads allowing specific capture of MHC class II expressing exosomes. The proteins from these magnetic bead coupled exosomes were then subjected to separation by 1DE and protein band excision followed by MALDI-TOF MS or quadrupole-TOF (Q-TOF) with a nanoelectrospray (ESI) source. Only proteins identified with two or more peptides were reported. Amongst these identifications were heat shock proteins, cytoskeletal proteins, and enzymes involved in glycolysis [87].

The exosome proteomics work to date (Table 1.5) demonstrates the numerous ways in which exosomes have been isolated from CM and biological fluids for this type of analysis and furthermore what sort of proteomics approaches have been taken by various research groups to analyse their exosome samples. Yates *et al.*, (2009) and others present good reviews of the general MS-based proteomics approaches used and advances made [138, 139, 142]. A very common workflow in the field is 1DE coupled with either MALDI-TOF MS or more recently, with the advances in technologies, liquid chromatography (LC)-MS/MS. This later method, 1DE LC-MS/MS has yielded the highest number of protein identifications. However 1DE LC-MS/MS is likely to be the most expensive in both time and money. In one study identifying 1132 proteins 40 gel slices were analysed requiring extensive amounts of time for processing. The peptides from each slice were separated by reverse-phase LC followed by analysis using a linear ion trap mass spectrometer with a nano-electrospray ion source. For many researchers, this kind of equipment infrastructure and support funds for consumables and research time is not available. It is therefore essential to plan any proteomics study carefully and utilise the best available technology to identify as many proteins as possible.

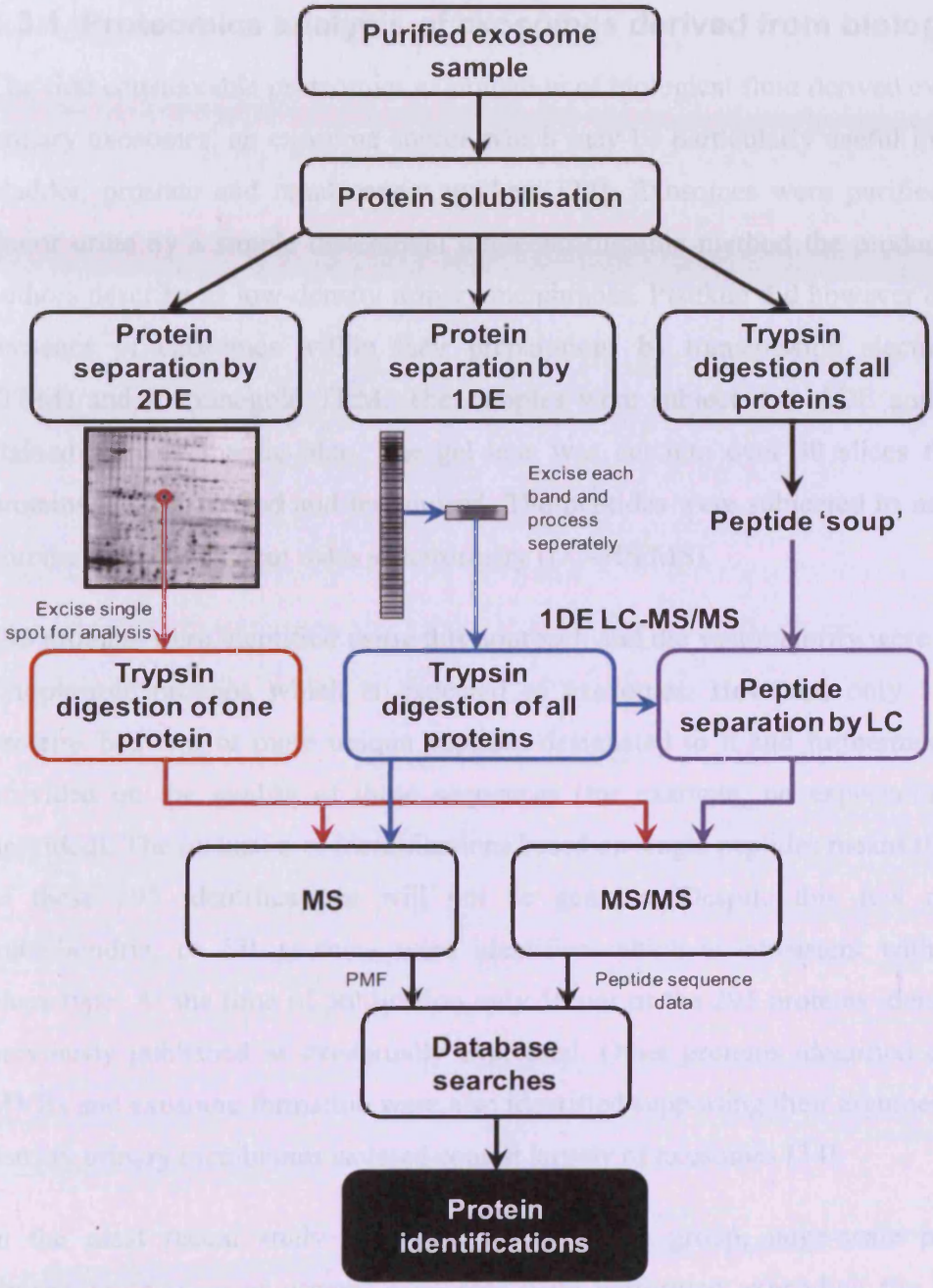


Figure 1.9: Summary of different workflows employed for the proteomics analysis of exosomes

The flow chart demonstrates the three main proteomics routes that have been used to date to identify exosomal proteins. 2DE: two-dimensional electrophoresis; 1DE: one-dimensional electrophoresis; 1DE LC-MS/MS: 1DE coupled with liquid chromatography (LC) and tandem mass spectrometry (MS/MS). Adapted from Brewis and Gadella (2010) [126].

1.3.1 Proteomics analysis of exosomes derived from biological fluids

The first considerable proteomics examination of biological fluid derived exosomes was of urinary exosomes, an exosome source which may be particularly useful in the search for bladder, prostate and renal cancer markers [34]. Exosomes were purified from healthy donor urine by a simple differential ultracentrifugation method the product of which the authors describe as low-density urinary membranes. Pisitkun did however demonstrate the presence of exosomes within their preparations by transmission electron microscopy (TEM) and immunogold TEM. The samples were subjected to 1DE and the gels were stained with coomassie blue. The gel lane was cut into over 30 slices from which the proteins were extracted and trypsinised. The peptides were subjected to nanospray liquid chromatography tandem mass spectrometry (LC-MS/MS).

295 proteins were identified using this approach and the vast majority were membrane and cytoplasmic proteins which is expected of exosomes. However, only 184 of the 295 proteins had two or more unique peptides designated to it and furthermore no data was provided on the quality of these sequences (for example, no expectation values were provided). The inclusion of identifications based on single peptides means that a proportion of these 295 identifications will not be genuine. Despite this few nuclear, Golgi, mitochondria, or ER proteins were identified which is consistent with an exosomes phenotype. At the time of publication only 50 out of the 295 proteins identified had been previously published as exosomally expressed. Other proteins identified associated with MVBs and exosome formation were also identified supporting their argument that the low-density urinary membranes isolated consist largely of exosomes [34].

In the most recent study published by the same group, large-scale proteomics and phosphoproteomics of urinary exosomes were performed expanding the known urinary exosome proteome. A total of 1132 proteins were reported to be identified using 1DE LC-MS/MS. Out of these 205 proteins had been previously identified [59]. Again their dataset includes identifications based on single peptides and the author also states that the proteomic profiling performed is "of a low-density membrane fraction from human urine

consisting chiefly of exosomes" which suggests, as before, a certain degree of non-exosomal contaminating proteins [34].

Out of all the protein identifications, 177 proteins were found to be associated with disease according the Online Mendelian Inheritance of Man (OMIM) database. Twenty-four of these are known to be associated with renal disease. The authors concentrated on one particular protein NKCC2 in which the gene coding for this protein is known to be mutated leading to the Bartter syndrome type 1. They demonstrated in a few patients the total absence of the protein NKCC2 in their urinary exosomes even though it was present in healthy donor urine [59]. Their earlier study also identified 21 proteins associated with renal or systemic diseases each of which has the potential to be a biomarker for their respective diseases. One protein in particular they identified as putative biomarker of autosomal dominant polycystic kidney disease was polycystin-1. They describe how it has low abundance in kidney tissue but is readily detectable in urinary exosomes [34].

In 2006 Zhou *et al.*, utilised comparative 2D gel based proteomics to specifically search for non-invasive urinary biomarkers of acute kidney injury using a cisplatin-induced AKI rat model. The exosomes isolated, by the same method as Pisitkun *et al.,* (2004) [34, 143], from urine prior to cisplatin treatment and post treatment were compared using 2D difference gel electrophoresis (DiGE). Unfortunately only 20% of the proteins picked were identified highlighting potential issues in using 2D gel methods for exosome proteomics analysis. However, one protein was identified to be of interest as a kidney injury biomarker, Fetuin A. The levels of Fetuin A increased after kidney injury. Urinary exosomes from normal healthy donors did not identify Fetuin A. Furthermore, the evaluation of urine from acute kidney injury patients demonstrated a 50-fold increase in the levels of Fetuin A [34, 144].

Utilising urinary exosomes or exosome enriched urinary sediment as a sub-proteome of the biological fluid has yielded some interesting results giving insight into the complexity and potential clinical value of the urinary exosome proteome. The information obtained may be useful in investigating the biogenesis and function of the exosomes as well as identifying

potential biomarkers for disease. All of these studies [34, 59, 144] highlight the need for more work to validate the potential biomarkers and that there is huge potential for these exosomes to be used as a non-invasive source of biomarkers. Improved urine collection and purification is however needed to ensure the samples are of highest quality.

Although urinary exosome proteomics has yielded the most protein identifications for body fluid-derived exosomes a large numbers of identifications have also been achieved from saliva exosomes. In this instance a shotgun proteomics approach (multidimensional protein identification technology; MudPIT) was utilised. This study highlighted the presence of exosomal proteins which have links to specific diseases [61]. Gonzalez-Begne utilised a similar purification method to Pisitkun *et al.*,(2004) [34]. A total of 491 proteins were detected (false discovery rate of 5%). Numerous proteins associated with exosome biogenesis were identified as well as proteins associated with salivary secretion potentially reflecting the unique characteristics of parotid exosomes. This work suggests salivary exosomes as a potential source of disease markers and this would again offer a good non-invasive source for clinical exosome analysis.

Biological fluids allow researchers to study the proteome of exosomes secreted *in vivo* potentially giving rise to protein information pertaining to the *in vivo* function of exosomes and to biomarkers for disease. Biological fluids may be a source of potential biomarkers but unfortunately the nature of the sample may hinder the identification of less abundant proteins significant to disease. Exosomes derived from any biological fluids are likely to have been secreted from many of if not all the cells in which the fluid comes in contact with. Furthermore, there may be difficulties with variation between individuals or between samples as the likely diverse physiological parameters that modulate exosome production/composition are essentially unknown. It may therefore be prudent to perform exosome proteomics analysis on *in vitro* derived exosomes with the potential to apply any knowledge gained to developing a test using *in vivo* derived exosomes to detect disease.

1.3.2 Identifying exosomal proteins of interest in disease

In vitro exosome proteomics studies have been performed identifying a multitude of proteins that may be of interest in disease or may be suitable biomarkers. For example Hegmans *et al.*, (2004) isolated exosomes by differential centrifugation from seven different mesothelioma cell lines established from human tissue. The proteins were analysed using a 1DE MALDI-TOF MS approach. The authors used a protein identification cut off of five or more matching peptide masses to give high quality proteomics data. Protein information was deduced from PMF analysis identifying 38 proteins. Included amongst these were previously identified exosomal proteins such as MHC Class I, HSPs, annexins, and cytoskeletal proteins. Interestingly the protein identifications included developmental endothelial locus-1 (DEL-1), a tumour associated protein. DEL-1 is involved in angiogenesis and may also be involved in targeting exosomes to dendritic cells (DCs) for cross-presentation [57]. Although empirical evidence is shown this has not yet been published.

With proteomics analysis of any biological sample the quality of the sample going into the workflow will reflect the quality of the data coming out. In the case of exosome based proteomics studies problems with the data quality may not only be reflected by the quality of the MS data reported but also the sample being analysed. In order to identify truly exosomal proteins the method used for exosome purification needs to be verified as producing high quality exosome preparations. The higher quality preparations of exosomes are likely to come from isolation procedures which involve a sucrose cushion or gradient [103, 145] or immunoisolation [87, 96] as each of these techniques utilise inherent properties of exosomes eliminating most if not all contaminating proteins.

Unfortunately many exosome proteomics studies do not go to these lengths and use the simple differential centrifugation technique [34, 40, 53, 59, 61, 99]. This may lead to the incorporation of more protein aggregates and other smaller cellular material in the final pellet and ultimately the identification of both exosomal and contaminating proteins. In an article focussed on protocols for isolating and characterising exosomes the inclusion of an

additional purification step, to remove more contaminants, is suggested for applications such as proteomics analysis [133]. As a general rule any published exosome manuscript should present convincing data (for example flow cytometry, immunoblotting analysis and/or electron microscopy images) characterising the sample to demonstrate the quality of the samples used but unfortunately this data is more often lacking.

Overall the exosome proteomics studies carried out to date have brought to light the capability of exosome proteomics to identify proteins involved in their biogenesis and likely function as well as identifying potential biomarkers for disease. With further proteomics investigations it may be possible to identify clinically relevant information from exosomes that may be developed into an exosome based diagnostics platform or for disease monitoring.

Table 1.5: Methods of isolation and characterisation of exosomes from cell lines and body fluids

	Isolation strategies [a)]	Validation [b)]	Proteomic strategies	Proteins identified	Ref.
Cell lines					
Hematopoietic cells					
B cell (RN HLA-DR15$^+$)	DC, DG and immunobeads (MHC-II)	WB (MHC-II)	1-DE, MALDI-TOF MS, and nESI/MS/MS	21 including: MHC-I and II, CD45, integrin α4, hsc70, hsp90, Giα2, actin, tubulin, moesin, clathrin, GAPDH, enolase, and EF1α1	[87]
B cell (RN HLA-DR15$^+$)	DC, DG	IEM (MHC-II, CD53, and CD82), WB (MHC-II, HLA-DM, CD37, CD63, CD81, CD82, and CD86)		Including: CD37, CD53, CD63, CD81, CD82, and CD86	[46]
DCs (D1)	DC, F	EM, WB (MHC-II and Lamp2) FCS (MHC-I and -II, CD9, Mac-1, and CD86)	1-DE, MALDI-TOF MS, and nESI/MS/MS	37 including: actin, tubulin, cofilin, MFG-E8, annexins, rabs, CD9, hsp90β, TSG101, syntenin, histones, Alix, 14-3-3 proteins, galectin-3, gag, reverse transcriptase/pol, and Mac-1 αβ	[99]
Dendritic cells (MD-DC)	DC, DG	EM, WB	1-DE, MALDI-TOF MS	~35 including alix, annexins, ICAM-1 and cofilin	[146]
DCs (D1 and BM-DC)	DC, DG	EM, IEM (MHC-II), WB (MHC-II)	1-DE, MALDI-TOF MS	9 including CD9, gag, Mac-1, MFG-E8, hsc73, and annexin-II	[85]
Dendritic cells (D1 and BM-DC)	F, UC, DG	EM, WB (Clathrin, hsc70, annexin II, CD9, flotillin 1, ICAM-1, MHC-II, TSG101, MHC I, MFGIE8), FACS	1-DE, LC-MS/MS	~150 including: CD9, annexin-II, ICAM-1 and TSG101	[147]
Mast cell (MC/9, HMC-1, BMMC)	DC, F, DG	EM, FACS (CD63)	1-DE, LC-MS/MS	271 including: mast carboxypeptidase A, tubulins, TCP proteins, ezrin, moesin, 40S ribosomal proteins, 14-3-3 proteins, CD43, CD63, CD97, annexins, MHC-I, histones, hsc70, and integrin-α6	[111]
Mast cell (MC/9, HMC-1, BMMC) and mastocytoma (P815)	DC	EM, IEM (polyclonal Abs to exosomes)	1-DE, MALDI-TOF MS, ELISA	Including: MHC-II, CD40, CD40L, CD86, LFA-1, ICAM-1, CD13, annexin-VI, actins, and CDC25	[53]
T-cells (Jurkat cells, T cell blasts, E$^+$ cells, and MART-1$^+$ T cell)	F, UC	EM, IEM and WB (TCR β and CD3ε), FACS (CD63, TCR β, CD3ε, MHC-I and -II)	WB, FACS	Including: TCRβ, CD3ε, and ζ, MHC-I and -II, CD2, CD18, chemokine receptor CXCR4, c-Cbl, tyrosine kinase Fyn, and Lck	[48]

Taken from Simpson *et al.*, (2009) [141]

Table 1.4: continued

	Isolation strategies [a]	Validation [b]	Proteomic strategies	Proteins identified	Ref.
Tumour cells					
Breast adenocarcinoma (BT-474 and MDA-MB-231)	DC, F, DG, immunobeads (HER2)	EM, FACS (HER2), WB (HER2, actin)	WB, FACS	HER2 identified	[135]
Colorectal cancer (HT29)	DC, diafiltration (100 K), DG	EM, WB (CD63 and CD81)	1-DE, LC-MS/MS	547 including: annexins, ARFs, Rabs, ADAM10, CD44, NG2, ephrin-B1,MIF, β-catenin, Junction plakoglobin, galectin-4, RACK1, and tetraspanin-8	[101]
Colon carcinoma cell lines (SW403, 1869col, and CRC28462)	DC	EM, IEM (CD63, FasL, and TRAIL), WB and FACS (CD63, FasL, TRAIL, CEA, and MHC-I)	WB	FasL and TRAIL identified	[148]
Colorectal cancer (LIM1215)	F, diafiltration (5 K), UC, immunobeads (A33)	EM, IEM (A33), WB (CD9, A33, TSG101, and hsc70)	1-DE, LC-MS/MS	~400 including: A33, CEA, EGFR, ADAM10, dipeptidase 1, ephrin-B1, hsc70, tetraspanins, ESCRT proteins, integrins, annexins, Rabs, and GTPases	[96]
Mammary adenocarcinoma (TS/A, H-2^d), P815 mastocytoma (H-2^d), melanoma (Fon and Mel-888)	DC, DG	EM, WB (hsc70 and MHC-I)	WB, IEM	MHC-I, hsp70, MART-1, and TRP identified	[58]
Melanoma (MeWo and SK-MEL-28)	F, UC	WB (MHC-I, MART-1, Mel-CAM, and annexin II)	2-DE, MALDI-TOF/TOF MS	41 including: Alix, hsp70, Giβ2, Giα, moesin, GAPDH, malate dehydrogenase, p120 catenin, PGRL, syntaxin-binding protein 1 and 2, septin-2, and WD repeat-containing protein 1	[40]
Mesothelioma (PMR-MM7 and 8)	DC	IEM (CD63)	1-DE, MALDI-TOF MS	30 including: annexins, actins, actinin-4 tubulins, hsc70, hsp90, integrins, fibronectin, GAPDH, MHC-I, PLVAP, and DEL-1	[57]
Brain tumour (EGFRvIII- transfected SMA560)	DC, DG (Optiprep)	EM, WB (Alix, GAPDH, α-anti-trypsin, CD9, PD1, CRT, transferrin, GPNMB, TGF-β1 and EGFRvIII), Acetylcholinesterase assay	2DE, MALDI-TOF/TOF		[105]

Taken from Simpson *et al.*, (2009)

Table 1.4: continued

	Isolation strategies [a)]	Validation [b)]	Proteomic strategies	Proteins identified	Ref.
Primary and normal cells					
Cortical neurons (8-day primary culture)	DC, DG	EM, WB (Alix, TSG101, and flotillin)	1-DE, LC-MS/MS	19 including: GLAST1, brain-specific cerulaplasmin, L1 cell adhesion molecule, GPI-anchored prion protein, and GluR2/3	[54]
Intestinal epithelial (HT29-19A and T84-DRB1*0401/CIITA)	DC, DG	EM, IEM and WB (CD26, CD63, MHC-I, and IIα), WB (TfR and Na^+K^+ -ATPase)	1-DE, MALDI-TOF MS	28 including: syntaxin-3, syntaxin-binding protein 2, EPS8, microsomal dipeptidase in AM and A33, epithelial cell surface antigen, and major vault protein BM	[149]
Microglia (N9 and primary culture from SJL/J mice)	DC, DG	EM, WB (CD9, CD63, syntaxin-8, rab7, rab11, clathrin, Lamp-1 and -2, Vti-1A, and -1B)	1-DE, LC-MS/MS	59 including: aminopeptidase N (CD13), MCT-1, cathepsin S, MHC class II-associated chaperone Ii, CD14, NAP-22, FcR for IgE, and GP42	[97]
Oligodendrocytes (primary culture and Oli-neu)	DC, DG	EM, WB (Alix, PLP, CNP, and TSG101)	LC-MS/MS	143 including CD81, 14-3-3 proteins, actins, tubulins, histones, EF1 and 2, hsp90, hsc70, Na^+K^+ATPase α chains, PLP, CNP, MBP, and MOG	[145]
Human tracheobronchial epithelial cell	DC, F, DG	EM, WB (MUC1, EBP50, CD133, Annexin II, TSG101, CD63)	1DE, LC-MS/MS	~40 including: CD63, TSG101, Mucins, actins and tubulins	[150]
Keratinocytes (2-day culture from foreskin)	DC, diafiltration (100K), DG	EM, WB (Hsc70 and LAMP2)	WB	14-3-3σ (stratifin)	[151]
Hepatocytes	DC, DG	EM, WB (TSG101, Alix, integrin-β1, CD63, CD81, ICAM-1 and lactadherin)	1DE, LC-MS/MS	251 including: tetraspanins, ASGR, cytochromes P450, cytoskeletal proteins, apolipoprotein-E and -AV, paraoxonase-1 and -3 regucalcin, UDP-glucuronosyltransferases	[56]
Virus-infected cells					
Rov epithelial and Mov neuroglial calls infected with PrP	DC, DG	IEM and WB (PrP, flotillin, TSG101, and TfR), WB (hsc70)	1-DE, LC-MS/MS	93 including 14-3-3 proteins, annexins, hsc70, integrins, rabs, actin, tubulins, MFG-E8, Gi2α, histones, PrP, and PrPsc	[106]

Taken from Simpson *et al.*, (2009)

Table 1.4: continued

	Isolation strategies [a)]	Validation [b)]	Proteomic strategies	Proteins identified	Ref.
Body fluids					
Blood					
Activated platelets	DC, DG	EM, IEM and WB (CD63)	WB, FACS	CD63 identified	[63]
Blood (2 day old-PBMC and plasma)	DC, F, DG	EM, IEM (CD63), FACS (CD9, CDE41a, CD63, CD81, Lamp-2, and MHC-II), WB (CD9, CD63, MHC-1 and -II, and TfR)	WB, FACS	CD81, CD41a, CD3ζ, and Lamp-2 identified	[64]
Pregnancy (blood)	Size exclusion, UC, immuno-beads (CD3, CD19, CD56, CD83, and PLAP)	EM	WB	PLAP, FasL, and PD-L1	[152]
Serum from patients with high grade glioma	DC	EM, WB (HSP, HSC70, EGRF and TGF-β1)	WB	HSP, HSC70, EGFR, and TGF-β1	[105]
Plasma	Size exclusion, DG	IEM, WB	1-DE, LC-MS/MS	66	[153]
Other body fluids					
Breast milk	DC, F, DG	IEM (CD63 and HLA-DR), WB and FACS (HLA-DR, CD81, and hsc70), FACS (MUC-1)	In-solution trypsinisation, SCX-LC-MS/MS	73 proteins including: MFG-E8, MUC1, hsp70, ARF-1, EH domain-containing protein 1, CD36, butyrophillin, and polymeric-Ig receptor	[62]
Bronchoalveolar lavage fluid	DC, immuno-beads (MHC-II)	IEM (HLA-DR and CD63), FACS (HLA-DR, CD54, CD63, and CD86)	-	-	[104]
Malignant pleural effusions	DC, DG	EM	1-DE, MALDI-TOF MS	50 including: MHC-I, actin, G protein, hsp90, BTG1, Bamacan, PEDF, BTG-1, TSG14, and TSP2	[68]

Taken from Simpson *et al.,* (2009)

Table 1.4: continued

	Isolation strategies [a]	Validation [b]	Proteomic strategies	Proteins identified	Ref.
Malignant pleural effusions and malignant ascites	DC, DG	EM, IEM (MHC-I and -II, TRP, gp 100, and CD81), WB (MHC-I and -II, MART-1, HER2, and hsc70)	WB	MART-1, TRP, gp100, and HER2	[67]
Synovial fluid (RA, OA and reactive arthritis)	DC, DG	EM, IEM (IgG)	2-DE, WB, MALDI-TOF MS	Citrullinated fibrin α-chain, CD5 antigen-like, fibrinogen fragment D, and β-chain	[154]
Urine and amniotic fluid	DC, DG	EM, WB (hsp70, AQP2, annexin-1 and CD9)	WB	CD24	[155]
Urine	UC	EM, IEM (APN, AQP2, CD9, and NCC), WB (TSG101, Alix, CD9, Rab-4, -5B and -11, SNX18, and others)	1-DE, LC-MS/MS	295 including: VPS protein, AQP2, polycystin-1, carbonic anhydrase II, and IV	[34]
Urine	DC		1-DE, LC-MS/MS	1132 including: AQP2, vacuolar H^+-ATPase subunits and ESCRTs. 14 phophorylated protein identified, including NCC, GPRC5B and GPRC5C	[59]
Urine (prostate cancer patient)	SC	WB (TSG101, 5T4, PSA, PSMA, GAPDH, CD9)	WB	TSG101, 5T4, PSA, PSMA, GAPDH and CD9	[36]
Saliva	UC	EM, WB (Alix, Aquaporin 5, CD81, and CD63)	1-DE, LC-MS/MS	491 including: Alix, AQP5, UBA1, VPS28 and annexins	[61]

Taken from Simpson *et al.,* (2009)

a) DC: differential centrifugations; DG: sucrose density gradient; SC: sucrose cushion; F: filtration (0.1μm and/or 0.2 μm filter; UC: ultracentrifugation.

b) WB: western blot; IEM: immunoelectron microscopy; FACS: fluorescence-activated cell sorted; EM: electron microscopy

1.4 *Exosomes as a source of novel bladder cancer markers*

Bladder cancer patients would benefit from the identification of novel disease biomarkers that could be used to help diagnose and monitor their disease. Various aspects of exosome biology suggest they may be a good source for novel disease biomarkers. Firstly exosomes can be purified from CM and biological fluids including urine [34, 96]. Therefore it may be possible to collect exosomes from the urine of BCa patients and healthy donors which could be used to identify and/or verify putative markers of BCa.

Exosomes are also a sub-proteome of the whole cell which presents an advantage in identifying lower abundance protein particularly membrane proteins. They are also enriched in tumour associated antigens and may reflect the stress status of their parent cell. The tumour cell environment may be subject to stresses such as hypoxia which may increase stress proteins that can be detected in exosomes. The presence and enrichment of tumour associated antigens and stress proteins particularly on the cell surface may offer a panel of markers, available from a single source. These could potentially be used in a multiplex diagnostic test.

Therefore, BCa exosome proteomics may be able to identify numerous marker proteins, associated with this under-investigated disease, that have potential to be used in the clinic to help diagnose and monitor the disease.

1.5 *Study Aims*

In this ambitious study we wanted to use exosomes as a platform for biomarker discovery in bladder cancer.

The approach from the outset was to isolate exosomes from urine specimens collected from BCa patients and healthy donors, and to use proteomics approaches to identify differentially expressed proteins. The proteins identified would subsequently be validated for their suitability to discriminate health from disease.

However, in order to pursue this objective it was necessary to develop methods for urine exosome purification and quality assurance, and devise a suitable proteomics workflow for analysis of exosome specimens. BCa cell lines in culture would provide a reliable stable source of exosomes to assist in the development of the methods required.

Chapter 2:

Materials and methods

2.1 *Materials*

All general reagents, unless otherwise stated, were analytical grade and purchased from Sigma-Aldrich Co. Ltd (Poole, UK), Invitrogen Ltd (Paisley, UK), Lonza Group Ltd (Basel, Switzerland), GE Healthcare (Bucks, UK), Fisher Scientific UK Ltd (Loughborough, UK). All water described was purified using a Milli-Q Biocel system (Millipore UK Ltd, Watford, UK).

2.2 *Culture of human cell lines*

All cells were maintained at 37°C at 95-98% humidity. They were tested monthly and confirmed negative for mycoplasma contamination using MycoAlert® mycoplasma detection kit (Lonza). Culture media and supplements for each cell line are detailed in Table 2.1. All cultures to be used for exosome preparations were supplemented with FBS (Invitrogen) depleted of bovine exosomes (FBS^{exo-}). FBS^{exo-} was produced by ultracentrifugation at 100,000 *g* for 16 h at 4°C and filtering using a 0.22 μm filter. Aliquots were stored at -20°C.

2.2.1 Monolayer culture

Monolayer cultures were established in 75 cm^3 culture flasks (Greiner Bio-One Ltd, Stonehouse, UK) and sub-cultured once confluent. This was typically at a ratio of 1:3 following 5 min incubation with 2 ml 0.05% (v/v) trypsin and 0.53 mM EDTA solution (Invitrogen). The protease activity was neutralised by the addition of FBS^{exo-}. The cell suspension was subsequently pelleted at 300 *g* for 5 min. The cells were then resuspended in the required medium with supplements (Table 2.1) and seeded into fresh 75 cm^3 culture flasks.

Table 2.1: Details of cell lines used their growth media and supplements

Cell line	Original cell source	Medium	Supplements		
T24 *	Bladder cancer [156]	RPMI-1640	Pen/Strep	L-glutamine	
HT1376 *	Bladder cancer [157]	DMEM	Pen/Strep		
HT1197 ¥	Bladder cancer [157]	EMEM	Pen/Strep	L-glutamine	1% NEAA
RT112 ¥	Bladder cancer [158, 159]	RPMI-1640	Pen/Strep	L-glutamine	
RT4 ¥	Bladder cancer [158, 159]	RPMI-1640	Pen/Strep	L-glutamine	
LnCAP §	Prostate cancer [160]	RPMI-1640	Pen/Strep	L-glutamine	
Caco-2 ¥	Colon cancer [161]	EMEM	Pen/Strep	L-glutamine	1% NEAA
MCF7 ¥	Breast cancer [162]	EMEM	Pen/Strep	L-glutamine	1% NEAA
#15 ¤	Mesothelioma	RPMI-1640	Pen/Strep	L-glutamine	
HFFs §	Human foreskin fibroblasts	RPMI-1640	Pen/Strep	L-glutamine	
SKOV3 ¥	Ovarian cancer	RPMI-1640	Pen/Strep	L-glutamine	1% Sodium pyruvate
AG02262 ‡	Human lung fibroblasts	DMEM F12	Pen/Strep	L-glutamine	

All cells were supplemented with 10% FBS^{exo-} (v/v)

Pen/Strep -penicillin (100 U/ml), streptomycin (100 µg/ml) (Lonza)

L-glutamine - 2mM (Invitrogen)

Company

* Cancer Research UK (CRUK)

¥ European Collection of Cell Cultures (ECACC) (Health Protection Agency, Salisbury, UK)

§ American Tissue Culture Collection (ATCC, LGC Standards, Middlesex, UK)

¤ Established by Dr Zsuzsanna Tabi, Section of Oncology and Palliative Medicine, School of Medicine, Cardiff University, UK

‡ Coriell Cell repositories (Coriell Institute for Medical Research, NJ)

2.2.2 Bioreactor culture in Integra CELLine™ flasks

Obtaining sufficient quantity of exosomes from adherent culture cells is difficult. Ideally, large cell numbers in a low volume of medium is needed but such conditions are unfavourable for maintaining cells with good viability. One approach adapted by our laboratory involves the Integra CELLine bioreactors (Integra Biosciences AG, Chur, Switzerland) originally designed for hybridoma cultures. These flasks have two compartments a cell compartment and a nutrient medium compartment (Figure 2.1). The cell compartment is small (maximum volume around 20 ml) and the cells are attached to woven polyethylene terephtalate (PET) matrix, providing a large surface area. The semi-permeable membrane allows nutrient and waste exchange with the medium compartment which holds a significant volume (up to 1000 ml). The cells are therefore maintained in 500-1000 ml of culture medium whilst present within the cell compartment which also retains the exosomes. Exosomes can therefore be purified from a much smaller volume thus allowing higher exosome yields to be processed from a lesser volume of liquid. The yields from these bioreactors can be 8 to 10 times greater than the traditional monolayer cultures thus reducing costs, labour and time needed to obtain enough exosomes for experimentation [163].

In total eight cell lines were grown in Integra CELLine™ AD (adhere) 1000 flasks including five transitional cell carcinomas (TCC) of the bladder and three non-TCC carcinomas. The five TCC lines (HT1376, HT1197, T24, RT4, and RT112) were selected as they are well characterised in the literature [8, 158, 164, 165]. They are also from varying stages and grades of the disease giving an *in vitro* representation of human TCCs of varying severity (Table 2.3). In addition, the patients from which all five cell lines were established had no prior chemotherapy or radiotherapy. Therefore there should be no alterations in cell phenotype caused by medical intervention(s). The three non-bladder human carcinoma cell lines were chosen to represent other carcinomas. These were breast (MCF7) [162], colon (Caco-2) [161], and prostate (LnCAP) [160] (Table 2.2).

Cells were seeded into the cell compartment of Integra CELLine™ flasks at an initial density of 1.5-3 x10^7 cells in 15 ml of required supplemented culture medium plus 10% (v/v) FBS^{exo-}. The outer chamber was filled with 500 ml cell line specific medium and 10% (v/v) FBS. Cell conditioned media (CM) were collected from the cell compartment each week and prepared for exosome purification (see section 2.6). The cell compartment was washed three times with medium to remove any non-adherent cells or dead cells/debris before the addition of fresh of FBS supplemented medium to both compartments. After six or more weeks in culture (giving the cells time to fully acclimatise to the growth conditions) the amount of FBS used was reduced to 5% (v/v) to reduce the protein content of the CM that could interfere with downstream analysis.

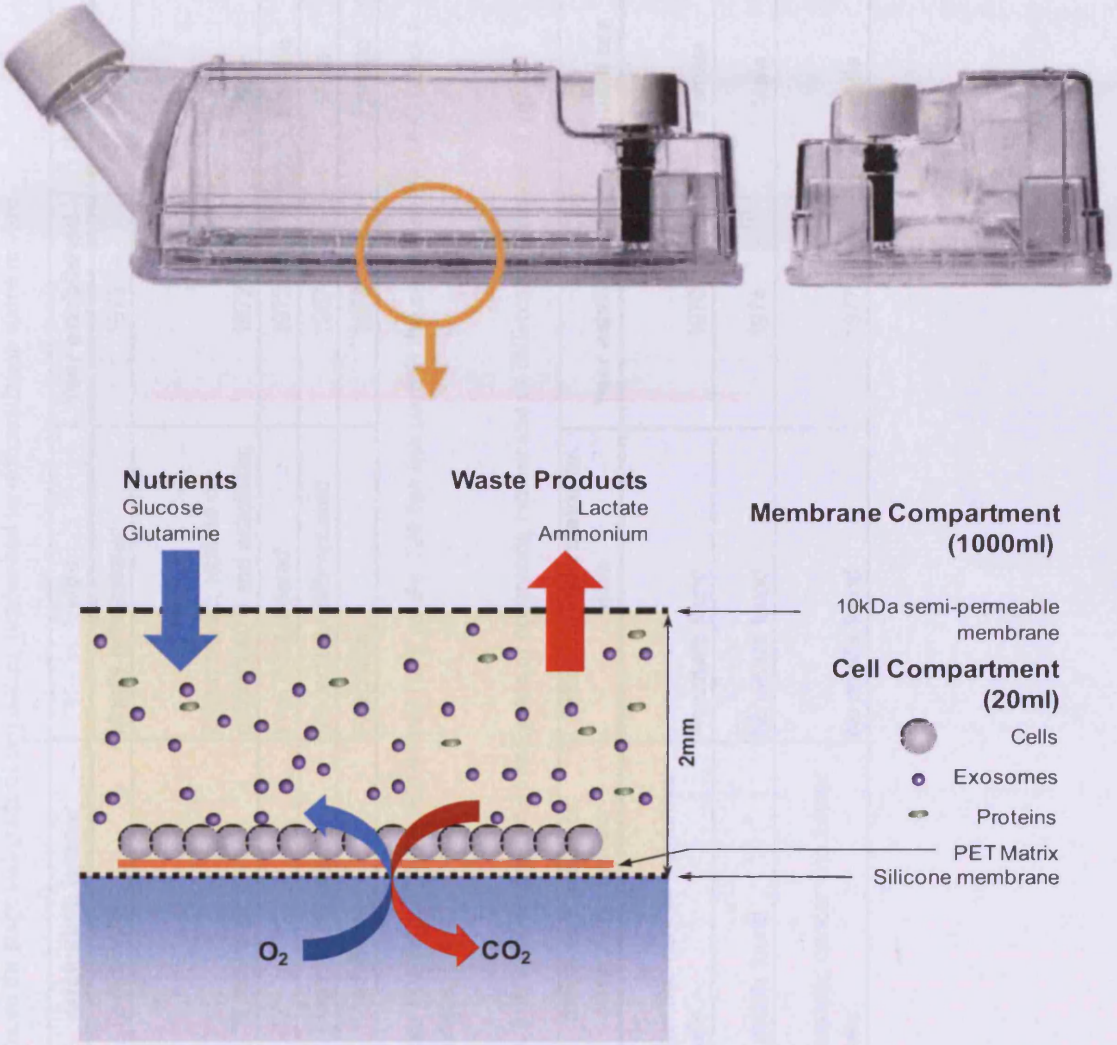


Figure 2.1: Integra CELLine™ flask

Originally designed for hybridoma culture, Integra CELLine™ flasks have two compartments a cell compartment and a nutrient medium compartment. The cell compartment is much smaller but allows the cells to attach to a PET matrix providing a large surface area for cell attachment. The 10 kDa semi-permeable membrane allows nutrient and waste exchange with the medium compartment and efficient gas exchange is achieved through a silicone membrane at the base of the cell compartment. Along with the cells and proteins (>10 kDa), exosomes are also retained within the cell compartment. A comparison of exosome quality and levels from CELLine™ flasks compared to traditional culture flasks is described in Mitchell *et al.*, (2008) [163]. Adapted from Integra CELLine™ Flyer (INTEGRA Bioscience AG, Switzerland) [166].

Table 2.3: Bladder TCC cell lines. Information on the stage and grade of originating tumour and its differentiation status in vitro

Cell line	Origin of tumour biopsy	originating tumour	*vitro*	Year established	Patient sex
HT1376	Bladder primary	G3, T2 minimum	Mostly differentiated	1973	Female
HT1197	Recurrent in bladder	G4, T2 minimum	Pleiomorphic; mixture of differentiated and anaplastic	1972	Male
RT112	Bladder primary	G1, T2	Well differentiated	1973	Female
RT4	Recurrent in bladder	G2, stage not reported	Moderately differentiated	1967	Male
EJ*	Recurrent in bladder	G3, T2 minimum	Anaplastic	1970	Female

*(*EJ cell line confirmed to be same MHC Class I haplotype (A1, A3/B18/Cw5) [165] as the T24 cell line used in the current study (MHC Class I haplotype determined by the Welsh Blood Service, UK))*

Table 2.2: Non-bladder carcinoma cell lines. Information on the stage and grade of originating tumour and its differentiation status in vitro

Cell line	Origin	Stage and grade of originating tumour	Differentiation status *in vitro*	Year established	Patient sex
MCF7	Breast adenocarcinoma pleural effusion	Metastatic cancer - no further details	No details found	1970	Female
Caco-2	Colorectal adenocarcinoma	No details found	No details found	1974	Male
LnCAP	Metastatic lesion of prostate adenocarcinoma	Metastatic cancer - no further details	No details found	1977	Male

2.3 *Light microscopy of live cells*

Light microscopy was used to demonstrate the general morphology of each cell line when growing in monolayer culture. 80-100% confluent live cells in 75 cm^3 culture flasks were imaged using a Zeiss Axiovert 40 CFL microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). Phase contrast images were taken using the 20x objective lens, Canon Powershot G6 digital camera and Canon utilities remote capture (v.2.7.5.27).

2.4 *Immunohistochemistry of fixed cells*

The wells of 8 chambered cover glass slides (Fisher) were seeded with 50-60,000 cells/well and incubated overnight or until ~50% confluent. The cells were washed with phosphate buffered saline (PBS) (Lonza) then fixed in ice cold 1:1 acetone /methanol (v/v) (both Fisher Scientific) for 10 min and subsequently air dried.

The cells were washed three times with PBS and then blocked with 1% (w/v) Bovine serum albumin (BSA) in Hanks' balanced salt solution (HBSS) (both Sigma) for 1 h at room temperature (RT). The wells were washed twice with 0.1% (w/v) BSA in HBSS. The cells were incubated in 40 µg/ ml primary antibody (Ab) (Table 2.5) in 0.1% (w/v) BSA in HBSS overnight at 4°C followed by 3 washes in 0.1% (w/v) BSA in HBSS. Samples were then incubated for 1 h in 25 µg/ ml fluorescein isothiocyanate (FITC) conjugated F(ab')2 Ab (DAKO UK Ltd, Ely, UK) in 0.1% (w/v) BSA in HBSS, at RT in the dark, followed by 2 washes in 0.1% (w/v) BSA in HBSS. The cells were incubated with a solution of 4',6-diamidino-2-phenylindole (DAPI) (14.3 mM) (Invitrogen) diluted to 1:40,000 in 0.1% (w/v) BSA in HBSS for 30 s followed by 3 further washes in 0.1% (w/v) BSA in HBSS. The wells were subsequently filled with PBS (300 µl per well) and imaged using a Zeiss Axiovert 40 CFL microscope fitted with 20x and 40x oil lenses, a UV lamp and filters at emission wavelengths of 518 nm (FITC) and 458 nm (DAPI).

2.5 *Phenotyping of cells by flow cytometry*

Trypsinised cells were prepared for flow cytometry by washing in PBS followed by centrifugation at 300 *g* for 5 min at RT. The cell pellet was resuspended in autoMACS™ (Milltenyi Biotec Ltd, Bisley, UK) running buffer at a density of approximately 100,000 cells per 100 µl. For each antibody test 100 µl of cell suspension was incubated with 10 µg/ml of primary Ab (Table 2.5) for 45 min on ice. Cells were washed and centrifuged at 300 *g* for 5 min at RT and then incubated with secondary antibody in 100 µl autoMACS™ for 30 min on ice. A final wash was performed and the cells resuspended in 300 µl autoMACS™ running buffer for flow cytometry. Cells were analysed using a BD FACSCanto™ (Becton Dickinson, Oxford, UK) and BD FACSDiva v6 software (Becton Dickinson).

2.6 *Purification of exosomes using a sucrose cushion*

The CM was pre-cleared by serial centrifugation to remove cells, 400 *g* for 5 min at 25°C, followed by centrifugation at 2000 *g* for 15 min at 5°C to remove any large cell debris. The supernatants were then frozen at -80°C.

Samples were defrosted in a water bath heated to 37°C and mixed briefly. The supernatants were subjected to a further clearing step of 10,000 *g* for 45 min [42, 67]. The samples were underlain with 4 ml of 30% sucrose/ deuterium oxide (D_2O) (density of 1.2 g/ml) and ultracentrifuged at 100,000 *g* for 2 h (with a SW32 rotor, and an Optima LE80K Ultracentrifuge, Beckman Coulter, High Wycombe, UK). Around 2 ml of the centre most part of the sucrose cushion was then collected and diluted in excess PBS to wash away the sucrose [39, 42, 67]. The exosomes were then pelleted by ultracentrifugation at 100,000 *g* for 2 h (with a fixed angle 70Ti rotor, and an Optima LE80K, Beckman Coulter). Exosome pellets were typically resuspended in 50-150 µl PBS and stored at -80°C [46, 47].

Exosome samples were quantified by protein concentration determined using a Micro BCA protein assay (Thermo Fisher Scientific Inc, UK). A standard curve was performed by serial dilution of 1 µg/ml BSA to 0 µg/ml by 10 points. Typically exosome preparations

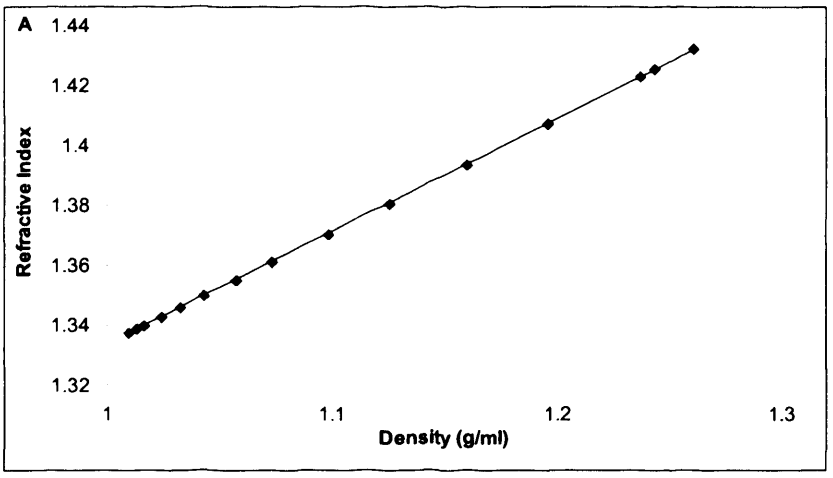
were diluted 1:8 with PBS. Absorbance values were extrapolated from the standard curve to calculate the protein content of the exosome preparations.

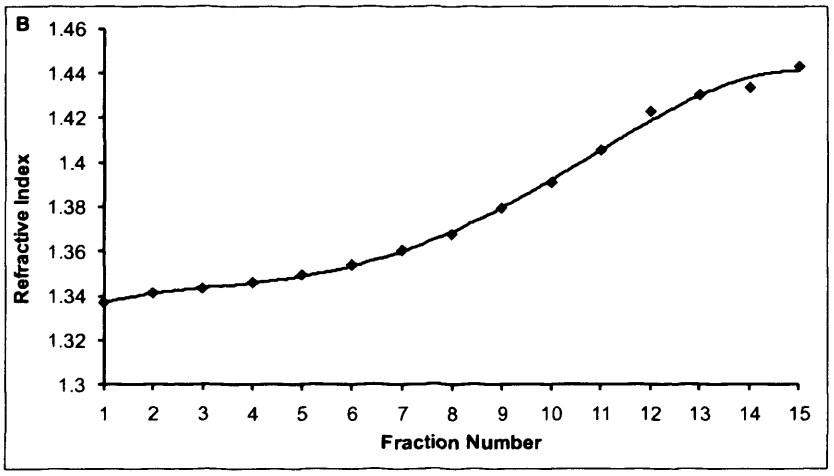
2.7 *Purification of exosomes using a continuous sucrose gradient*

Pre-cleared samples were defrosted at 37°C and mixed briefly. The supernatants were subjected to ultracentrifugation at 10,000 *g* for 30 min at 4°C using a TLA110 rotor in an Optima-Max ultracentrifuge (Beckman Coulter). The supernatant was transferred to fresh centrifuge tubes and subjected to a further ultracentrifugation at 150,000 *g* for 30 min at 4°C. The supernatant was then removed and discarded and the remaining pellet was resuspended in 200 µl PBS.

Continuous sucrose gradients were created using a gradient maker (Hoefer S614, GE Bioscience). The chamber adjacent to the outflow aperture was filled with 0.2 M sucrose solution and the second chamber was filled with an equal volume of 2.5 M sucrose solution. The gradient was then poured in an open top polyallomer centrifuge tube (Beckman Coulter). Two gradients were always made in order to provide a balance. The resuspended pellet was the overlaid on top of the gradient and the samples subjected to ultracentrifugation at 210,000 *g* for 16 h at 4°C (using an MLS-50 rotor in an Optima-Max ultracentrifuge). Once centrifugation was complete 330 µl aliquots were carefully taken from the top of tube until all liquid had been removed (usually 15 aliquots).

The refractive index of collected fractions was measured at 20°C using an automatic refractometer (J57WR-SV, Rudolph Scientific) and from this the density was calculated as described previously [47], using the conversion table in the Beckman Coulter ultracentrifuge manual. Typical refractive index measurements can be seen in Figure 2.2. The relationship between density and refractive index is linear (at fixed temperature and pressure).





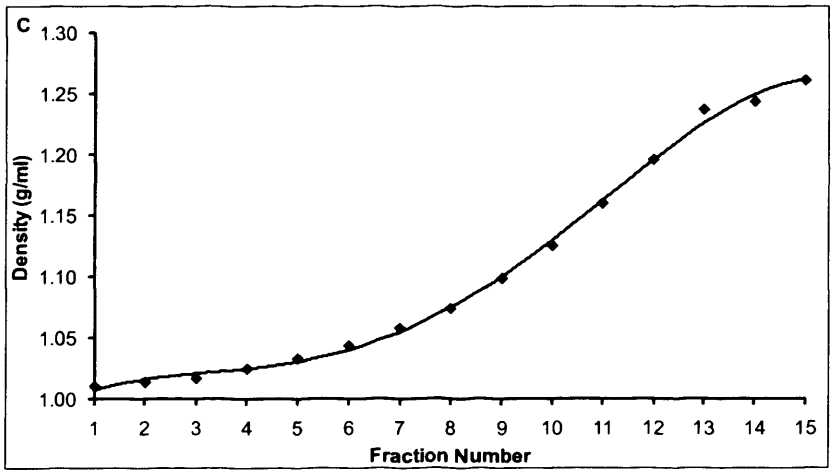


Figure 2.2: Typical refractive index measurements for fractions of sucrose gradient separated exosomes

A. demonstrates the linear relationship between density and refractive index. B and C show typical plots of refractive index and densities of sucrose gradient fractions.

2.8 *Preparation of cell lysates*

Cell lysates (CL) were prepared by resuspending $1x10^6$ cells in 100 µl lysis buffer (1x protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) plus 2% NP40) and incubating on ice for 30 min. Cells were vortexed and homogenised using a 1 ml syringe and 25 gauge needle. Samples were centrifuged twice at 10,000 *g* for 10 min. The subsequent supernatant was then split into aliquots and stored at -80°C. Protein concentration was determined by Micro BCA protein assay (Thermo Fisher Scientific Inc, UK).

2.9 *Analysis of samples by immunoblotting*

Samples were diluted a minimum of 1:1 sample to sample buffer (0.5 M Tris pH 6.8, 25% Glycerol (BDH Chemicals Ltd, Poole, UK), 1% SDS, Bromophenol blue), either reducing, with the addition of 20 mM Dithiothreitol (DTT) (Sigma) or non-reducing. All samples were heated for 10 min at 96°C using a DNA amplifier (DNA Amplifier MIR-D30, Sanyo electric Co. Ltd, Japan).

Using the XCell SureLock™ Novex Mini-Cell system (Invitrogen) 12 well 1 mm, or 15 well 1.5 mm pre-cast NuPAGE 4-12% Bis-Tris gradient gels (Invitrogen) were loaded with a ladder marker (Precision Plus Protein™ Standards, Invitrogen) and the samples. The gels were run, using 1x NuPAGE® MOPS SDS running buffer (Invitrogen) and Invitrogen PowerEase™ 500 power supply until the dye front reached the bottom of the gel (200 V constant, start: 100-115 mA/gel, end: 60-70 mA/gel).

Proteins were transferred onto methanol activated PVDF membranes (GE Healthcare) using 25 mM Tris, 192 mM glycine (both Sigma) pH 8.3 transfer buffer and a BioRad Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad Laboratories Inc, Hemel Hempstead, UK). In addition to the recommended cooling conditions (frozen BioIce cooling unit in the tank) the tank assembly was placed in ice. The blots were run for 1 h at a constant 80 V and the membranes were then blocked overnight at 4°C in WesternDot™ blocking buffer (Invitrogen).

The membranes were incubated for 1 h at RT with primary Ab between 0.2 µg/ml and 1 µg/ml, where the optimal dose was empirically determined for each antibody (Table 2.4). The membranes were then washed 3x 5 min with 1x WesternDot™ wash buffer and incubated with a Biotin-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at RT. After a further 3x 5 min washes membranes were incubated Qdot® 625 streptavidin conjugate for 1 hour. The membranes were washed 3x 5 min in wash buffer followed by 1 wash in water. The bands were detected using a MiniBIS-Pro (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel) imager fitted with a UV lamp drawer.

Table 2.4: Antibody details and concentrations for immunoblotting

Specificity	Clone	Isotype	Size (kDa)	Working concentration (µg/ml)	Conditions	Company	Cat. No
5T4	H8	IgG_1	72	0.5	Non-reducing	Oxford bioMedica	Gift from R. Harrop, Oxford bioMedica
Basigin	8D6	IgG_1	55	1.3	Reducing	Insight Biotech	sc-21746
Calnexin	AF18	IgG_1	90	1	Reducing	Insight Biotech	sc-23955
CD44	DF1485	IgG_1	90-95	1	Non-reducing	Insight Biotech	sc-7297
CD63	MEM-259	IgG_1	40-60	1	Non-reducing	Serotec	MCA2142
CD73	2B6	IgG_{2b}	67-71	2	Reducing	Insight Biotech	sc-130006
CD81	1D6	IgG_1	22-26	0.2	Non-reducing	Serotec	MCA1847EL
CD9	209306	IgG_{2b}	24	0.2	Non-reducing	R&D	MAB1880
CK 17	Q-09	IgG_1	46	1	Reducing	Insight Biotech	sc-100930
CK 18	DC-10	IgG_1	45	1	Reducing	Insight Biotech	sc-6259
Galectin-3	9C4	IgG_1	31	1:200 culture supernatant	Reducing	Insight Biotech	sc-56108
GAP DH	1A10A11	IgG	36-38	0.1	Reducing	BioChain	Y3322GAPDH
GRP 94*	N/A	IgG_{2a}	94	1	Reducing	Stressgen	SPA-850
Her2/neu	F-11	IgG_{2a}	185	2	Reducing	Insight Biotech	sc-7301
HLA-G	4H84	IgG_1	39	2.5	Reducing	Insight Biotech	sc-21799
hnRNP K	D-6	IgG_{2a}	65	0.04	Reducing	Insight Biotech	sc-28380
HSP 90 α/β	F-8	IgG_{2a}	90	0.75	Reducing	Insight Biotech	sc-13119
LAMP-1	H4A3	IgG_1	110	2	Reducing	Insight Biotech	sc-20011
LAMP-2	H4B4	IgG_1	120	2	Reducing	Insight Biotech	sc-18822
MHC Class 1	HC10	IgG_{2b}	46	1:1000 hybridoma supernatant	Reducing	MRC co-operative	Cardiff University
THP**	D-20	IgG	85	1.5	Reducing	Insight Biotech	sc-19552
TSG101	C-2	IgG_{2a}	45	2	Reducing	Insight Biotech	sc-7964
α Tubulin	B-7	IgG_{2a}	50-55	2.5	Reducing	Insight Biotech	sc-5286
β-catenin***		Polyclonal	94	1:10,000 whole antiserum	Reducing	Sigma	C 2206

All primary antibodies were raised in mice unless otherwise indicated. Primary antibodies were compatible with anti-mouse or anti-goat horseradish peroxidase conjugated secondary antibodies or mouse immunoglobulins HRP. The Ab clone, isotype, specific protein size, working concentration, conditions to be run under, company and catalogue number are indicated in the table. Secondary Ab was either Anti-mouse immunoglobulins horseradish peroxidise (HRP) conjugated secondary antibody (Insight Biotechnology Ltd., UK), Anti-rabbit immunoglobulins HRP, anti-goat immunoglobulins HGP or an anti-mouse immunoglobulins HRP (DAKO), which is cross-reactive for rat antigens (* raised in rat, **raised in goat, ***raised in rabbit).

2.10 *Exosome quality assurance assay (ExoQA)*

This method was used to evaluate the purity of exosome preparations. White surfactant free aldehyde sulphate 3.9 μm diameter latex micro-beads (Interfacial Dynamics, Portland, Oregon) were washed twice in MES buffer (0.025 M MES, 0.154 M NaCl (both Sigma), pH 6) at 2000 *g* for 10 min at RT. The subsequent bead pellet was resuspend in MES buffer and the equivalent of 1 μl of stock micro-beads were incubated with 1 μg of purified exosomes made up to a total volume of 100 μl with MES buffer. The samples were shaken for 1 h at RT followed by rolling overnight at 4°C. The exosome micro-bead complexes were then washed at 2000 *g* for 10 min at RT and blocked for 2 h in 1% BSA MES (wt/vol). The blocking buffer was washed away and the exosome micro-bead complexes were resuspended in 1 ml 0.1% BSA MES.

For each condition to be tested 50 μl of exosomes coupled to micro-beads were plated out onto a 96 well plate. To each well 1 μg of primary Ab (Table 2.5) was added and after 1 min on a plate shaker the samples were incubated for 1 h on ice. The exosome micro-bead complexes were subsequently washed twice with 0.1% BSA MES (2000 *g*, 10 min, RT) and incubated with 50 μl FITC conjugated secondary Ab (1:50) (DAKO) for 1 h on ice, before two more washes. The exosome micro-bead complexes were finally resuspend in 100 μl per well of MES buffer and analysed by flow cytometry using a FACSCanto instrument configured with a high throughput sampling module running FACSDiva v6.1.2 software. A graphical summary of the method can be seen in Figure 2.3.

Micro-bead populations were identified by forward and side scatter properties and a gate (P1; Figure 2.3) was drawn around the single-bead population, thus ignoring bead doublets and triplets. Histograms on gated events were used to determine median fluorescence values for each antibody tested.

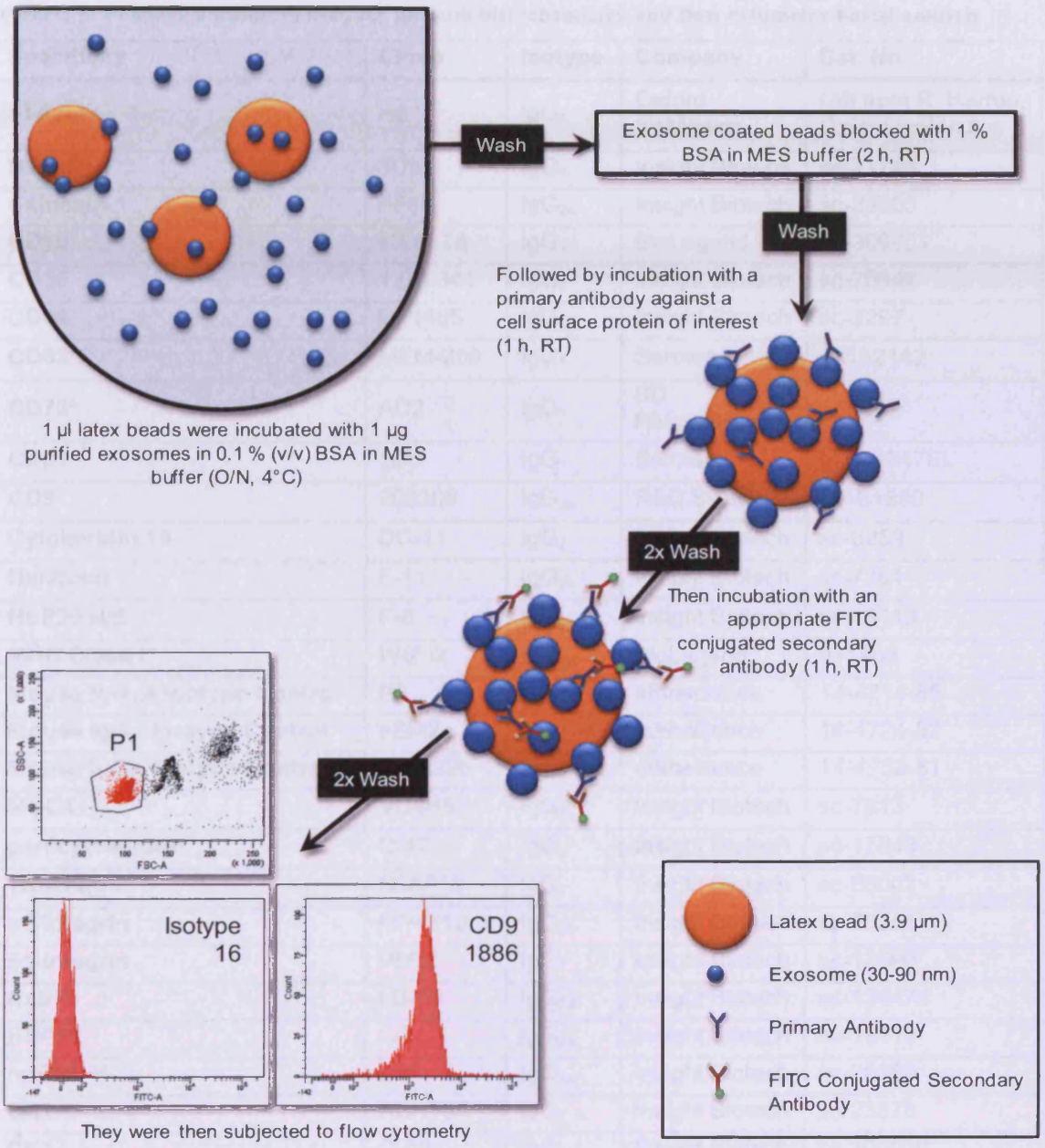


Figure 2.3: Flow diagram of flow cytometry of exosome coated latex micro-beads

Latex micro-beads were incubated with purified exosome sample in MES buffer and then washed and blocked. After a further wash the exosome coated micro-beads were then incubated with primary antibody followed by two washes and incubation in the FITC conjugated secondary. After a further two washes the exosome bead complexes were subjected to flow cytometry, where a population of single micro-beads (P1) was identified and all analysis was performed on this gated population.

Table 2.5: Primary antibodies used for immunohistochemistry and flow cytometry based analysis

Specificity	Clone	Isotype	Company	Cat. No
5T4	H8	IgG_1	Oxford bioMedica	Gift from R. Harrop, Oxford bioMedica
Basigin	8D6	IgG_1	Insight Biotech	sc-21746
Calnexin	AF18	IgG_{2a}	Insight Biotech	sc-23955
CD10	MEM-78	IgG_1	BioLegend	sc-309901
CD36	1.BB.344	IgG_1	Insight Biotech	sc-70642
CD44	DF1485	IgG_1	Insight Biotech	sc-7297
CD63	MEM-259	IgG_1	Serotec	MCA2142
CD73*	AD2	IgG_1	BD Pharmingen	550257
CD81	1D6	IgG_1	Serotec	MCA1847EL
CD9	209306	IgG_{2b}	R&D Systems	MAB1880
Cytokeratin 18	DC-11	IgG_2	Insight Biotech	sc-6259
Her2/neu	F-11	IgG_{2a}	Insight Biotech	sc-7301
HSP90 α/β	F-8	IgG_{2a}	Insight Biotech	sc-13119
MHC Class I*	W6/32	IgG_{2a}	BioLegend	311406
Mouse IgG_1,k Isotype Control	P3		eBioscience	14-4714-85
Mouse IgG_{2a} Isotype Control	eBM2a		eBioscience	14-4724-82
Mouse IgG_{2b} Isotype Control	eBMG2b		eBioscience	14-4732-81
MUC1	VU4H5	IgG_1	Insight Biotech	sc-7313
pan-cytokeratin	D-12	IgG_1	Insight Biotech	sc-17843
Vimentin	5G3F10	IgG_1	Insight Biotech	sc-66002
α6-integrin	MP4F10	IgG_{2b}	Insight Biotech	sc-53356
β1-integrin	P5D2	IgG_1	Insight Biotech	sc-13590
Rab1b	LD-S3	IgG_{2a}	Insight Biotech	sc-130474
HSP90	F-8	IgG_{2a}	Insight Biotech	sc-13119
hnRNP K	D-6	IgG_{2a}	Insight Biotech	sc-28380
CK7	RCK105	IgG_1	Insight Biotech	sc-23876
CK17	Q-09	IgG_1	Insight Biotech	sc-100930
CK18	DC-10	IgG_1	Insight Biotech	sc-6259
CK19	BA16	IgG_1	Insight Biotech	sc-53257

All primary antibodies were mouse derived monoclonal Ab detected with Polyclonal Goat anti-mouse immunoglobulins/ FITC Conjugated F(ab')2 Ab (F047902; DAKO UK Ltd, Ely, UK). The Ab clone, isotype, company and catalogue number are indicated in the table. Note: Not all Ab were used for both techniques. (*PE conjugated)

2.11 *Analysis of exosome sucrose gradient purified exosomes by flow cytometry*

The same procedure (section 2.10) was also used for exosome sucrose gradient fractions but one third of each fraction was incubated with the equivalent of 0.4 µl original stock beads. The rest of the procedure remained the same.

2.12 *Analysis of exosome micro-bead complexes using fixation and permeablisation*

In some instances a fixation and permeablisation (IntraPrep kit™, Beckman Coulter) procedure was used to examine the expression of intraluminal proteins. In this instance, once the wash buffer had been washed away, the samples were incubated in fixative solution for 10 min at RT. Exosome micro-bead complexes were then washed as for ExoQA. The beads were resuspended in permeabilising solution plus primary Ab and incubated on ice for 45 min. The procedure then remained the same as for the ExoQA method apart from incubating the exosome micro-bead complexes with secondary Ab in permeabilising solution for 45 min on ice. The flow cytometry analysis remained the same.

2.13 *Imaging exosomes using transmission electron microscopy*

Previously frozen exosomes were thawed on ice and resuspend in 1% (v/v) glutaraldehyde (Sigma) in PBS (pH 7.4). A 5 µl drop of suspension was transferred on to pioloform-coated copper grid and incubated at room temperature for 5 min. The grid was transferred into a 50 µl drop of water for 2 min. This process was repeated seven times (total of eight washes). The sample was stained with a 5 µl drop of 2% methyl cellulose containing 2% uranyl acetate (both Sigma) and incubated on ice for 10 min. Any excess fluid was removed with filter paper (Whatman Ltd, UK) and allowed to air dry for 10 min before viewing by transmission electron microscopy (Philips EM 208, FEI Co, Eindhoven, The Netherlands).

2.14 *Separation of exosomal proteins by two dimensional electrophoresis*

Proteins were separated by two dimensional electrophoreses (2DE) by firstly rehydrating the IPG (immobilised pH gradient) strips (Immobiline DryStrip pH 3-10 NL, 24 cm, GE Healthcare) in with rehydration buffer (7M Urea, 2M Thiourea (both GE Healthcare), 4% (w/v) CHAPS (Sigma), 0.005% (v/v) bromophenol blue and 0.5% (v/v) immobilised pH gradient (IPG) buffer pH 3-10NL) (both GE Healthcare) in Immobiline™ DryStrip reswelling trays (GE Healthcare). Strips were covered with DryStrip cover fluid (GE Healthcare) and left at RT for 12 h to rehydrate.

The exosome samples were then prepared for isoelectric focussing (IEF) by re-pelleting at 100,000 *g* for 45 min at 4°C in order to increase the efficiency of solubilisation and to remove interfering substances such as salt. The subsequent pellet was resuspended in lysis buffer (rehydration buffer plus 20 mM DTT) and incubated at RT for 1 h with vortexing every 10 min. The sample was then centrifuged at 12,000 *g* for 10 min to pellet any remaining insoluble material. The supernatant containing the solubilised proteins were subjected to protein precipitation using a 2D clean-up kit (GE Healthcare) performed according to the manufacturer's instructions and subsequently resuspend in 50-150 µl lysis buffer. The sample was then loaded on to the IEF strip via anodic cup loading in a manifold using an Ettan™ IPGphor™ 3 (GE Healthcare) IEF system. The strip(s) were then subjected to isoelectric focussing for 1 h at 500 V, 7 h up to 1,000 V, 3 h up to 10,000 V (gradient), 4 h at 10,000 V, 1 h down to 500 V and 6 h at 500 V. Overall current limit was 75 µA per strip.

A minimum of five hours prior to preparing the second dimension 10% polyacrylamide gels were poured into the DALT*six* Gel Caster (GE Healthcare). Once the gels were set the IPG strips were rinsed with water and then equilibrated in equilibration buffer (50 mM Tris-HCL pH 8.8, 6 M urea, 2% SDS (GE Healthcare), 30% Glycerol, 0.002% bromophenol blue) supplemented with 1% (w/v) DTT (Sigma). This was followed by equilibration with equilibration buffer supplemented with 2.5% (w/v) iodoacetamide (GE

Healthcare). The strips were then carefully placed on top of the second dimension gel and sealed in place with an agarose (GE Healthcare) solution (5%) ensuring there were no bubbles between the first and second dimension gels.

The gels were then placed in an Ettan DALT*six* Electrophoresis Unit (GE Healthcare) with the lower chamber was filled with anodic buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) and the upper chamber with cathodic buffer (50 mM Tris, 384 mM Glycine, 0.2% SDS). An electric current was applied to the gels overnight at 12°C using the following settings: Step one 10 mA/gel, 80 V, 1 W/gel for 1 h; Step 2 12 mA/gel, 150 V, 2 W/gel for 14-16 h. The gels were run until the bromophenol blue dye front had reached the bottom of the gel. The gels were then separated from the glass plates and placed in fixative for 30 min (10% acetic acid, 40% ethanol (both Fisher Scientific) and subsequently silver stained using PlusOne™ Silver Staining Kit (GE Healthcare) with minor adjustments to the manufacturers' protocol. No glutaraldehyde was used and formaldehyde was only used in the developing stage of the protocol. Gels were scanned using an image scanner (UMAX PowerLook 1120, GE Healthcare) and saved as 256 greyscale, 600 dpi .tiff files.

2.14.1 Gel plug sample preparation for mass spectrometry

In order for peptides to be recovered from a 1DE or 2DE gels for MS analysis several objectives have to be met. This includes de-staining of protein spots, reduction and alkylation of the protein, fragmentation of proteins into peptides and transfer of the peptides onto a MS plate for analysis.

In brief gel plugs (1.5 mm diameter) were excised using a manual spot cutting pipette (Spot Picker OneTouch Plus; Web Scientific Ltd) and the gel spots placed into Ettan Digestor 96 well plates the peptides were recovered following trypsin digestion using a slightly modified version of the Shevchenko *et al.,* (1996) method [167]. Sequencing grade modified trypsin (Promega UK Ltd) was used at 6.25 ng/µl in 25 mM NH_4HCO_3 and incubated at 37°C for 3 h. Finally the dried peptides were resuspended in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA; 5µl) for mass spectrometry (MS)

analysis. An aliquot corresponding to 10% of the material (0.5 µl) was spotted onto a 384 well MS plate. The samples were allowed to dry and were then overlaid with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma; 0.5 µl prepared by mixing 5 mg matrix with 1 ml of 50% (v/v) acetonitrile in 0.1% (v/v) TFA).

2.14.2 Mass spectrometry and data analysis of 2DE separated proteins

Mass spectrometry was performed using a matrix assisted laser desorption ionisation time of flight-time of flight (MALDI TOF/TOF) mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyzer; Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355 nm [168-171]. MALDI mass spectra and subsequent tandem MS (MS/MS) spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other were excluded from the selection and the peaks were analysed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6 x 10-6 Torr) and default calibration.

Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed using the MASCOT Database search engine v2.1 (Matrix Science Ltd, London, UK) embedded into Global Proteome Server (GPS) Explorer software v3.6 (Applied Biosystems) on the Swiss-Prot database [172]. Searches were restricted to the human taxonomy with trypsin specificity (one missed cleavage allowed) and the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identifications confirmed if there was high quality tandem MS (good y-ion) data for two or more peptides (expect (e) value $p < 0.05$ for each peptide; overall $p < 0.0025$) or one peptide (only if the e value was $p < 0.0001$).

2.15 *Separation of exosomal peptides by nano-liquid chromatography*

2.15.1 Preparation of exosome-derived peptides for nano-LC

Exosome preparations were repelleted at 118,000 *g* for 45 min at 4°C (using a TLA-110 rotor and Optima-Max ultracentrifuge). The pellets were solubilised in 100 µl triethylammonium bicarbonate (TEAB) lysis buffer (20 mM TEAB) containing 20 mM DTT and 1% (w/v) SDS. Samples were incubated at RT for 10 min then heated to 95°C for 10 min and then left for a further 10 min at RT. They were subjected to an additional ultracentrifugation step (118,000 g for 45 min at RT) and supernatants (now free of insoluble material) were subjected to solvent precipitation to remove salts, lipids and detergent (using 2D clean-up kit). The resulting pellets were resuspended in 20 mM TEAB and left overnight at 4°C.

The protein content was then determined using a BCA protein assay kit (Sigma). Samples were then reduced, denatured and alkylated using an Applied Biosystems iTRAQ labelling kit and standard protocol. The proteins were subjected to digestion with trypsin (0.8 µg per sample) and incubated at 37°C for 12-16 h. The samples were then dried and resuspended in water with 0.1% (v/v) TFA.

2.15.2 LC-MALDI and protein identification

Digested peptides were separated on a nano-LC system (UltiMate 3000, Dionex, Sunnyvale, USA) using a two-dimensional salt plug method as described by Brennan *et al.*, (2009) as follows. Peptides corresponding to 2 µg of undigested protein were separated on a nano-LC system (UltiMate 3000, Dionex, Sunnyvale, USA). Peptides were applied to a strong cation exchange (SCX) cartridge (Bio-SCX, 500 µm, 15 mm, 5 µm, Dionex) that was plumbed upstream of the reverse phase (RP) desalting cartridge (PepMap100, 300 µm, 5 mm, 100 Å, Dionex).

The samples were separated on the SCX cartridge using 20 µl step elutions with increasing concentrations of NaCl (Breakthrough, 100 mM, 200 mM, 400 mM, 800 mM, and 1M).

Each step elution was loaded onto the RP column where the eluting peptides were desalted. Peptides were then separated using a C18 column (PepMap75 μm id, 30 cm, 3 μm, 100 Å, Dionex) at a flow rate of 300 nL/min. The buffers used were: A: 2% acetonitrile in water with 0.05% (v/v) TFA and B: 90% acetonitrile in water with 0.01% (v/v) TFA. Peptides were separated using a two-step gradient with the first step from 5 to 20% solvent B for 25 min and the second step from 20% to 50% solvent B for 21 min. Fractionation of the peptides into 8 second spots on an LC-MALDI sample plate was performed with a Probot microfraction collector (Dionex). CHCA (Sigma) was used as MALDI matrix (2 mg/ml in 70% (v/v) acetonitrile in 0.1% (v/v) TFA containing 10 fmol/μl Glu-Fib) which was continuously added to the column effluent via a μ-tee mixing piece at a flow rate of 1.4μL/min [169].

Mass spectrometry was performed using an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer with a 200 Hz solid state laser operating at a wavelength of 355nm. The protocol used was the same as that described by Brennan *et al.*, (2009). After screening of all LC-MALDI sample positions in MS positive reflector mode using 800 laser shots (mass range 700-4000 Da; focus mass 2000) the fragmentation of up to 6 automatically selected precursors was performed (most intense ion signals per spot position with S/N above 50 and strongest analysed first). Internal calibration of each spot in MS was achieved against the Glu-Fib added to the matrix. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolutions of each other were excluded from the selection. In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6 x 10-6 Torr) and default calibration [169].

The MS/MS data was used to search the Swiss-Prot database (Version 57.7; release date 20090901; 497293 sequences; human taxonomy) using the MASCOT Database search engine v2.1.04 (Matrix Science Ltd, London, UK), embedded into GPS Explorer software v3.6 Build 327 (Applied Biosystems). Default GPS parameters were used and the following MASCOT parameters were set:1 missed cleavage allowed, fixed modification of MMTS(C), variable modifications of oxidation (M), pyro-glu (N-term E) and pyro-glu (N-

term Q), 150 ppm mass tolerance in MS and 0.3 Da mass tolerance for MS/MS. These are recommended published tolerances for LC-MALDI [169-171].

In order for a protein to be identified there needed to be a minimum of two peptides with MASCOT e-values less than 0.05. There was a false discovery rate (FDR) of 0% which was determined using the same Swiss-Prot database with the entire sequence randomised. Where more than one protein was identified the protein with the highest MOWSE score in MASCOT was reported.

2.16 *Bioinformatics analysis of LC MALDI MS-identified proteins*

The MS-identified protein dataset was analysed for any biological enrichment against previously defined lists using MetaCore GeneGO (Version 5.4) and selected ExoCarta submissions (MS-based data containing 10 or more matching gene identifiers) [140]. Using 44 studies from ExoCarta gene sets, our protein list was converted from Swiss-Prot accession numbers to EntrezGene identifications using BioMart. Overrepresentation analysis (ORA) was then performed using the hypergeometric distribution in R against a background of all human genes with EntrezGene identifications. For ORA in MetaCore the data was first converted into Swiss-Prot identifications (using BioMart) before analysis using hypergeometric tests.

Chapter 3:

Purification of exosomes from cancer cell lines

3.1 *Introduction*

Exosomes are known to be secreted from a large number of cell types by fusion of multivesicular bodies (MVB) with the cell membrane [94]. Exosomes contained with the MVB are then released into the extracellular fluid space from which they can be purified. Most exosome research groups utilise ultracentrifugation as a principal tool for exosome purification. This is true of samples obtained *ex-vivo* (such as urine, malignant effusions and saliva) and from cell-conditioned media (CM). Biological fluids, given their micro-particulate and molecular complexity, present a major challenge in terms of exosome purification. Using cell culture systems presents certain drawbacks such as culture-related artefacts. However, it does offer an opportunity to obtain exquisite pure exosomes as CM are significantly less complex, less variable and more easily obtained.

Even though CM offers an accessible and consistent source of exosomes, the use of traditional cell culture means high volumes of CM needs to be processed in order to obtain good exosome yields. This is because it is difficult to grow adherent cells in high density culture in traditional cell culture flasks due to issues with surface area to volume ratio (SA: V) making it very difficult to obtain CM rich in exosomes. For example in one study 3.2 L of CM was required to yield 250 μg of exosomes which was sufficient for just one two dimensional electrophoresis gel (2DE) experiment [98]. Processing such large volumes of CM is time consuming, expensive and inefficient.

Another approach to culturing cells in order to obtain high exosome yields efficiently involves the use of bioreactor flasks (Integra CELLine™ flasks) a technique for cell culture for exosome analysis developed in our department [163]. Exosomes can subsequently be purified from this concentrated sample source. For this study the 30% sucrose cushion method was used as this method offers good exosome recovery and eliminates more of the contaminating proteins than the basic pelleting method [133], without the lengthy time and effort involved with isolation via a linear sucrose gradient (section 1.2.6).

Once the exosomes have been isolated it is paramount that the quality of the exosome preparations is checked in order to establish whether a sample is pure enough for proteomics analysis. One method of analysing sample quality is immunoblotting for known exosome markers and for proteins not exosomally expressed; such as nuclear, Golgi, mitochondrial and endoplasmic reticulum (ER) proteins [133]. Proteins known to be expressed and enriched in exosomes include the tetraspanins CD9, CD81, and CD63 [42, 46, 63], also the MVB associated protein TSG101 [108]. These and more are used by our lab [39, 44, 134] and others [42, 64] to characterise preparations as exosomal.

In addition, transmission electron microscopy (TEM) of a typical sample should be performed giving detail on the size and morphology of any exosomes and highlight the presence of visible non-exosomal debris. Both immunoblotting and TEM offer useful information about the purified exosomes but they are both time and sample intensive. Using an exosome quality control assay, where exosome coated latex micro-beads are stained for known exosomal surface proteins and then subject flow cytometry (FC), may allow efficient monitoring of sample purity while retaining most of the sample for subsequent analysis by proteomic methods.

3.2 *Aims*

The aims of this chapter were to:

- establish a work flow to generate large quantities of pure exosomes from adherent cancer cell lines
- establish a reliable method to purify highly pure exosomes from CM
- investigate the exosome phenotypes of each cell line
- develop a reliable method for quality controlling exosome samples
- establish a stock of exosomes from different cell sources that can be used to help verify any putative biomarkers identified

3.3 *Characterisation of cell lines*

Some straightforward checks were performed to confirm the cells exhibited the expected morphology and phenotype compared to published material [8]. In addition, we assessed the level of expression of some characteristic exosome markers.

3.3.1 Cell morphology

The morphology of each cell line growing in monolayer was assessed by light microscopy. The images of HT1376, T24, and RT112 (Figure 3.1a, c, and e) show a typical polygonal morphology seen in transitional epithelium not under stretch. The only TCC cell line to appear particularly different was the RT4 cells (Figure 3.1d). In this case the cells did not form a monolayer by adhering to the plastic of the culture flask but grew in islands of a more dome like shape of densely packed cells in polygonal formation. However, this is normal for this cell line (personal communication, Professor J Masters of University College London) [8]. The HT1197 cell line did appear morphologically to have more than one cell population, which has been described in the literature [157]. Many of the cells appeared to have the typical cobble stone like appearance, but others were larger and more stellate in appearance accounting for approximately 50% of the cells.

The three non-bladder carcinoma cell lines all had a different morphology to the TCC cell lines. MCF7 and LnCAP both appeared to grow without contact inhibition and formed overlapping cell layers after reaching confluence. The LnCAP cells appeared slightly spindle-like consistent with the literature. Caco-2 cells had morphology closer to that of the TCC cells but the cells were less compact and many contained vacuoles. It has been noted in the literature that Caco-2 cells can have varying morphologies [173]. In summary, the morphology of all the cell lines was consistent with the literature.

3.3.2 Cell phenotyping by immunohistochemistry and flow cytometry

Immunohistochemistry (IHC) and flow cytometry (FC) were performed on all cell lines to qualitatively and quantitatively check for epithelial phenotypes. In addition, the expression of tumour associated and other antigens reportedly enriched in exosomes isolated from other cell types were assessed. A panel of antibodies was used including exosomally expressed proteins such as CD63, -81, and -9 [42, 46, 63].

We first tested whether the carcinoma cell lines expressed cytokeratin (CK) a classical epithelial marker [174]. We initially used a pan-cytokeratin antibody and expected strong filamentous staining across all epithelial cells lines. We also examined fibroblasts as a negative control. However, this antibody demonstrated weak and heterogeneous staining across the various cell types including the fibroblasts (Figure 3.1). We concluded that overall staining was largely non-specific with this particular Ab and that this Ab was unsuitable for its intended purpose.

We then examined a particular cytokeratin CK18 which is known to be expressed by the transitional epithelium of the urinary bladder, colon, and the MCF7 cell line [174]. The staining was much stronger and more specific than the pan-cytokeratin (Figure 3.1) and its presence was confirmed by FC (Figure 3.3). Two cell lines HT1376 and MCF7 demonstrated particularly strong staining for CK18 (Figure 3.1a, f).

The mesenchymal cell marker vimentin was used as it is not expressed by normal epithelial cells but is expressed for example by fibroblasts. Therefore we used human foreskin fibroblast cells (HFFs) as a positive control (Figure 3.1i), which demonstrated specific structural staining whereas only staining of a low-level non-specific nature was observed in the other cell lines (Figure 3.1). In conclusion based on this very simple epithelial cell phenotyping the data agrees with the morphological information that these cell lines are indeed epithelial.

We also looked at the expression of tumour associated antigens Mucin1 (MUC1) and Human epidermal growth factor receptor 2 (Her2/neu) which are commonly over-

expressed by many carcinomas. MUC1 expression is normally seen on the apical surface of epithelial cells but altered expression is seen in numerous carcinomas and is known to promote tumour progression through its anti-adhesive affects [175, 176].

Two cell lines (HT1376 and MCF7; Figure 3.1a, f) were shown to have particularly strong IHC staining for MUC1. Cell surface expression was confirmed by FC (Figure 3.3) for HT1376 but not MCF7. This may be due to the heterogeneous IHC staining in the MCF7 cell population. Around 75% of the cell population demonstrated perinuclear staining of MUC1 whereas the remainder of the cells showed diffuse staining of MUC1 within the cell. However, little expression of MUC1 was observed in six of the cell lines which did not correspond to the literature [177-179]. This may have been caused by problems during the fixation process.

Her2/neu is normally involved in cell growth and differentiation but it has been noted to be over expressed in breast and other carcinomas. IHC staining was observed for many of the cell lines (Figure 3.1) although in most cases the staining was weak which does not correspond with data presented by others [180, 181]. This may have been caused by an incompatibility of the Ab with the acetone: methanol fixation method used in our IHC or the levels of the protein may have been below detection levels.

In terms of expected exosome markers the molecular chaperone HSP90 (heat shock protein 90) showed uniform expression by all the cell lines observed by IHC and FC (Figure 3.1) [94]. Low levels of tetraspanin staining by IHC were observed which was disappointing because other studies have found high levels of tetraspanins in exosomes [42, 46, 63]. However, FC on unfixed cells (Figure 3.2 and Figure 3.3) revealed strong staining for the three tetraspanins examined. These differences may be caused by an incompatibility of the IHC fix-perm method with these tetraspanin antibodies particularly CD9 and -81. The IHC staining for CD63 did show some instances of distinct perinuclear binding of the antibody (Figure 3.1a, c-d).

In summary, the cell lines appear to be epithelial confirming their expected morphology/phenotype. In addition we have evaluated the levels of some known exosomal proteins, including two tumour associated antigens, giving us an indication of what we might expect to be expressed in exosomes derived from these cells-lines.

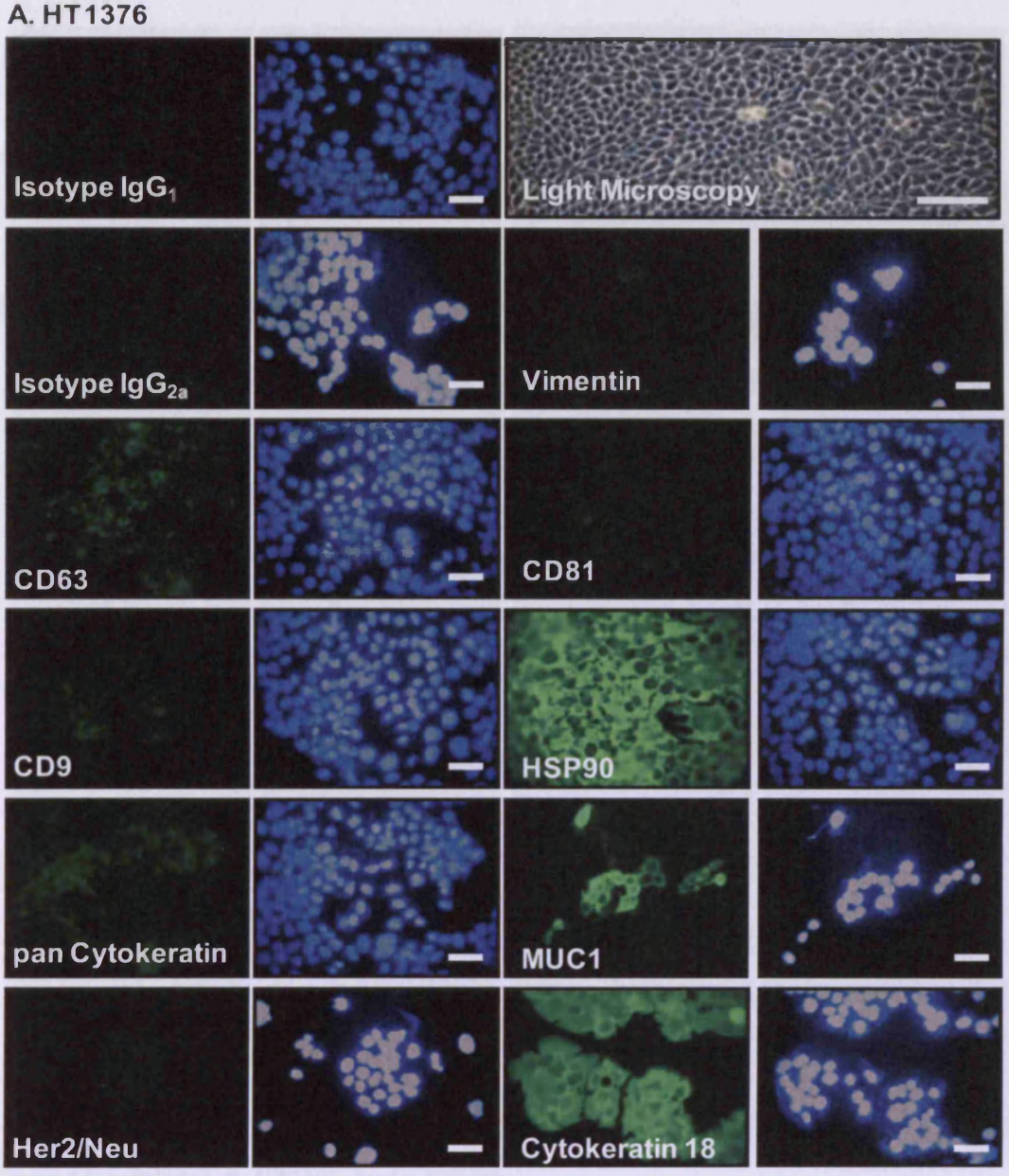


Figure 3.1: Morphological and immunohistochemical characterisation of bladder cancer and other carcinoma cell lines

A. HT1376. Light microscopy images were taken of live cells under phase contrast. Immunofluorescence images were taken of fix-perm cells labelled for the target protein followed by a goat anti-mouse FITC conjugated secondary (left hand image of each pair). The cells were additionally stained with DAPI (right hand image of each pair). (Scale: light 100μm; IHC 50μm)

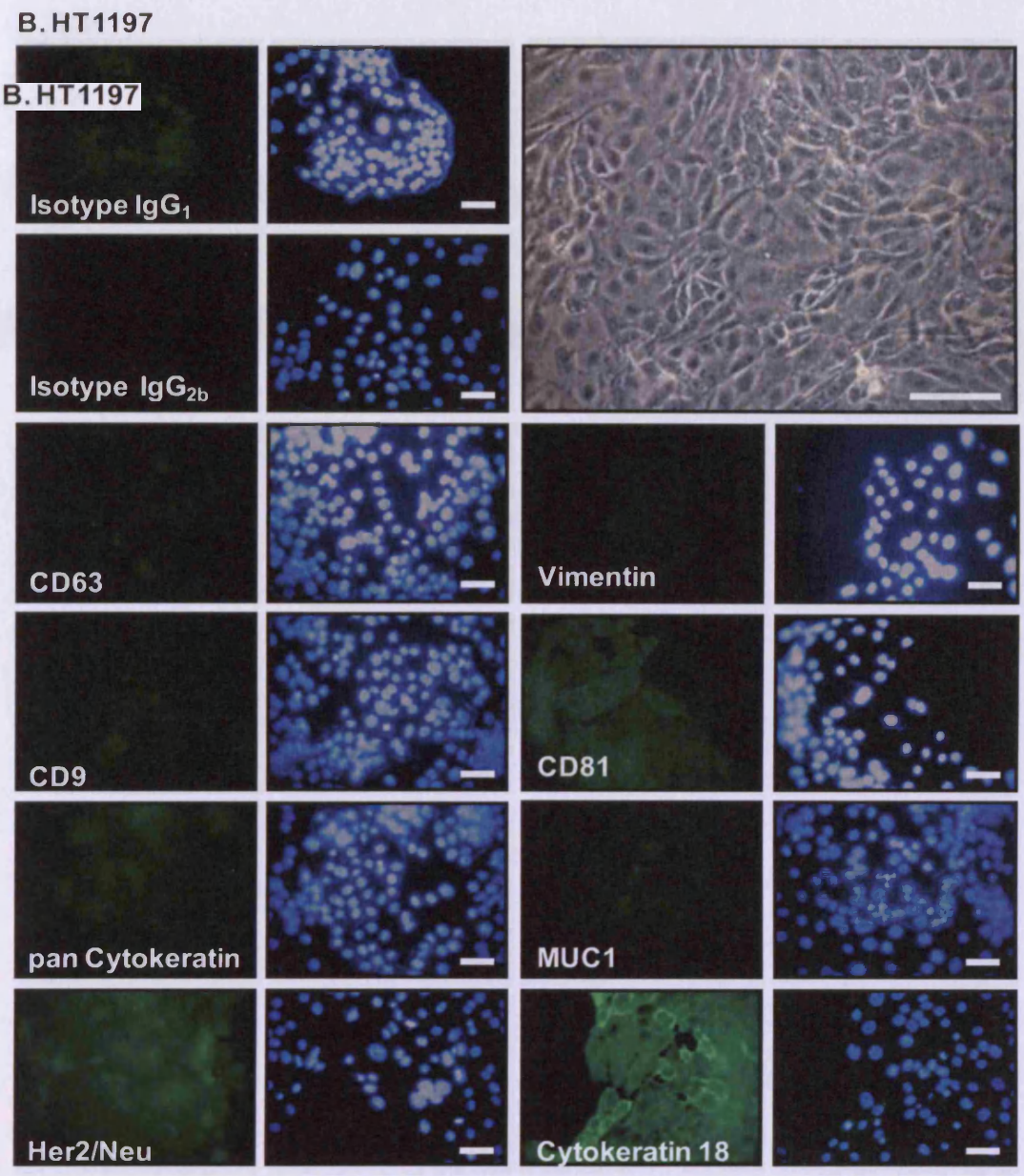


Figure 3.1. continued. B. HT1197 (Scale: 100μm)

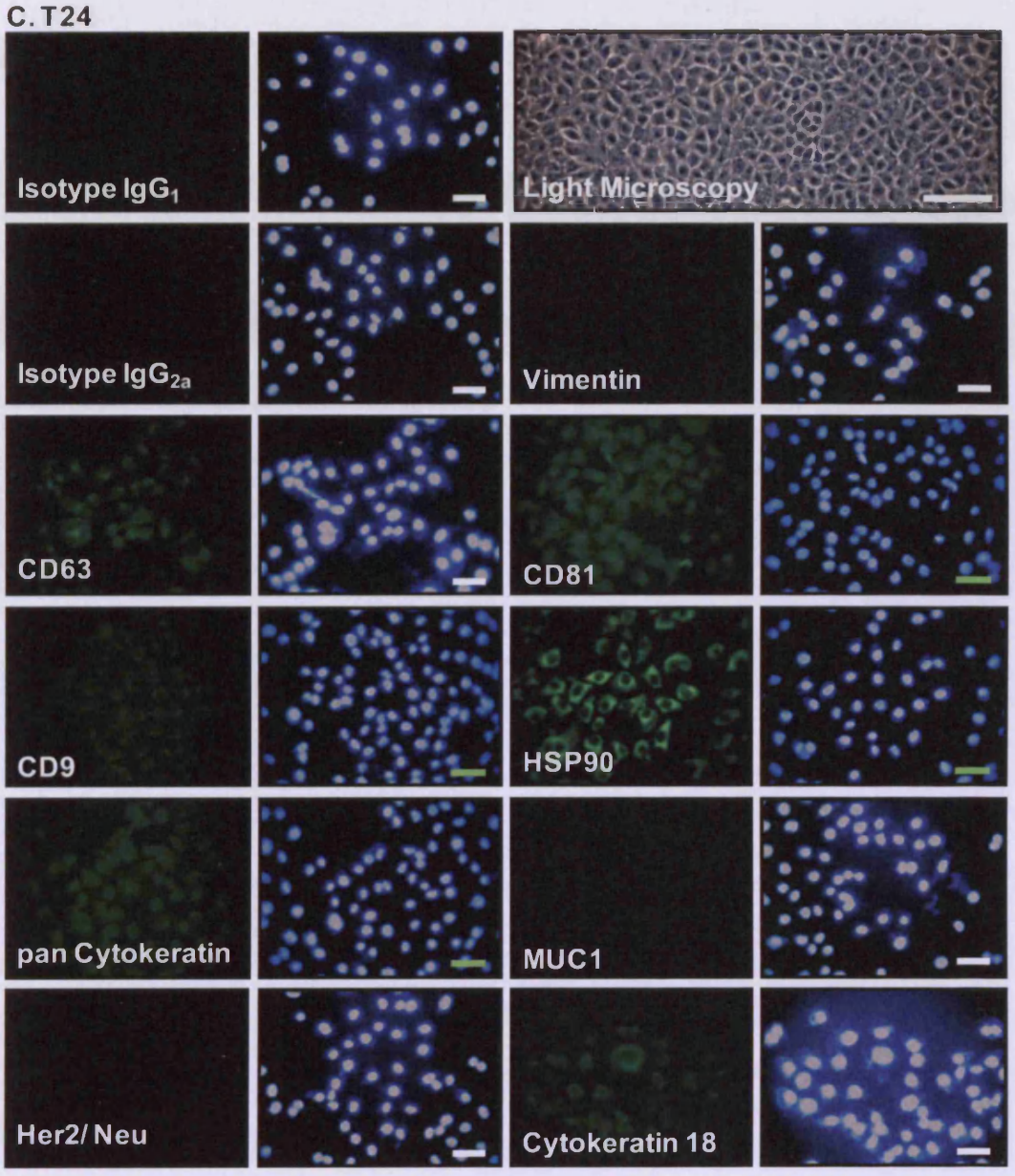


Figure 3.1. continued. C. T24 (Scale: light 100μm; IHC white 50μm; IHC green 100μm)

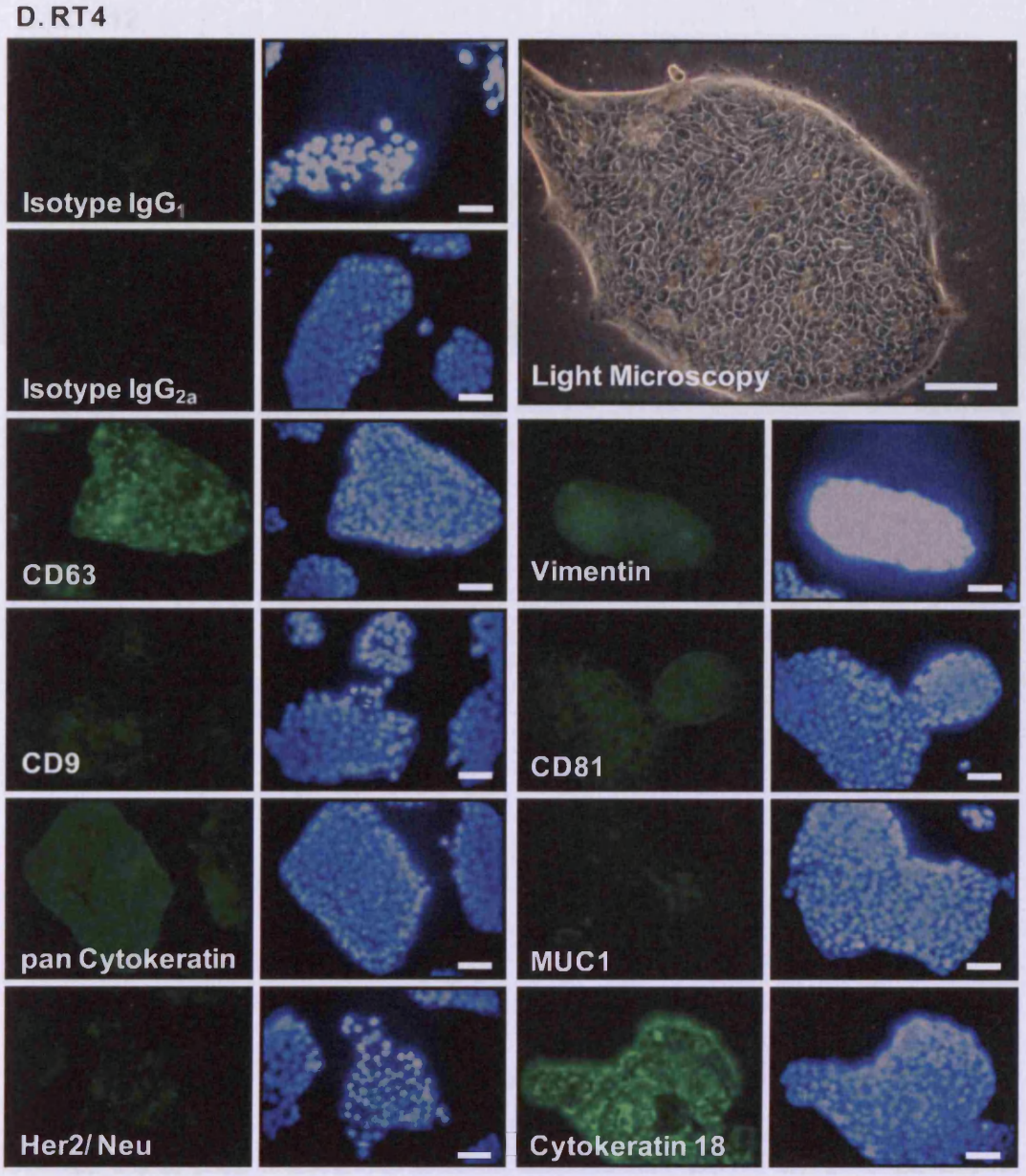


Figure 3.1. continued. D. RT4 (Scale: light 100μm; IHC 50μm)

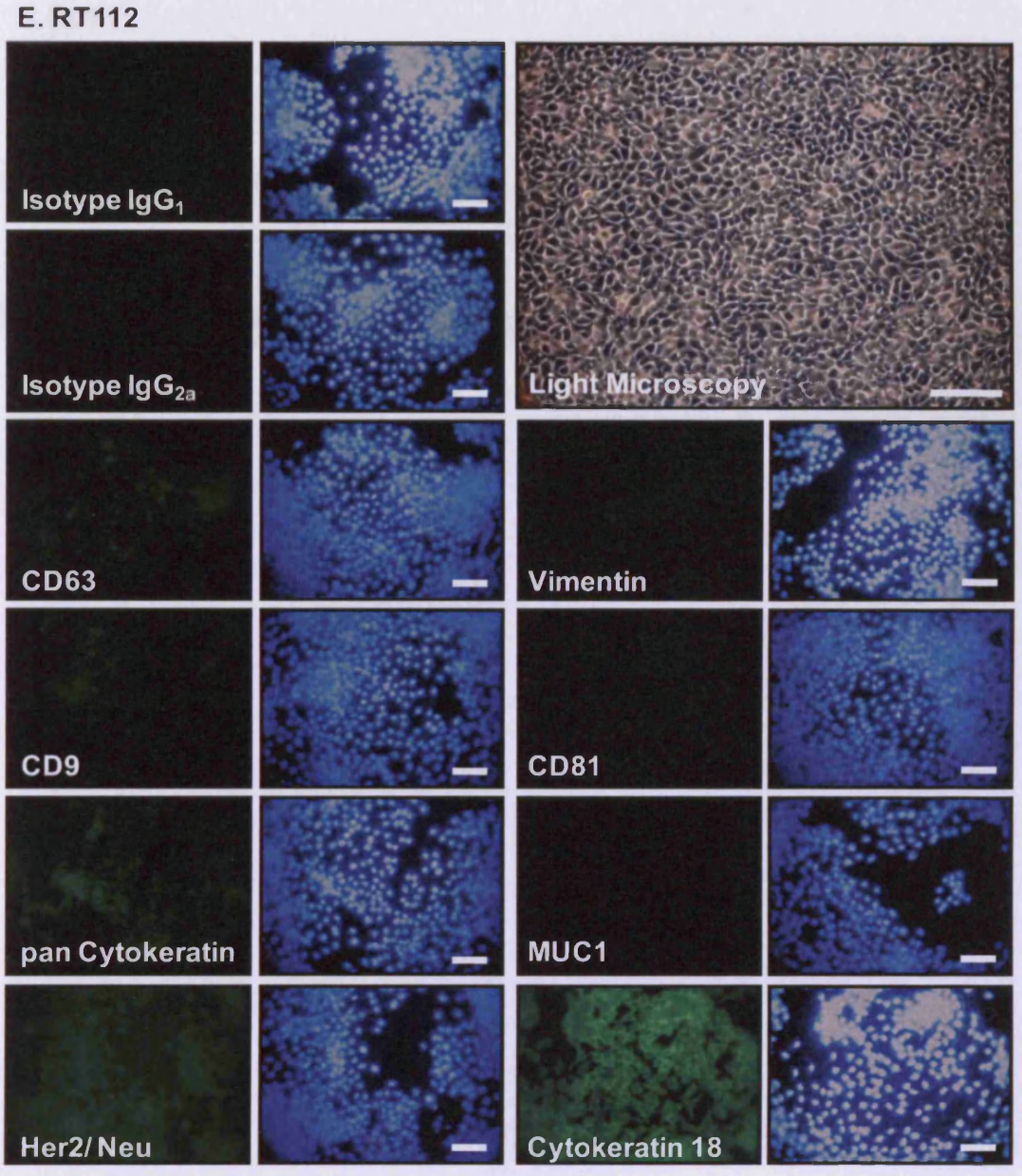


Figure 3.1. continued. E. RT112 (Scale: 100μm)

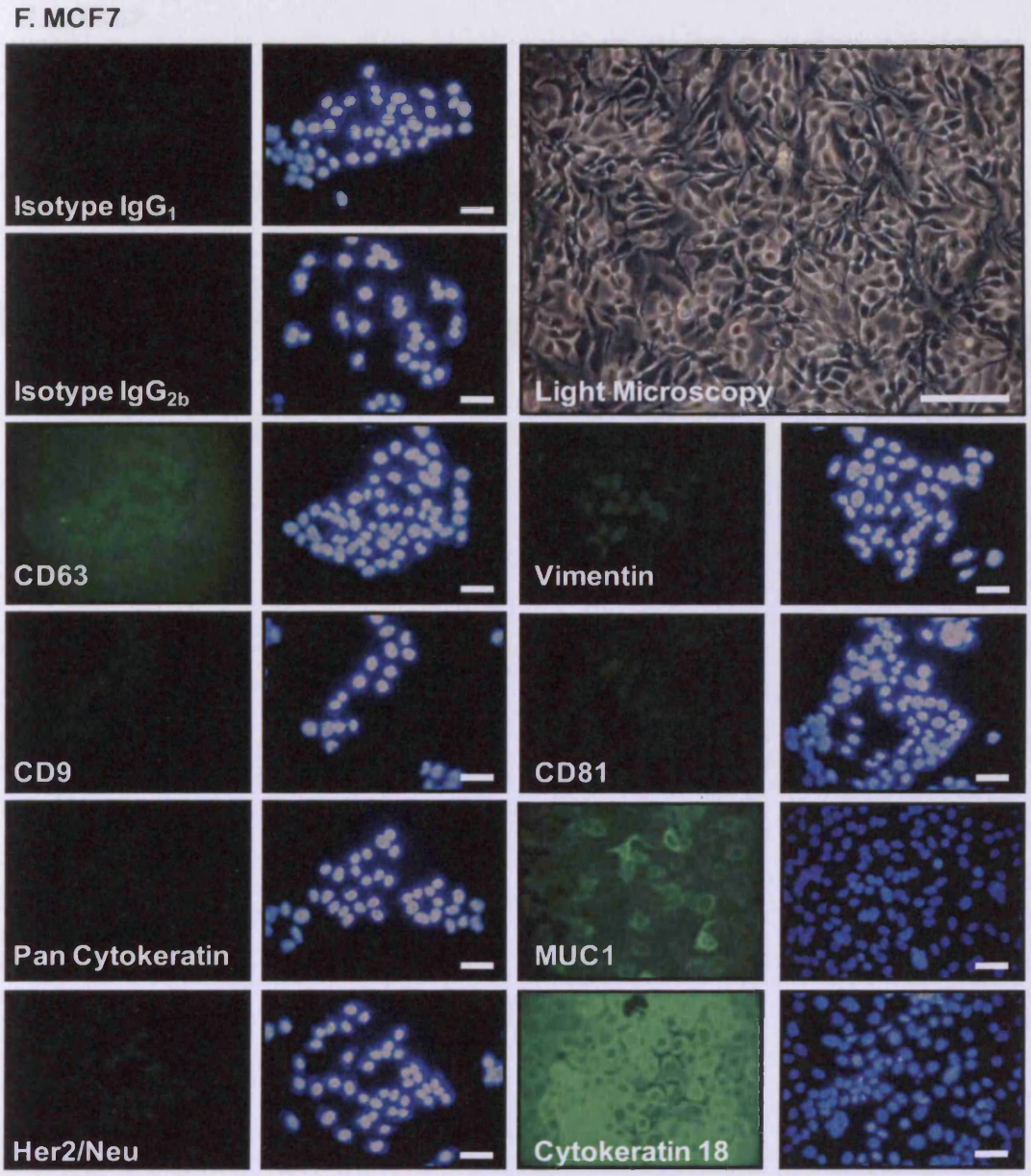


Figure 3.1. continued. F. MCF7 (Scale: light 100; IHC 50μm);

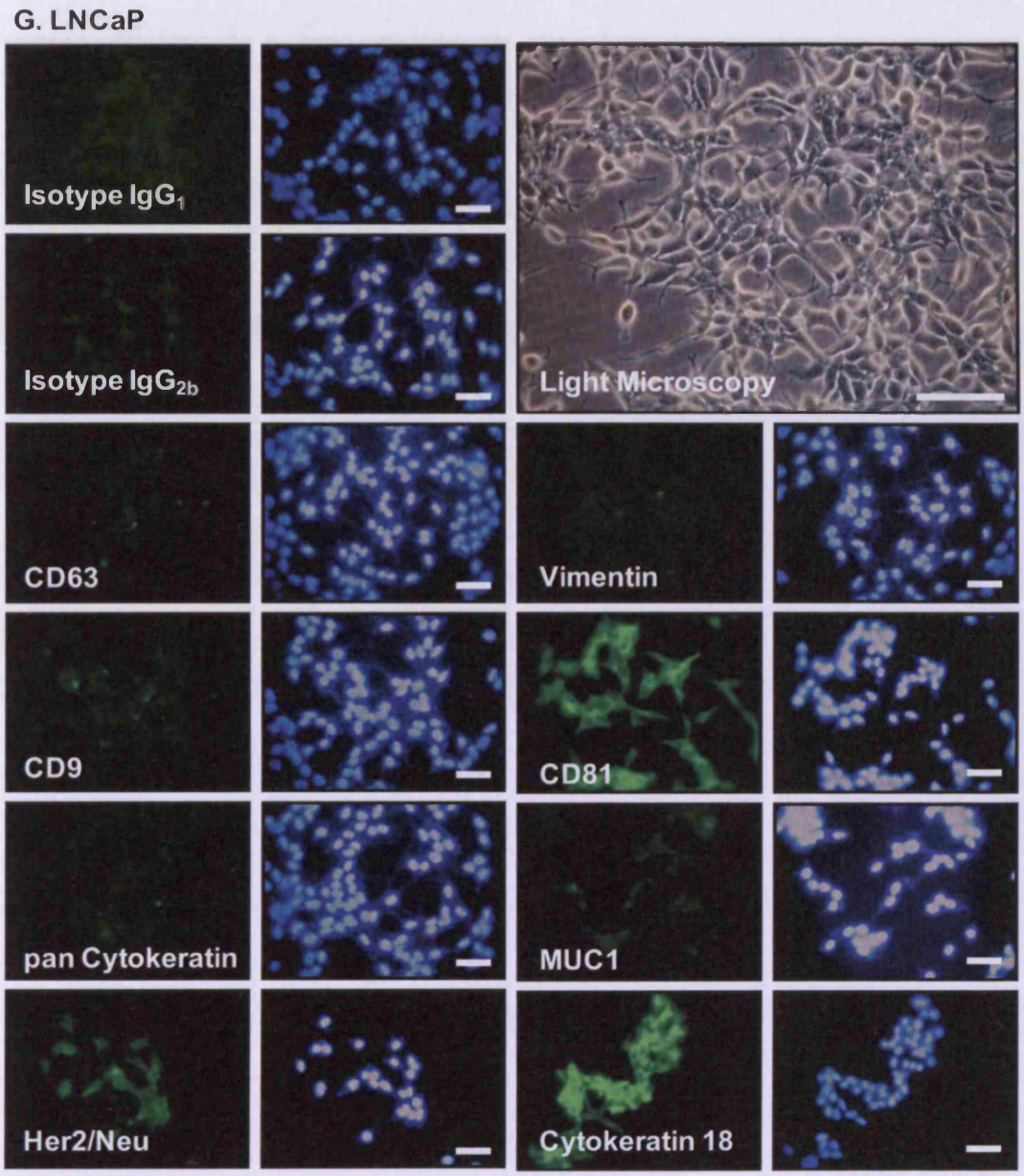


Figure 3.1. continued. G. LnCAP (Scale: 100μm);

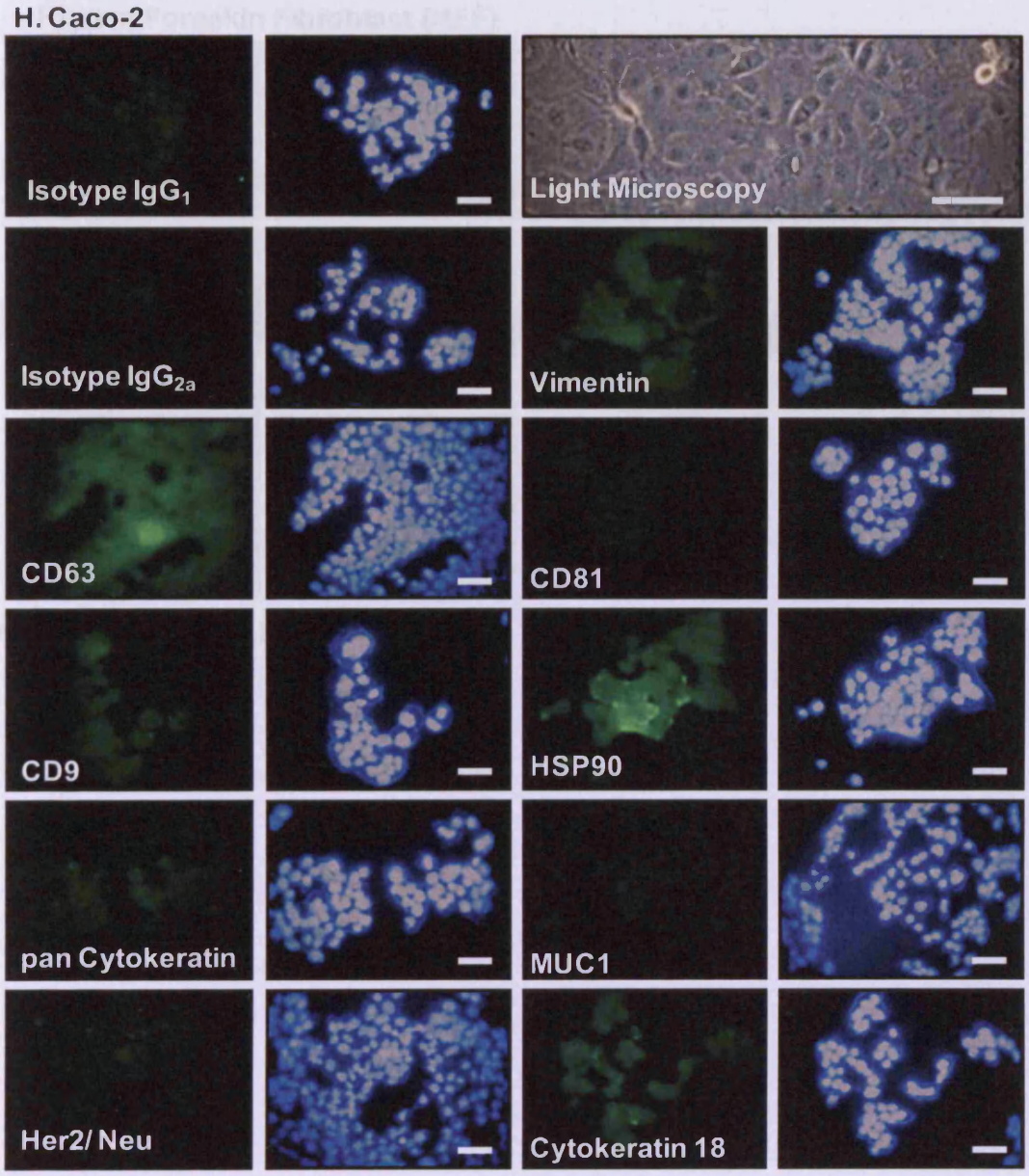


Figure 3.1. continued. H. Caco2 (Scale: 100µm);

I. Human Foreskin Fibroblast (HFF)

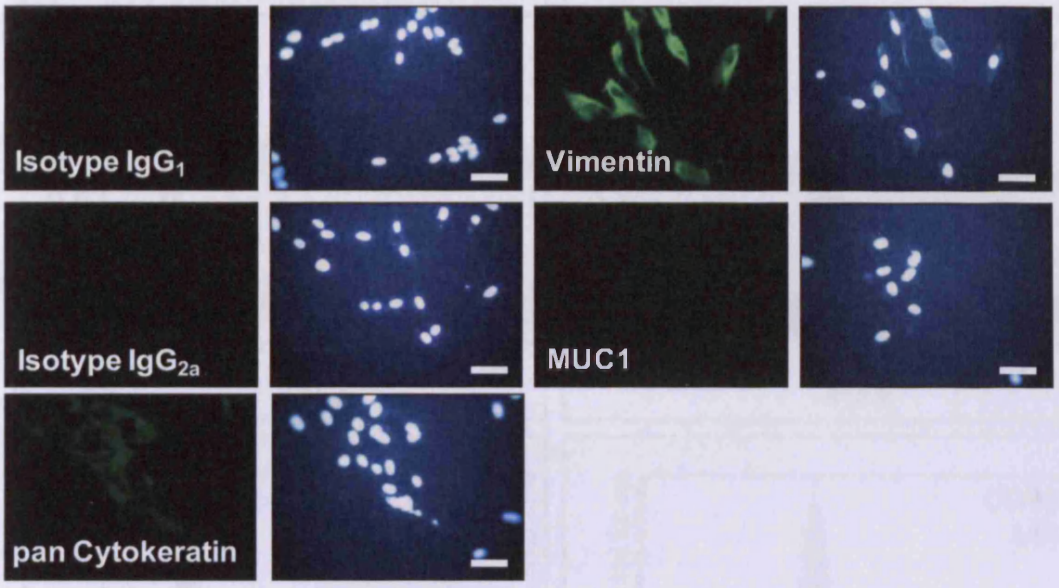


Figure 3.1. continued. I. HFF (Scale: 50μm).

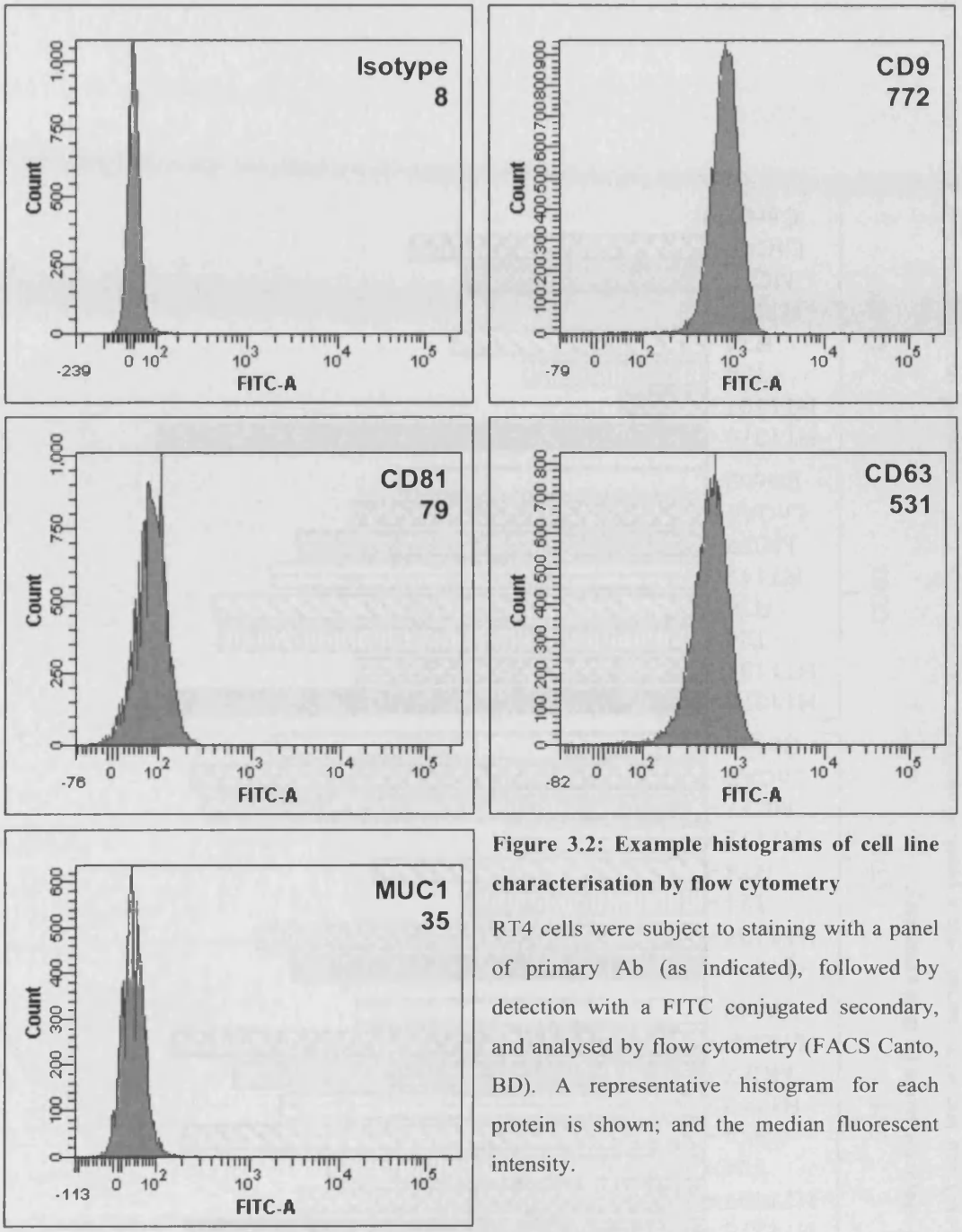


Figure 3.2: Example histograms of cell line characterisation by flow cytometry

RT4 cells were subject to staining with a panel of primary Ab (as indicated), followed by detection with a FITC conjugated secondary, and analysed by flow cytometry (FACS Canto, BD). A representative histogram for each protein is shown; and the median fluorescent intensity.

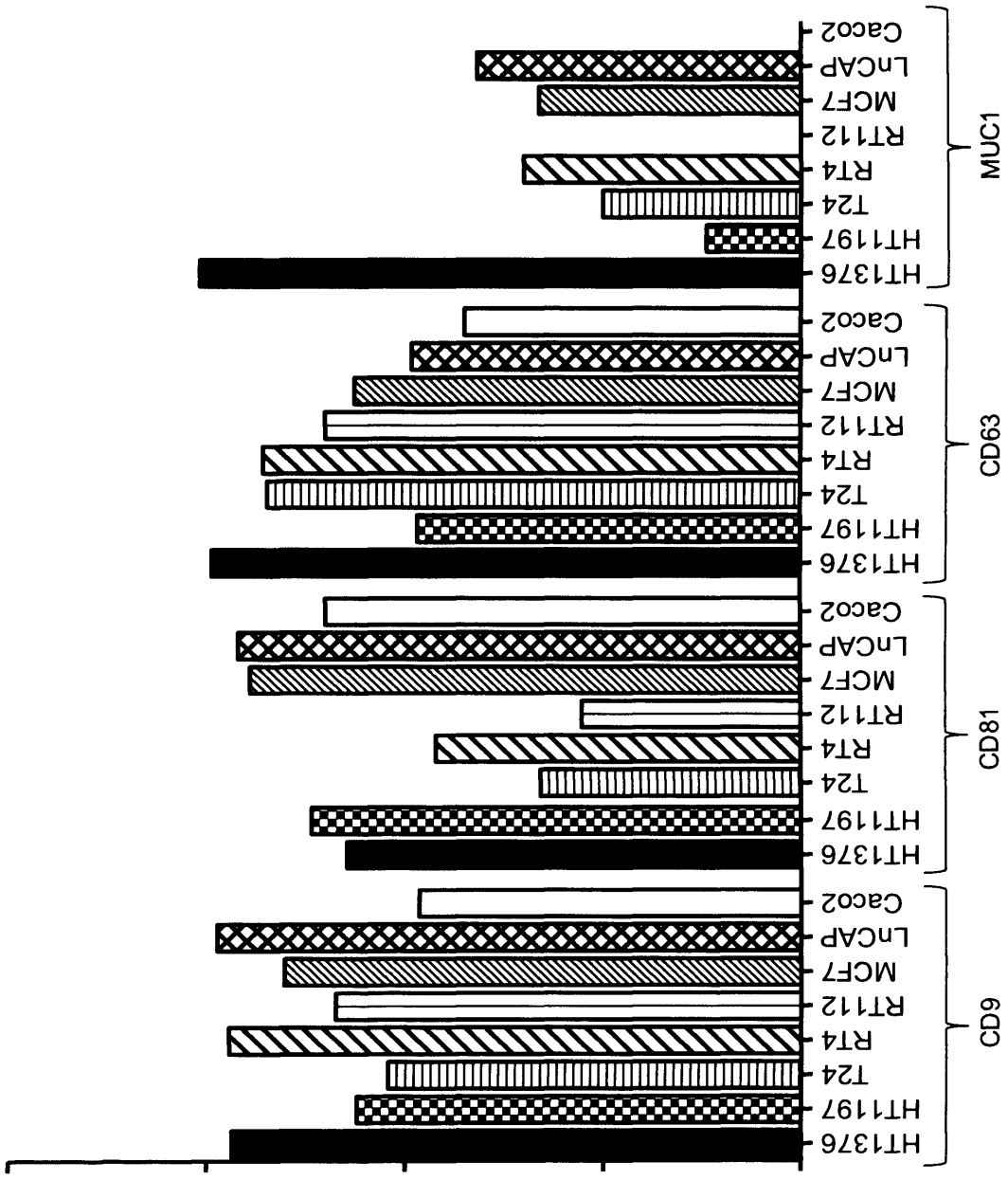


Figure 3.3: Cell characterisation by flow cytometry

Live cells from each cell line were stained with a panel of antibodies followed by a FITC conjugated secondary antibody and subsequently flow cytometry. The chart shows the log median fluorescence (with isotype subtracted) for each cell line for a panel of antibodies.

3.4 *Isolating exosomes from cultured cancer cells*

Once the cell line phenotypes had been confirmed the cells were seeded into Integra CELLine™ bioreactors (section 2.2.2). As soon as the cells were established and growing well in the bioreactors each cell lines exosomes were isolated from the CM collected by the sucrose cushion method. This method of exosome purification has been used by this laboratory [38, 44, 134] and others [42, 67, 163] as a suitable method for separating exosomes from non-exosomal cellular debris in CM and is detailed in the general materials and methods.

In order to examine the efficacy of the CELLine™ flasks in producing concentrated exosomes the exosome concentration of the CM had to be determined. Firstly the protein concentration of the final exosome product was ascertained by BCA-protein assay. To calculate the exosome concentration of the CM the quantity of purified exosomes was divided by the volume of CM used.

We have established multiple cell types successfully in long-term culture in the CELLine™ bioreactor flasks. In addition we are able to generate significant quantities of exosomes using this approach. The results however demonstrated that the exosome concentration of CM varied greatly between the cell lines (Figure 3.4). This may reflect different cell numbers in the flasks and/or different exosome production capacity among the cell lines. As the number of cells per CELLine™ flask is not known and is technically very challenging to determine for adherent cell cultures in this system, measuring the exosome content of the CM was the best comparator for determining the exosome production of each cell line. There are several other variables that may influence exosome content of CM including the potential effect of the originating tumour grade/stage but this does not appear to be the case. The different cell lines may also have different exosome production capacities or the number of days between feeding influencing the CM exosome content a vital parameter which effects the CM exosome content [163]. Lastly some variation will also occur during purification even though a standardised method was used.

To summarise, we have shown that it is possible to isolate good quantities of exosomes from cell lines grown in CELLine™ bioreactor flasks purifying 10-12 times more exosomes than the same volume of CM from traditional cell culture. This demonstrates that using CELLine™ bioreactor flasks is a more time and cost effective way of culturing cells for exosome analysis.

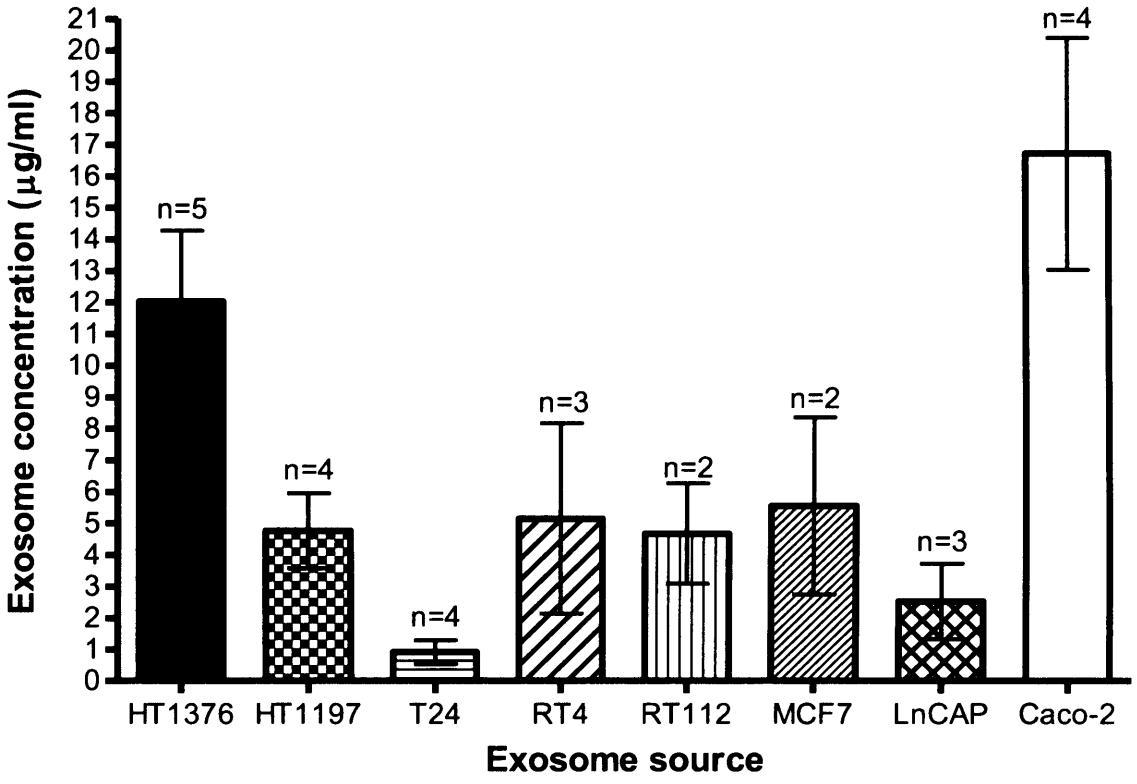


Figure 3.4: Exosome concentration of cell-conditioned media

The exosome content was determined by dividing the quantity of purified exosomes (within the final exosome pellet) by the volume of CM used in the purification to give the exosome concentration of the CM in µg/ ml. n=number of purifications; error bar = SEM.

3.5 *Analysis of exosome preparations by flow cytometry*

Although we had measurable protein present in the 'exosome pellet' it was important to confirm that this protein was principally exosome-related and not due to non-exosomal constituents. Purity of exosome preparations is usually determined by immunoblot staining for known exosomal proteins and for suspected contaminating proteins. TEM may also be used to determine whether the sample contains exosomes and/or other material. Both immunoblotting and TEM are time consuming and use sizable amounts of limited sample. With this in mind we developed a flow cytometric approach for analysis of exosome coated latex micro-beads, a method first described by Blanchard *et al.*, (2002) [48].

Our method utilises only 1 μg of purified exosomes thus using a minimal amount of sample [86]. Exosomes and non-exosomal contaminants are able to couple to the micro-beads. It is expected that if a sample is poor quality (containing many contaminants) the signal strength for exosome related proteins will be considerably lower than that of a good preparation (based on the ratio of contamination to actual exosomes). In addition to estimating contamination the method also highlights that the molecules detected are present on the outer exosome surface and is therefore a flexible tool to aid understanding of exosome structure.

A good exosome preparation was deliberately contaminated with increasing concentrations (0.0001 to 10%) of FBS, the most likely source of contamination in our preparations, to test the theory that the purity of a sample could be determined by the signal of known exosomal proteins. The samples were then coupled to micro-beads and stained for CD9 and matching isotype, and analysed by flow cytometry.

The data showed 0.1, 1, and 10% FBS contamination to have significant changes in CD9 signal intensity ($p<0.001$). With just 0.01% FBS contamination CD9 signal intensity was reduced by around 25% (Figure 3.5). This shows the assay is sensitive in detecting changes in the availability of CD9 which is likely to be exosomal. We felt this level of contamination to be unacceptable for downstream proteomic applications hence a decision was made to discard any exosome samples with a median FITC fluorescence value below

1000. This was chosen as the optimal threshold because samples with lower FITC values would have significantly more contaminants. Conversely having a threshold above 1000 would yield too few samples for downstream analysis. N.B. in later sections of the thesis the FITC conjugated secondary was substituted for an Alexa Fluor® 488 conjugated secondary giving an overall (fivefold) brighter stain for this assay. Therefore the threshold was raised to 5000.

An example of a good and poor exosome sample (Figure 3.6) shows the dramatic differences in sample quality the assay is able to detect. The low CD9 fluorescence in the poor sample is caused by a high contamination to exosome ratio therefore fewer exosomes could become coupled to the micro-beads thus decreasing the CD9 signal.

The results of the ExoQA for all the cell lines show the samples are generally of high quality with the scores for CD9 typically over 1000 (Figure 3.8). However, three cell lines (HT1197, MCF7, and Caco-2) had CD9 signals below the cut-off point. Unfortunately due to limited data it was not possible to determine whether these were truly poor samples caused by poor preparation. Alternatively, the results may highlight biological variance between exosomes from different sources. In addition, as we don't know whether tetraspanin levels within the cells are highly variable or stable therefore it was useful to examine all three tetraspanins. In general they were similarly expressed across the cell lines but did not reflect the expression pattern of the parent cells. The MUC1 results were variable and did not correspond well with the expression in the cells themselves. However, MCF7 and HT1376 (Figure 3.7) had the highest expression of MUC1 in the exosomes and also have the highest expression out of all the cells.

To summarise, the theory behind the ExoQA assay appears to be sound showing it is possible to detect significant decreases in the fluorescence of known exosomal proteins with increasing levels of contaminants. The ExoQA data for the different cell lines showed the samples were principally of high quality. However, the occasional sample which was a little poor possibly may have been caused by poor preparation, for example by overloading the sucrose cushion. In addition, the assay further supports the use of the sucrose cushion method to purify exosomes of high quality compatible with assorted cell lines.

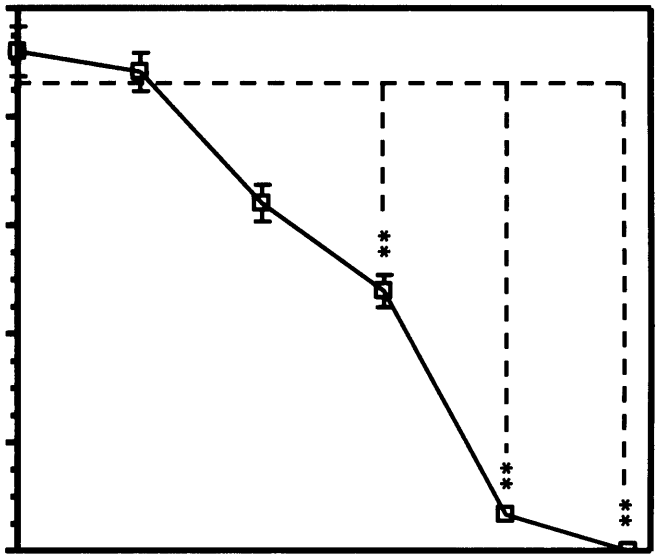


Figure 3.5: Testing of the exosome quality control assay by deliberate contamination

A previously quality assured HT1376 exosome preparation, shown to have positive expression of tetraspanin molecules on the surface, was contaminated with 0.0001, 0.001, 0.01, 0.1%, 1% and 10% FBS respectively and then incubated with latex micro-beads. The exosome bead complexes were stained for CD9 and a matched isotype and subject to flow cytometry, where the median fluorescence was measured. The graph shows a decrease in signal intensity for CD9 (mean $\pm$ SEM, n=6, **$p<0.001$, 1-way ANOVA with Tukey's post test), as a percentage of the highest peak fluorescence for CD9. The results are representative of over 5 experiments.

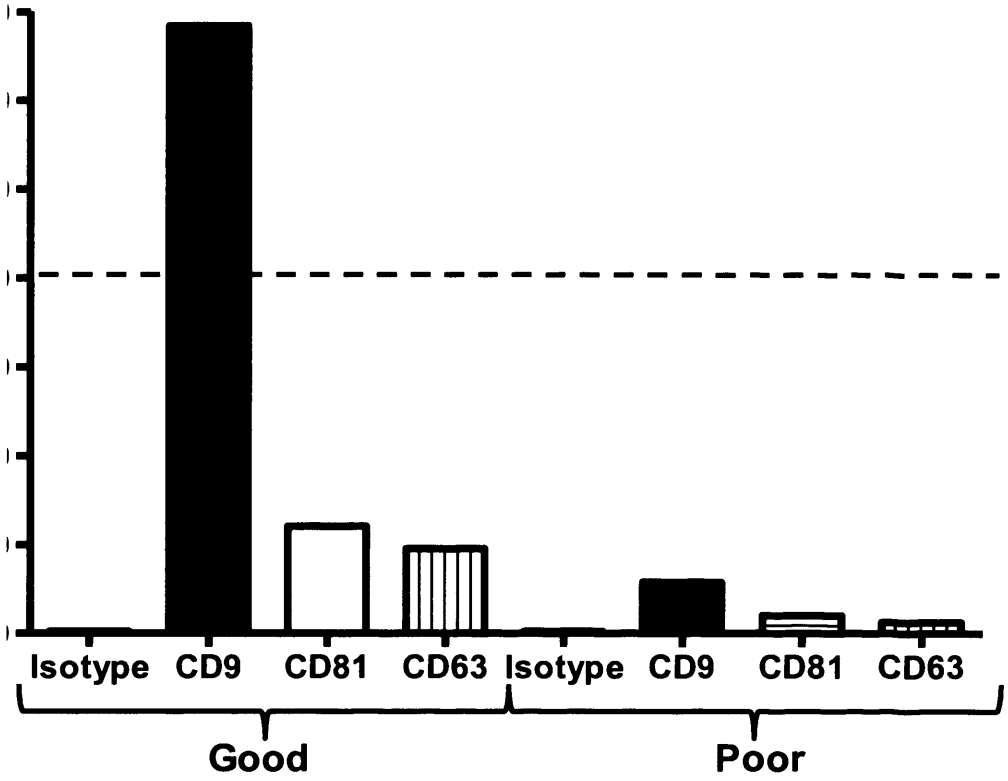


Figure 3.6: Differences in exosomal protein signal intensity between good and poor exosome preparations determined by ExoQA

Shown here are examples of exosome quality assurance assays (ExoQA) of a good and a poor exosome preparation, using exosome from the prostate cell line PC3 (CM provided by Dr Jason Webber). Displayed is the median fluorescence (FITC) of the protein of interest. The dashed line represents an arbitrary cut off point of 1000 for CD9 as a threshold for proteomics grade exosome preparations. Anything below this cut-off is deemed poor quality.

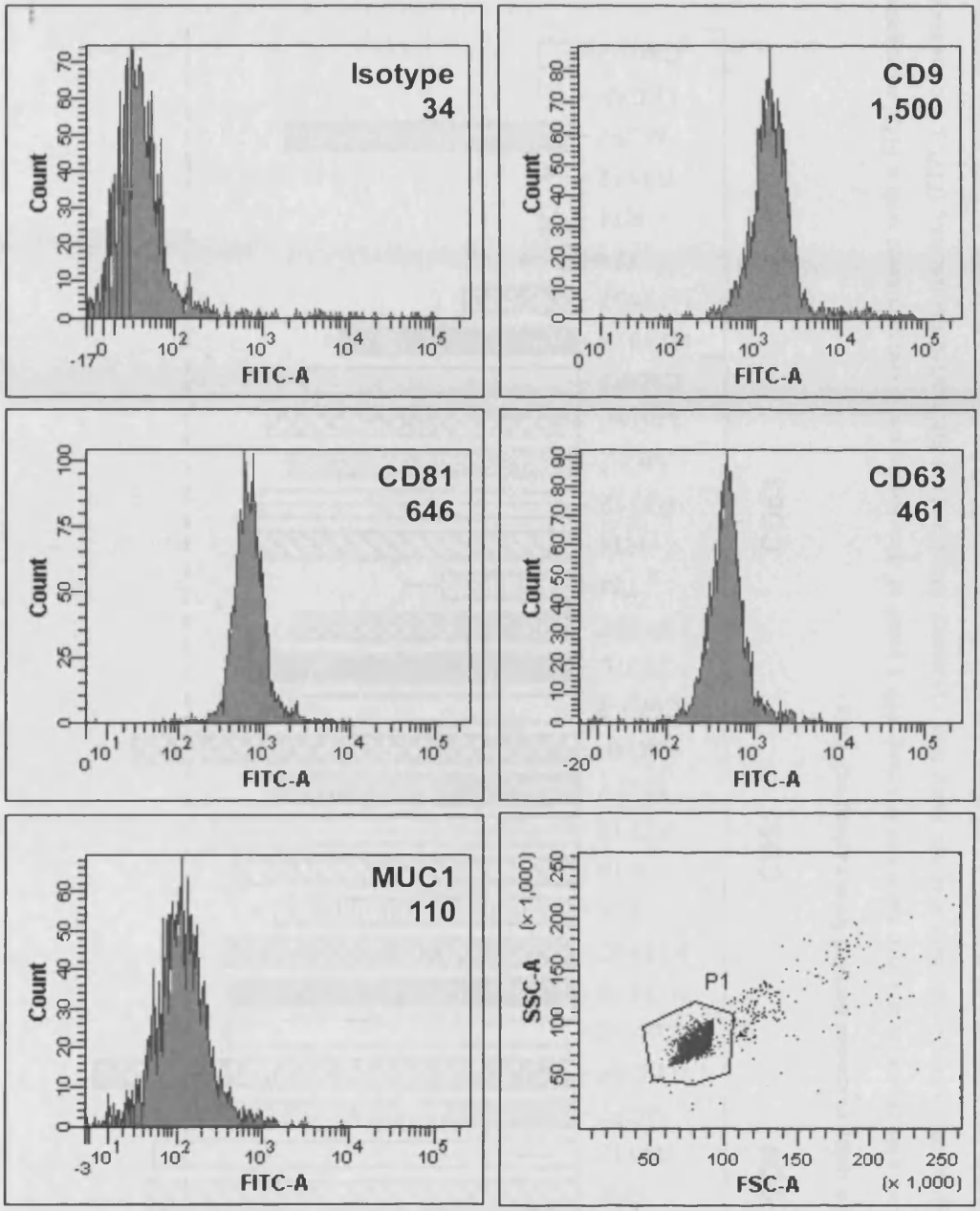


Figure 3.7: Representative flow cytometry histograms for the ExoQA antibody panel

The histograms shown are from a HT1376 exosome bead assay. Each histogram represents the median fluorescence (FITC-A) for each exosome coated bead on a logarithmic scale for 5 proteins stained for CD9, CD81, CD63, and MUC-1), plus an isotype control (IgG1). The median fluorescence value is shown. The dot plot, bottom right, shows the exosome coupled micro-bead population based on forward scatter (FSC) and side scatter (SSC).

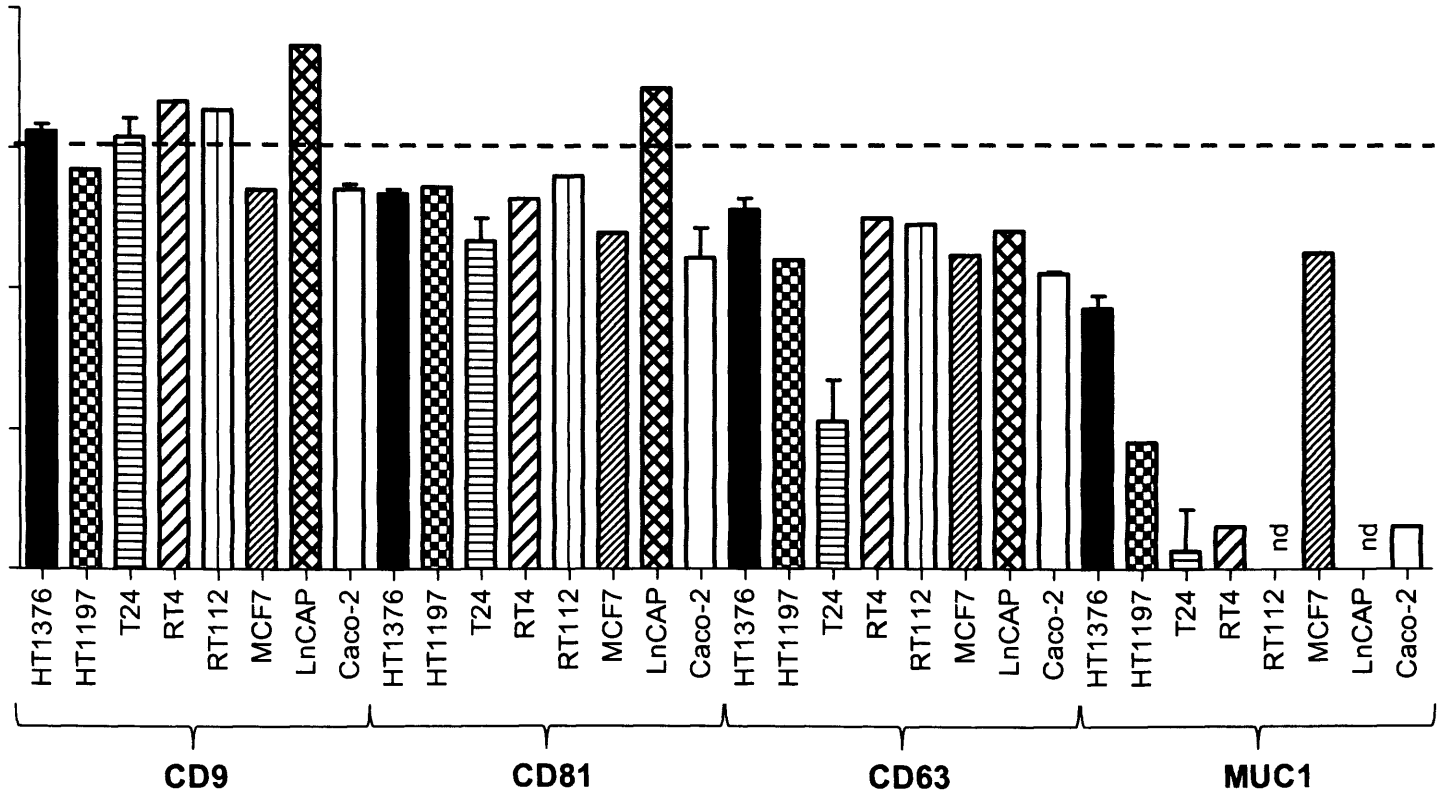


Figure 3.8: Results of ExoQA using exosomes derived from various cell lines

Latex micro-beads were coated with exosomes and then subject to labelling with a panel of Abs, subsequently counterstained with a FITC conjugated secondary. The exosome coupled micro-beads were then analysed using flow cytometry. Displayed are the median fluorescence (FITC), the exosome source, and the antigen, the dashed line represents an arbitrary cut off point of median fluorescence 1000 for CD9 as a threshold for high quality exosome preparations. Error bar = standard error, nd = not determined.

3.6 *Characterising exosomes by immunoblotting*

Exosomes from the cell lines used have not previously been examined apart from LnCAP [36, 37] and T24 [182]. Exosomes from each source were characterised by immunoblotting using a panel of antibodies to establish whether their exosomes expressed typical/expected exosomal proteins and whether these proteins were enriched compared with their parent cells. In addition, we determined whether proteins not normally expressed by exosomes could be detected in our samples.

Immunoblotting was performed on whole cell lysates (CL) and their corresponding exosomes allowing us to assess relative expression of proteins of interest in exosomes compared to the parent cell. Loading wells with equal quantity of protein was a straightforward means of achieving this.

Enrichment of the MVB protein TSG101 was seen in all but two of the cell lines. This is absolutely consistent with having purified something exosome like. T24 and Caco-2 showed expression approximately equivalent to that of the CL (Figure 3.9). This may indicate a poor exosome sample, minor enrichment, or possibly reduced incorporation of TSG101 into the exosomes during their formation.

Expression levels of LAMP1 (Lysosomal-associated membrane protein 1), a member of a family of membrane glycoproteins expressed within the endosomal/lysosomal system, differed between the cell types. Some cell lines showed increased expression in exosomes and some decreased relative to the CL. LAMP2 on the other hand was generally expressed to the same degree by the cells and their exosomes or there was some exosomal enrichment. The expression levels of the common cellular protein heat shock protein 90 (HSP90) were either similarly expressed or lesser in the exosomes.

When looking at the tetraspanins (CD9, -81,-63) it was clear to see enrichment of all three in all of the cell lines examined (Figure 3.9). In many instances dramatic enrichment was seen in the exosomes. Conversely, the cancer associated protein Her2/neu, which may be enriched in exosomes derived from bodily fluids [67, 135], was not identified in any of the

exosomes by immunoblot. As only 5μg of protein was loaded onto the gels as a result any Her2/neu might have been below detectable levels or it may be that these exosomes do not express Her2/neu. It is certainly not enriched in these exosomes.

MHC Class I was only examined in the five TCC cell lines where the expression differed greatly. Only HT1376 and RT4 (Figure 3.9a) showed classical exosomal enrichment for MHC Class I. HT1197 unusually only had positive staining in the CL, a unique discovery. Most exosomal MHC studies have focussed on EBV (Epstein Barr virus) immortalised B-cells or dendritic cells. Such cells, given their direct roles in immunity as antigen presenters are expected to produce exosomes replete with MHC molecules [47, 74]. However the MHC locus in cancer is often impacted with low levels of surface Class I expression common amongst cancer cell lines.

The cytosolic protein marker Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed equal expression in the main in exosomes and CL. The endoplasmic reticulum (ER) chaperone protein GRP 94 is not putatively expressed by exosomes so acted as a control for purity [58]. It was identified in all CL and stained very weakly in some of the exosome samples. This may indicate a small level of non-exosomal material present in these preparations or it possible that this protein is expressed somewhat by exosomes.

The immunoblotting results show that the end product of exosome purification from each cell line is consistent with previously published molecular phenotyping of exosomes. This shows that overall each of the proteins was expressed in the expected way [94, 133]. Furthermore, the high degree of enrichment of the tetraspanins in the exosomes strongly suggests very active selection/recruitment of these proteins into exosomes during their intracellular manufacture. In conclusion the data support very successful purification of exosomes from all cell lines which are either free/low in putative non-exosomal proteins like GRP 94.

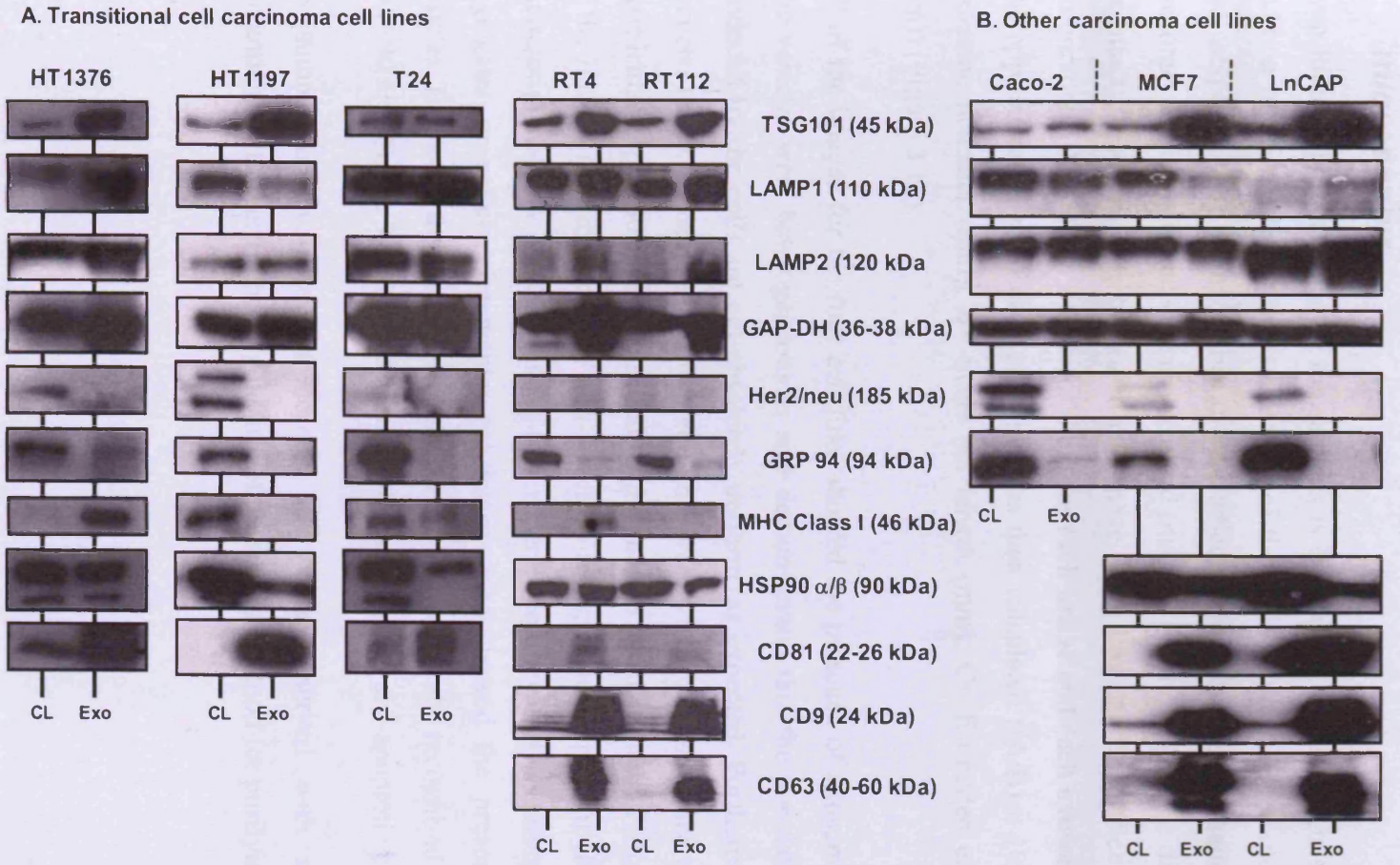


Figure 3.9: Immunoblot characterisation of cell line derived exosomes

Five µg of cell lysate (CL) or exosomal proteins (Exo) of each cell line was solubilised and then subjected to 1DE and immunoblotting with a panel of antibodies in order to determine whether the samples expressed a protein repertoire typical of exosomes. The protein labelled and its molecular weight is shown on the right-hand side. A. Transitional cell carcinoma cell lines. B. Other carcinoma cell lines.

3.7 *Analysis of exosome size by transmission electron microscopy*

Even though the phenotype of the samples is suggestive of the presence of exosome the addition of TEM allows the visualisation of the purified product thus permitting the size and morphology of the exosomes to be studied. Exosomes were visualised by TEM and exosome size was evaluated using scanned images (Figure 3.10). The diameters of up to six intact exosomes were measured per image using Powerpoint (Office 2007, Microsoft Corporation). The length of the scale bar for each image and each exosome was recorded. The approximate size of each exosome was then calculated ((A/B) x 100 = C where A= Exosome diameter (mm); B= Scale bar length (mm); C= Estimated exosome diameter (nm)) (Figure 3.11).

All of the images for the four cell lines showed the presence of exosomes (Figure 3.10). The vesicles were heterogeneous in size demonstrating that the population of exosomes produced by the cells are not absolutely uniform, as expected. Furthermore, the average diameter the exosomes from all four cell lines were within 15 nm of each other. This shows a particular population size is purified by the sucrose cushion method (Figure 3.11). Some of the images appeared to have non-vesicular/denser material present (Figure 3.10). The nature/origin of this is unknown but may reflect a low level of contamination by protein aggregates or cellular fragments. All images also showed the presence of damaged vesicles. However, the intact vesicles were all well within the recognised exosome size of 30 to 90 nm (Figure 3.10f) and distinct from microvesicles and apoptotic blebs/debris [94].

To summarise, exosomes of correct size were observed with minimal sample contamination further supporting the use of the sucrose method for purifying exosomes.

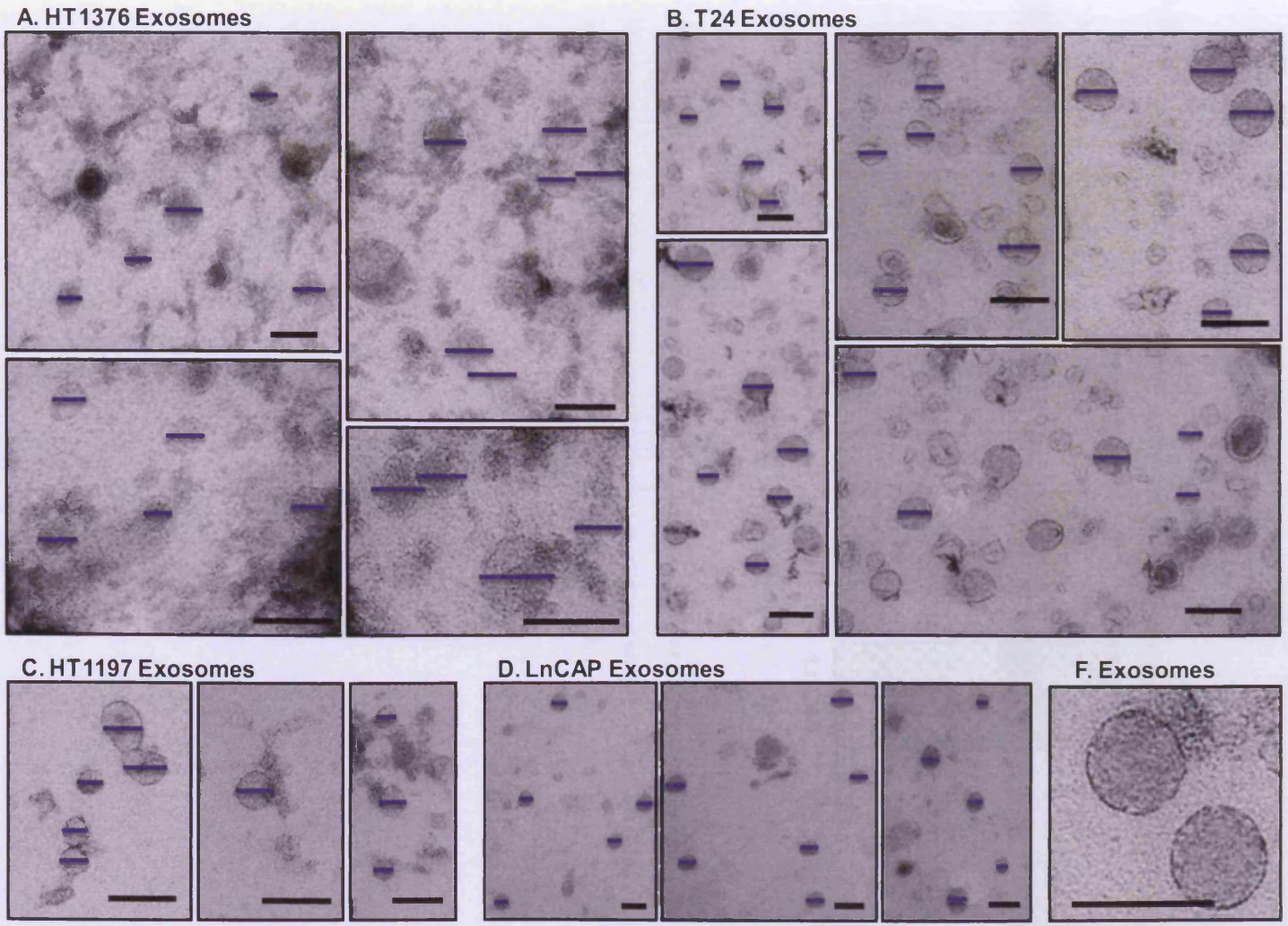


Figure 3.10: Imaging of exosomes by TEM and measurement of exosome diameter

The diameters of vesicles from each image of exosomes from four cell lines were measured as indicated by a purple line (A-D). A representative close up of an exosome is shown in F. The black line (bottom right of each image) is a 100 nm scale bar.

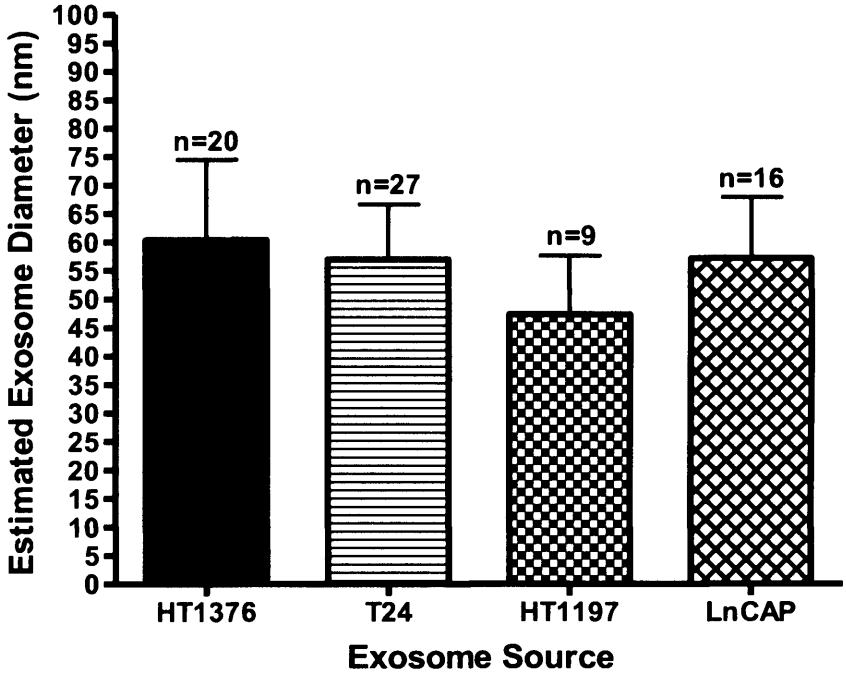


Figure 3.11: Estimation of the size of exosomes produced by four different cell lines

Measurements were taken from transmission electron microscopy images and the estimated average size was plotted. n = number of measurements, error bars = standard deviation.

3.8 *Discussion*

The main aims of this chapter were to develop methods for the purification of exosomes from CM and to evaluate the quality of exosomes obtained. Several experiments using both qualitative and quantitative techniques were performed on a number of different cell lines in order to optimise the methodologies. The culture conditions for each of the cell lines were optimised to achieve good quantities of exosomes efficiently from CM of CELLine™ culture flasks.

The cell lines (five TCC and three non-TCC) chosen for the study were confirmed as epithelial based on cell morphology, IHC and FC analysis of each cell line. Known epithelial cell proteins (CKs) and the non-epithelial intermediate filament protein vimentin were examined and all the cell lines were found to be CK18 positive and vimentin negative.

Two cell lines showed particularly strong staining for CK18, HT1376 and MCF7, compared to the other cell lines examined. This may be related to alterations in the cells normal expression as it is known to be up-regulated in several carcinomas including breast, colon, and TCC [164]. Although without a non-malignant primary urothelial cell for comparison it is hard to deduce whether this is normal or abnormal expression of CK18. It may be interesting to look for CK18 expression in exosomes particularly HT1376 and MCF7.

The cell lines were all successfully established in the specialised CELLine™ culture flasks. Furthermore, the HSP90 levels of exosomes produced within these flasks were not elevated indicating that the cells were not unduly subjected to stress such as hypoxia and/or starvation which is consistent with previous studies [163]. If they were subjected to stress one may expect to see HSP90 elevated in the exosomes [134].

The amount of exosomes produced in each of the flasks, isolated using the sucrose cushion method, varied from 16.74 ±7.37μg/ ml (n=3) to just 0.92 ±0.75μg/ ml (n=4). This was comparable to the yields from a mesothelioma cell line [163]. This mesothelioma cell line

yielded 12 times more exosomal protein from the CELLine™ flask compared to standard cell culture in $75cm^3$ flasks. Although this difference was not measured in the current study, as we did not do the direct comparison of exosome yields from traditional flasks here, it is expected that the results would be comparable for these cell lines. Variability in exosome content of the CM of the different cell lines, measured as protein captured within the sucrose cushion, could be caused by differing cell numbers in the flasks or possibly differences in the exosome production capacity of the cell lines. To further evaluate this one could trypsinise the flasks and quantify the ratio of cells to exosomes produced over a specified period of time. However, this would have set the study back by several months as it can take many weeks for a cell line to become well established in the CELLine™ flasks.

The exosome quality assurance assay developed by our laboratory based on an already established method [48] differs in a number of ways from techniques used by others. Other groups use a much higher saturating ratio of exosomes to micro-beads (3:1) compared to our 1μg:1μl ratio using much less sample to simply analyse quality [183]. Micro-beads have also been coupled with an antibody such as CD63 in order to immunoisolate the exosomes [64].

The initial theory that more contaminants in the sample would lead to reduced tetraspanin signal was confirmed. We established that as little as 0.01% contamination was required to reduce the signal intensity for CD9 by 25%. Increasing the contamination further to 0.1% significantly reduced the signal intensity, by over 40% ($p \leq 0.001\%$). This assay is a novel technique for assessing sample quality and will reduce the amount of sample and time needed to distinguish between excellent and poor quality preparations. As it is a quantitative technique the results are more easily interpreted than the usual immunoblots and as such the standardised method is well suited for batch to batch variation testing.

The theory behind the ExoQA assay was only tested using exosomes from the HT1376 cell line in which the 1000 threshold was based on. Most exosome samples from other cell lines performed well but certain cell lines did not perform as well using this assay. This may have been caused by inefficient clearing of the CM. For example, based on

observations, if there is a large pellet and debris stuck to the sides of the centrifuge tube after the 10,000 *g* clearing step more of this debris is likely to become re-suspended in the supernatant. Conversely, if the poor ExoQA results for these three cell lines (HT1197, MCF7, and Caco-2) were not due to contamination it could mean that the assay and arbitrary threshold may have to be adjusted to take this into account. Also, the level of a particular tetraspanin may be highly variable across the cell lines. For example some cell lines may express significantly less CD9 than HT1376 but when looking at a different tetraspanin (CD63) this difference is less marked. For instance the difference in the expression of CD63 in T24 cells compared to HT1376 cells is much less than CD9 (Figure 3.3). This suggests there is an advantage in looking at more than one tetraspanin to evaluate different cell types. In addition the arbitrary threshold (1000) for CD9 may not be appropriate for different cell types.

Each cell lines exosomes were characterised using a comprehensive panel of antibodies something which has not been previously performed on any of the cell lines. All the cell line derived exosomes were shown to express well known exosomal proteins (TSG101, CD9, and LAMP1) confirming the presence of exosomes within the sample and supporting the results of the ExoQA. In addition it suggests active recruitment of proteins such as TSG101, CD9, CD81, and CD63 into exosomes as indicated by the stronger staining observed in the exosome samples compared to whole cell lysates. Lastly minimal contamination with the ER protein GRP94 was observed demonstrating that the samples are low in contaminating cellular debris.

The results of the TEM also agree with this statement showing the presence of a heterogeneous population of vesicles within the accepted size range for exosomes. However, some of the vesicular structures did appear to be damaged perhaps by the techniques used. Microvesicles and/or apoptotic blebs were not observed but some images did show non-exosomal material which may be protein aggregates or cell debris.

Overall, this chapter has shown that reasonable quantities of exosomes of high quality can be obtained efficiently from the CM of the cell lines grown in the CELLine™ flasks using

the sucrose cushion method. A method for quality assuring exosome samples has been developed and is a flexible tool for routine sample analysis that preserves the bulk of each specimen for other downstream analysis. In addition, we now have a diverse bank of cancer exosomes of defined quality to use in subsequent studies of exosome constituents. This will prove a valuable tool for validating any newly discovered proteins accrued from proteomics studies.

Chapter 4:

Analysis of urine exosomes

4.1 *Introduction*

Several putative biomarkers for diseases of the genitourinary tract have been identified in exosomes supporting their use in biomarker discovery research [34, 144, 184]. To our knowledge urinary exosomes have not been studied in the context of renal or bladder cancer (BCa). However, there is one report describing microparticles derived from BCa patient urine. Here a complex mixture of microvesicles, exosomes and other urine constituents were analysed. Due to the large mixture of protein sources it is unknown whether the proteins identified, including eight with altered expression in cancer, are exosomal [33].

Exosomes were first isolated from urine by Pisitkun *et al.,* (2004) and were found to contain numerous proteins associated with renal disease and hypertension [34]. Since then urine exosome studies have mainly focussed on renal disease. In one study the use of differential proteomics methods identified exosomal Fetuin-A as a potential urinary biomarker for acute renal injury. Fetuin-A levels increased 50 fold following nephrotoxin exposure in rats. Exosomal-Fetuin-A was also found to be elevated in patients with acute renal injury before changes were seen in urinary creatinine [144]. Another study identified exosomal-aquaporin-1 (AQP1) as a potential biomarker for renal ischemia-reperfusion (I/R) injury. The researchers showed a reduction in the amount urinary exosome AQP1 just 6 h after renal I/R [184]. However, not all exosomally expressed proteins are informative markers of clinical value. For example exosomal sodium transporters did not alter in hypertensive patients [185].

Research has been performed on urinary exosomes with respect to prostate cancer (PCa) identifying mRNA for markers PCA-3 and the fusion protein TMPPRSS2-ERG [35]. Our group has also analysed urine exosomes from PCa patients. Exosomes were purified from fresh spot urine samples taken longitudinally during standard therapy and analysed for any changes in the levels of known prostate antigens PSA and PSMA. The cancer associated protein 5T4 was also identified in PCa patient urine exosomes but not healthy donor urine

exosomes [36]. This prostate study was carried out in parallel to the current study therefore some of the data was collected for both studies and will be discussed in this chapter.

Urine exosomes from BCa patients may offer a suitable non-invasive source of BCa biomarkers, as tumours are in direct contact with the urine they will likely secrete exosomes directly into the urinary space. Cancer cells have also demonstrated elevated exosome secretion due to aberrant p53-related pathways. As a consequence there may be more exosomes present in cancer patient urine compared with healthy controls. This is an aspect that will be examined in this chapter [186]. In addition, urinary exosomes from BCa patients and healthy donors will be examined to determine if it is feasible to analyse them for potential biomarkers.

This feasibility stage is important as we expect this, as any other study of biological fluid, to be challenging. Exosomes from biological fluids will be derived from heterogeneous cell types. For example, exosomes derived from plasma are likely to contain exosomes from lymphocytes, platelets, endothelial cells [64], as well a proportion from highly vascularised organs, such as the liver [56]. Exosomes present in urine will also be derived from multiple cell types including urothelial cells of the kidney and cells downstream of the renal tract [34-36]. Hence, it will be difficult to quantify the proportion of cancer-derived exosomes within the total urinary exosome pool.

The samples are further complicated by the presence of highly abundant non-exosomal proteins contaminating the preparations. For example, in a study where exosomes were prepared from malignant pleural effusions by sucrose gradient separation and only fractions containing exosomes (determined by electron microscopy) were analysed, soluble proteins including albumin, immunoglobulin and complement components were identified. These were not associated with the exosomes but had co-isolated with them [68]. Tamm-Horsfall protein (THP) is the most abundant protein in urine and has also been identified in exosome samples derived from urine [34]. It is therefore important to take into consideration the impact of the sample complexity when analysing data. Requiring particular attention when validating any newly discovered proteins of interest.

In order to maximise the potential to obtain exosomes rich in tumour antigens we intended to obtain urine specimens from patients with bulky bladder tumours following trans-urethral resection of bladder tumour (TURBT) but prior to the start of any other treatment. Analysing exosomes from this subset of transitional cell carcinoma (TCC) patients may increase the probability of identifying exosomal tumour associated proteins, while eliminating the effects of chemo-, immuno-, or radiotherapy that may have complex non-specific effects on the pelvic area. Currently there is little understanding about the stability of exosomes within the urinary tract (*in vivo* or *ex vivo*) and therefore aimed to process samples as rapidly as possible and certainly within 30 min of collection.

The use of differential ultracentrifugation with the addition of a sucrose cushion is considered to be a suitable method for isolating exosomes of good quality from CM as demonstrated in Chapter 3. Other studies examining exosomes utilise a simple pellet method for successful urine analysis (first described by Pisitkun *et al.*,(2004) [34]). We hypothesise that a sucrose cushion approach may improve sample quality, and yield clearer results, facilitating interpretation.

4.2 *Aims*

The overall aims of this chapter were to:

- Demonstrate it is possible to isolate exosomes from bladder cancer urine specimens
- Determine whether there are more exosomes present in the urine from patients compared with healthy donors
- Evaluate the quality of the urine exosomes
- Determine whether urine exosomes can be realistically used for proteomics analysis

4.3 *Collection and processing of spot urine samples*

Ethics permission was granted by the South Wales Local Research Ethics Committee (LREC) to undertake a pilot study to establish feasibility of using urine exosomes as a biomarker source for genitourinary cancers including prostate, bladder and kidney. Urinary exosomes of renal cancer patients were not examined as the affected kidney is usually removed early following diagnosis. We took the view that any residual disease would unlikely contribute greatly toward the total urinary exosome pool because only minimal tumour tissue would be exposed to the urinary space. However, fresh spot urine samples were collected from healthy donors and BCa patients. In addition PCa patient urine was collected for a study running in parallel and some of the data is included here for comparison.

Up to 180 ml spot urine samples were collected from 12 healthy donors (10 male (M) and 2 female (F)), and three BCa patients (2M, 1F) with bulky disease whom had had no prior chemo-, immuno-, or radiotherapy. The samples were brought to the laboratory for processing within 30 min of collection and various information was collected including urine dipstick test results (Combur5 Test®D; Roche Diagnostics Ltd, Burgess Hill, UK) detailing the blood, protein, glucose and ketone content of the urine as well as the pH (Table 4.1). This would highlight any unusual samples that may be affected by complex parameters such as diabetes or infection of the urinary tract (UTI).

The results (Table 4.1) demonstrate that none of the healthy donor samples exhibited gross protein- or haematuria. Haematuria was identified in bladder and prostate cancer donors, which was not unexpected. Gross proteinuria was not identified in any of the cancer patient samples. This data highlighted that the samples were typical as expected and therefore no samples were excluded from the study.

Samples were pre-cleared of cells and cell-debris (400 *g* 5 min 4°C and 3000 *g* 15 min 4°C) and filtered through 0.22 μm vacuum filters (Fisher Scientific UK Ltd, Loughborough, UK). Vacuum filtering, rather than our usual 10,000 *g* pre-clearing step, was utilised as the BCa specimens contained more visible debris than CM possibly due to haematuria.

Through observation, CM samples with high levels of visible contamination were not effectively cleared by the 10,000 *g* step highlighting potential issues with the technique when high levels of visible contamination were present. Vacuum filtration offered a viable alternative in removing this visible contamination and was also quicker than the 10,000 *g* centrifugation thus reducing the sample processing time. Filtration has been used in other exosome studies without any apparent problems [40, 187]. The filtrate was subjected to ultracentrifugation with the addition of a 30% sucrose/D_2O cushion as described in the general materials and methods. Protein content was determined using a Micro BCA protein assay. In addition, unlike other urine exosome studies, we did not use protease inhibitors as the exosomes were being prepared from fresh urine within half an hour of urination and not from urine stored long term (months) [143].

Table 4.1: Details of urine specimens collected from healthy donors (HD), bladder cancer patients (BC) and prostate cancer patients (PC)

Donor	Age	Sex	Dipstick					Specimen Volume (ml)	Total Exosomes Recovered (µg)	Exosome Concentration (ng/ml)
			Blood	Protein	Glucose	Ketones	pH			
BC01	86	Male	4	1	1	0	5	155	13.5	87.3
BC02	74	Female	2	1	0	0	5	180	10.9	60.4
BC03	75	Male	4	1	0	0	5	140	6.1	43.6
HD01	29	Male	0	0	0	0	7	180	9.8	54.4
HD02	37	Male	0	1	0	0	7	180	115.2	640.0
HD03	37	Male	0	1	0	0	7	180	32.3	179.4
HD04	65	Male	2	0	0	0	5	180	55.4	307.8
HD05	61	Male	0	1	0	0	7	180	154.7	859.4
HD06	50	Male	0	1	0	0	7	180	8.7	48.3
HD07	49	Male	0	0	0	0	6	150	61.2	408.0
HD08	55	Male	0	1	0	0	6	180	37.2	206.7
HD09	56	Male	0	0	4	0	7	145	28.5	196.6
HD10	57	Male	0	1	0	0	8	170	130.3	766.5
HD11	25	Female	0	0	0	0	5	130	18.2	139.8
HD12	27	Female	0	1	0	0	8	180	6.7	37.1
PC01	66	Male	1	1	0	0	7	90	72.9	810.0
PC02	62	Male	1	1	0	0	-	170	125.5	738.2
PC03	70	Male	4	2	4	0	5	180	72.9	405.3
PC04	65	Male	0	1	0	0	5	95	25.4	268.0
PC05	69	Male	4	0	0	0	5	180	38.4	213.6
PC06	70	Male	0	0	0	0	4	180	19.4	108.1
PC07	53	Male	3	1	1	0	6	97	39	402.1
PC08	61	Male	0	1	1	0	6	150	125.1	834.4
PC09	66	Male	0	1	0	0	5	120	8.2	68.3
PC10	71	Male	0	1	0	0	5	120	19.4	162.3

4.4 *Compatibility of the sucrose cushion method with urine as a source material*

We first examined whether or not our standard sucrose cushion method was compatible with urine as a source material. These experiments were performed using healthy donor (HD) urine specimens which were readily available.

Samples were collected from each stage of the exosome purification process: unprocessed urine, urine from 2000 *g* centrifugation, and urine from above the sucrose cushion after the ultracentrifugation. The wells of a one dimensional electrophoresis (1DE) gel were loaded with 10 µl of supernatant from one of these steps or 10 µl of purified urine exosomes. Samples were then subjected to electrophoresis and subsequently used for immunoblotting or silver staining.

The amount of sample used per gel was limited and therefore silver staining was needed. It is more sensitive at detecting proteins than coomassie staining and is able to detect proteins down to nanogram levels. Immunoblotting was performed using a panel of antibodies for known exosomal proteins and other gels were silver stained to broadly examine the protein profile of the samples.

The silver stained one-dimensional (1D) gel (Figure 4.1a) shows a principal band of ~80 kDa detected in the crude urine samples (lanes 1-3) is absent from the final exosome product (lane 4) as well as others in the lower molecular weight region. This suggests the elimination of this protein and perhaps other contaminants. On the other hand the complex banding observed in the purified exosomes demonstrates enrichment for a highly complex assortment of proteins spanning a wide molecular weight range. The results of the immunoblotting (Figure 4.1b) suggest the enrichment of exosomes. This is shown by the detectable levels of exosome markers (TSG101, CD9, HSP90, and LAMP1) only in purified exosomes. Overall, the data shows that the standardised sucrose cushion method is effective for purifying exosomes from spot urine samples of healthy donors.

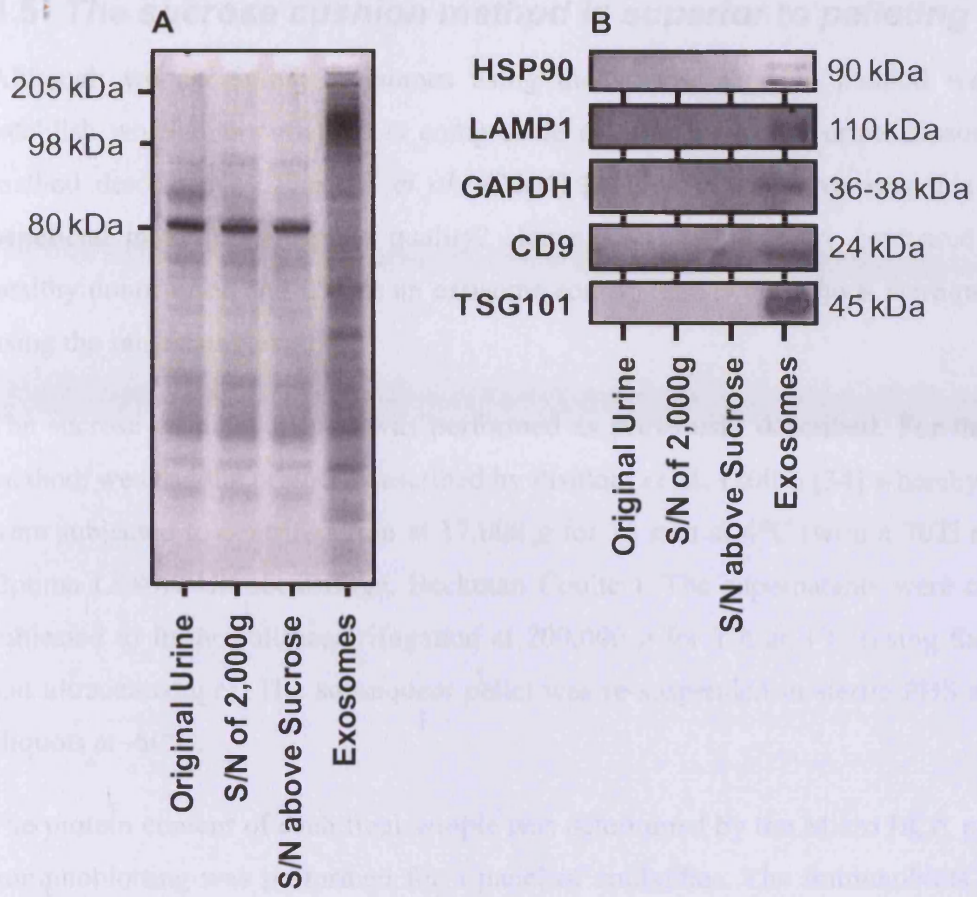


Figure 4.1: Purification of urine derived exosomes demonstrates concentration of proteins

Fresh healthy donor urine was subjected to exosome purification, and at each step, 10µl of sample was removed and then separated by 1DE. (A) Silver stained gel demonstrating effective removal of the principal non-exosomal protein bands. (B) Immunoblot analysis, using antibodies against typical exosome proteins as indicated, revealing effective concentration of expected exosomal proteins by this method.

4.5 The sucrose cushion method is superior to pelleting alone

Although we can purify exosomes using the sucrose cushion method we wanted to establish whether our method is comparable or superior to the urine exosome pelleting method described by Pisitkun *et al.,* (2004) [34]. In other words was this extra effort beneficial in terms of sample quality? Hence, the methods were compared using fresh healthy donor urine and CM as an exosome source. The two methods were run in parallel using the same samples.

The sucrose cushion method was performed as previously described. For the alternative method, we used the protocol described by Pisitkun *et al.,* (2004) [34] whereby the samples were subjected to centrifugation at 17,000 *g* for 15 min at 4°C (with a 70Ti rotor, and an Optima LE80K Ultracentrifuge, Beckman Coulter). The supernatants were collected and subjected to further ultracentrifugation at 200,000 *g* for 1 h at 4°C (using the same rotor and ultracentrifuge). The subsequent pellet was re-suspended in sterile PBS and stored in aliquots at -80°C.

The protein content of each final sample was determined by the Micro BCA protein assay. Immunoblotting was performed for a panel of antibodies. The immunoblots (Figure 4.2) show higher levels of all the exosomal proteins using the sucrose cushion method, purified from both CM and healthy donor urine. This suggests the pellet from the sucrose cushion method is richer in exosomes and poorer in non-exosomal contaminants compared with the alternative method. Good enrichment of the tumour associated antigen 5T4 (Figure 4.2a) was seen using the sucrose method, but was barely/not detected by using the simple pelleting protocol. However, whilst the non-exosomal endoplasmic reticulum (ER) protein calnexin was identified using both methods this band was much less prominent in the sucrose method. Similarly the abundant urine protein THP was only weakly present in the sucrose preparations.

The data presented supports the use of the sucrose method in purifying exosomes from fresh spot urine samples. Although practically more involved it showed significant benefits in terms of deriving exosomes of superior quality compared with competitor methods.

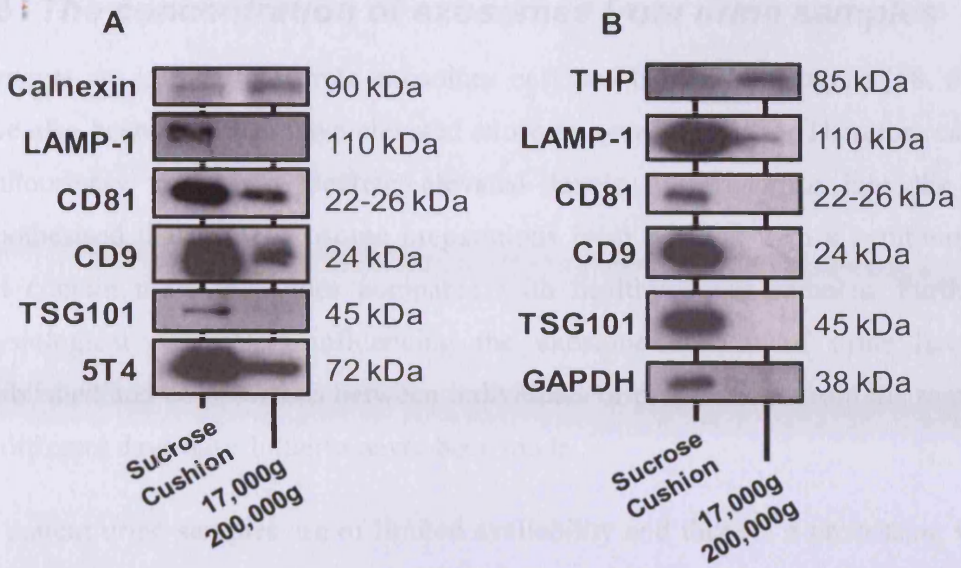


Figure 4.2: Comparison of exosome purification methods using immunoblotting with exosome markers

Two methods of exosome purification, ultracentrifugation using a sucrose cushion and differential ultracentrifugation, were compared using cell-conditioned media (A) and healthy donor urine (B). 5 µg of protein from each condition was solubilised and subjected to 1DE and immunoblotting with a panel of antibodies in order to compare the purification methods.

4.6 *The concentration of exosomes from urine samples*

Tumours are known to secrete exosomes enriched in tumour-antigens [58, 67], but they have also been shown to have elevated exosome secretion [186]. Therefore cancers of the genitourinary tract may secrete elevated levels of exosomes into the urine. We hypothesised that urine exosome preparations from patients with a genitourinary cancer will contain more exosomes compared with healthy donor samples. Furthermore, the physiological parameters influencing the exosome content of urine have not been established and comparisons between individuals or preparations from the same individual on different days have hitherto never been made.

As patient urine samples are of limited availability and there is a processing volume limit (180 ml) for any one time (because of the capacity of the ultracentrifuge), it was important to determine the likelihood of obtaining enough protein from fresh spot urine samples for proteomics analysis. The volume of urine for analysis was recorded (Table 4.1) and subjected to the standard sucrose cushion exosome purification protocol used in our laboratory. The total exosome content of each sample was determined using the Micro BCA protein assay and the urine exosome concentration subsequently calculated (ng/ml).

The exosome content of urine samples from 12 individual healthy donors, three BCa patients, and 10 PCa patients undergoing androgen deprivation therapy (ADT) were determined (Figure 4.3). The results show a large range and standard error of the mean (SEM) in the exosome content of urine from healthy donors (37.1 to 859.4 ng/ml, 320.3 ±82.8 ng/ml; n=12) but a smaller range for the BCa patients (43.6 to 87.3 ng/ml, 63.8 ±12.7 ng/ml; n=3) was observed. The very limited number of samples for the BCa patients made it difficult to perform statistical analysis. Therefore the PCa patient urine exosome results from another study (Table 4.1) were included for comparison [36]. Here the range was from 68.3 to 896.3 ng/ml (428.5 ±99.2 ng/ml; n=10). This again demonstrated the large variation observed between samples. No significant difference was detected between the exosome yields of HD and PCa samples (*t*-test; $p<0.05$).

The sample numbers were still very limited and in order to get a more accurate picture of the exosome yields from healthy donor, BCa, and PCa urine more samples would need to be processed. Variability in exosome content was also observed in preparations from different days from a single healthy donor varying from 45.6 to 182.2 ng/ml (102 ± 55.6 ng/ml; n=5) (Table 4.2). This signifies that variability is not just between different individuals but also between specimens from the same donor.

The results have shown that the amount of exosomal protein that can be isolated from a spot urine sample is very variable both between healthy donors and cancer patients as well as for an individual donor. Although a significant volume of urine was processed, often utilising the full capacity of the ultracentrifuge, the total exosomes available was very low. For example, 180 ml healthy urine may yield a total of just 6.7 μg of exosomes (Table 4.1). This variability along with the low exosome yields from fresh spot urine samples would pose problems for any further analysis and for future utility of urinary exosomes in the clinic. Other urine exosome studies appear to yield up to 30 times more exosomes from urine samples using alternative methods [143] and we were therefore surprised to see so little material in our samples.

The results do not support the hypothesis that urine exosome preparations from patients with a genitourinary cancer contain more exosomes than healthy donor samples. Instead, the results highlight the variability in the samples used. This may be caused by differences between individuals, the time of day the sample was given, the health of the individual, or the presence of blood or proteins in the urine. Other studies have also noted difficulties with yield variability suggesting THP as a cause. This study used the reducing agent dithiothreitol (DTT) to release exosomes from the THP polymeric network allowing more exosomes to be pelleted during the high speed (200,000 *g*) centrifugation step [188]. Although this method appears to increase the number of exosomes in the final pellet the original users of this method acknowledge that the THP is also pelleted, meaning this final pellet is not pure exosomes. Furthermore, DTT is likely to disrupt the disulphide bonds of exosomal proteins and aid in solubilising what would otherwise be exosomally associated proteins. Differences in the yields between our present study and others could be due to

many factors. These might include higher levels of contamination, the effect of proteases, or osmotic damage on the exosomes.

In conclusion, the comparisons made using urine from a range of sources show that there is no gross elevation in urine exosome quantity in disease. The unpredictably low yields achieved with some specimens may mean that proteomics analysis based on such samples is unfeasible.

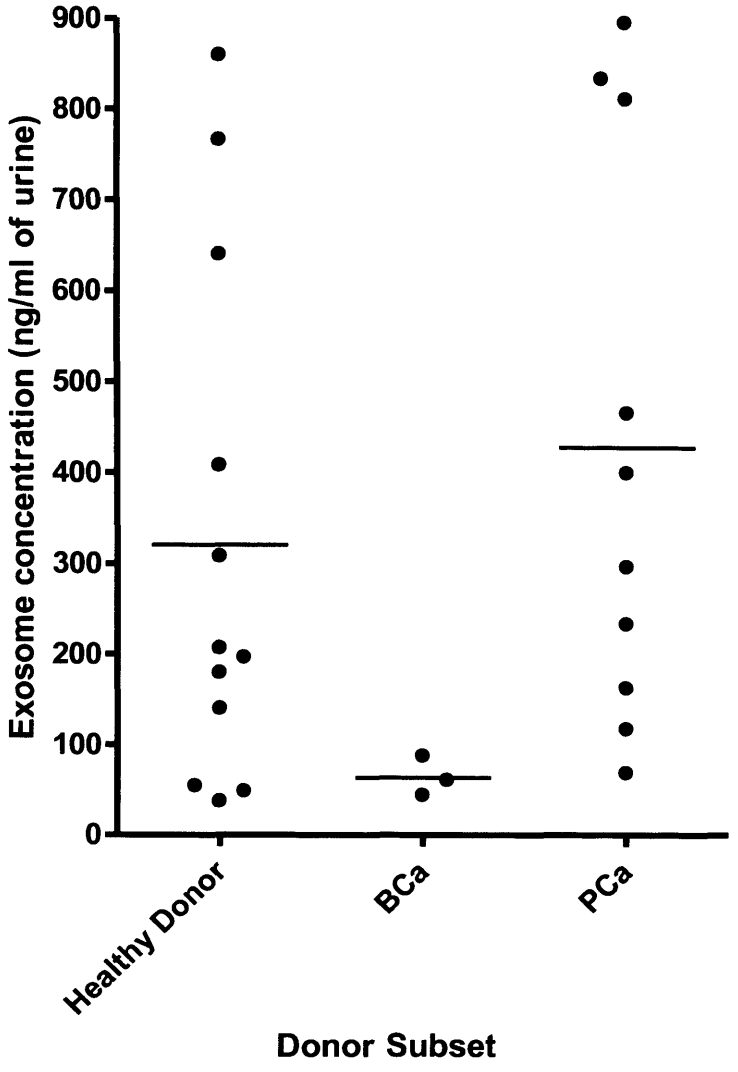


Figure 4.3: Exosome content of spot urine samples from healthy donors, bladder and prostate cancer patients

Spot urine samples from different individuals were processed and the exosome content of a urine sample was determined. The average exosome content of healthy donor urine was 320.3 ±82.8 ng/ml (SEM); n=12, bladder cancer patients (BCa) 63.77 ±12.71 ng/ml, n=3, and prostate cancer patients (PCa) 428.5 ±99.23 ng/ml; n=10.

Table 4.2: Details of urine specimens collected from one healthy donor on different days

Donor	Dipstick					Specimen Volume (ml)	Total Exosomes Recovered (µg)	Exosome Concentration (ng/ml)
	Blood	Protein	Glucose	Ketones	pH			
HD1	0	0	0	0	7	180	9.8	54.4
HD1a	0	2	0	0	6	180	18.79	104.4
HD1b	0	1	0	0	7	180	32.79	182.2
HD1c	0	0	0	0	6-7	180	22.22	123.5
HD1d	0	0	0	0	7	180	8.2	45.6

4.7 *Determination of the quality of urine exosomes*

We next examined the urine exosome samples for the presence of exosome markers. As described in the previous chapter the recognised method of analysing exosome sample purity is by immunoblotting using antibodies to known exosome markers. In addition to this we have developed an exosome quality assurance assay (ExoQA; sections 2.10 and 3.5) allowing the quality of limited samples to be examined while retaining the majority of the sample for further analysis. Both techniques were used to analyse the end product of urine exosome purification.

Urinary exosomes from six healthy donors were characterised by immunoblotting but unfortunately there was insufficient sample material to include the BCa exosome samples. The exosomes were probed for several known exosomal proteins (TSG101, GAPDH and CD9). These exosomal proteins were identified in most healthy donor (HD) samples, but at much lower levels than in exosomes purified from cultured PCa cell (LnCAP) derived exosomes (Figure 4.4). In most instances the CD9 staining intensity was higher than that of the LnCAP cell lysate (CL) suggesting there are at least some exosomes present in the samples and that the preparations were enriched in exosome markers compared with whole cell lysates. Even though the samples loaded on to the gel were all normalised by protein amount, there was substantial variability seen between all of the donors. This is likely to be caused by variation in the exosome to contaminant ratio in the preparation.

As with the cell line derived exosome samples BCa and HD urine exosome preparations were subjected to ExoQA by coupling 1 μg of sample to latex micro-beads and analysis by flow cytometry. Three BCa urine exosome preparations and six HD samples were analysed along with examples of HT1376 and T24 derived exosomes as positive controls. Only two of the urine derived exosome preparations, BC01 and HD1a, could be classed as good quality utilising the arbitrary CD9 fluorescence threshold of 1000 (Figure 4.5). This shows that the vast majority of the urine exosome samples were of poor quality compared with preparations obtained from cell lines.

Given the complexity and variability of the source material for the urinary exosome preparations it is perhaps not surprising that the samples do not match the quality achieved with cell culture sources. This suggests that the sucrose cushion method as it is may not be entirely suitable for urinary exosome preparation. It is possible that the urine exosomes may be predominantly at a density not compatible with the method so that we are only isolating a small proportion of the total exosomes present. Alternatively the exosomes floating within the sucrose cushion may be present at low levels compared with other soluble contaminants such as THP or albumin, which may coincidently co-localise with the exosomes within the sucrose. Another possibility is that urine may have an effect on the exosome membrane integrity and hence impact their flotation properties. Alternatively the conditions in the urine may be highly proteolytic and cause damage to the proteins of interest.

In conclusion we have found that, in addition to exosome quantity variation, there is variation in the actual exosome-expressed molecules within the sample (normalised for protein differences). This difference does not appear to correlate with exosome quantity. In other words high protein does not necessarily equate to low or high quality samples.

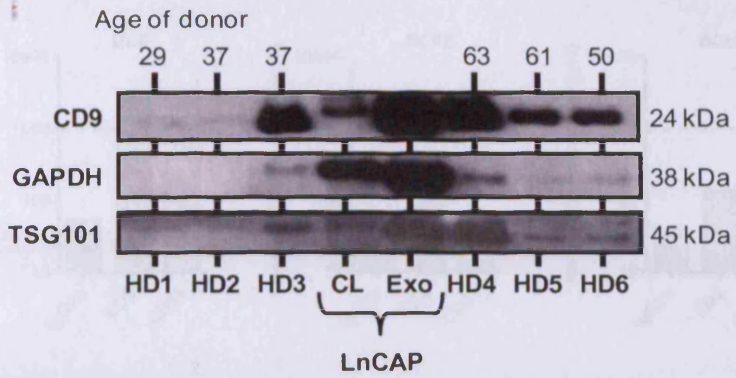


Figure 4.4: Characterisation of healthy donor urine exosomes using immunoblotting for exosomal markers

Six healthy donor (detailed in Table 4.1) fresh spot urine samples were subjected to exosome purification. Immunoblotting was performed using 5 µg of protein from each sample per well, with the addition of a well containing 5 µg LnCAP derived exosomes (Exo) and another containing 5 µg LnCAP whole cell lysates (CL). The blots were probed for CD9, GAPDH, and TSG101 as indicated.

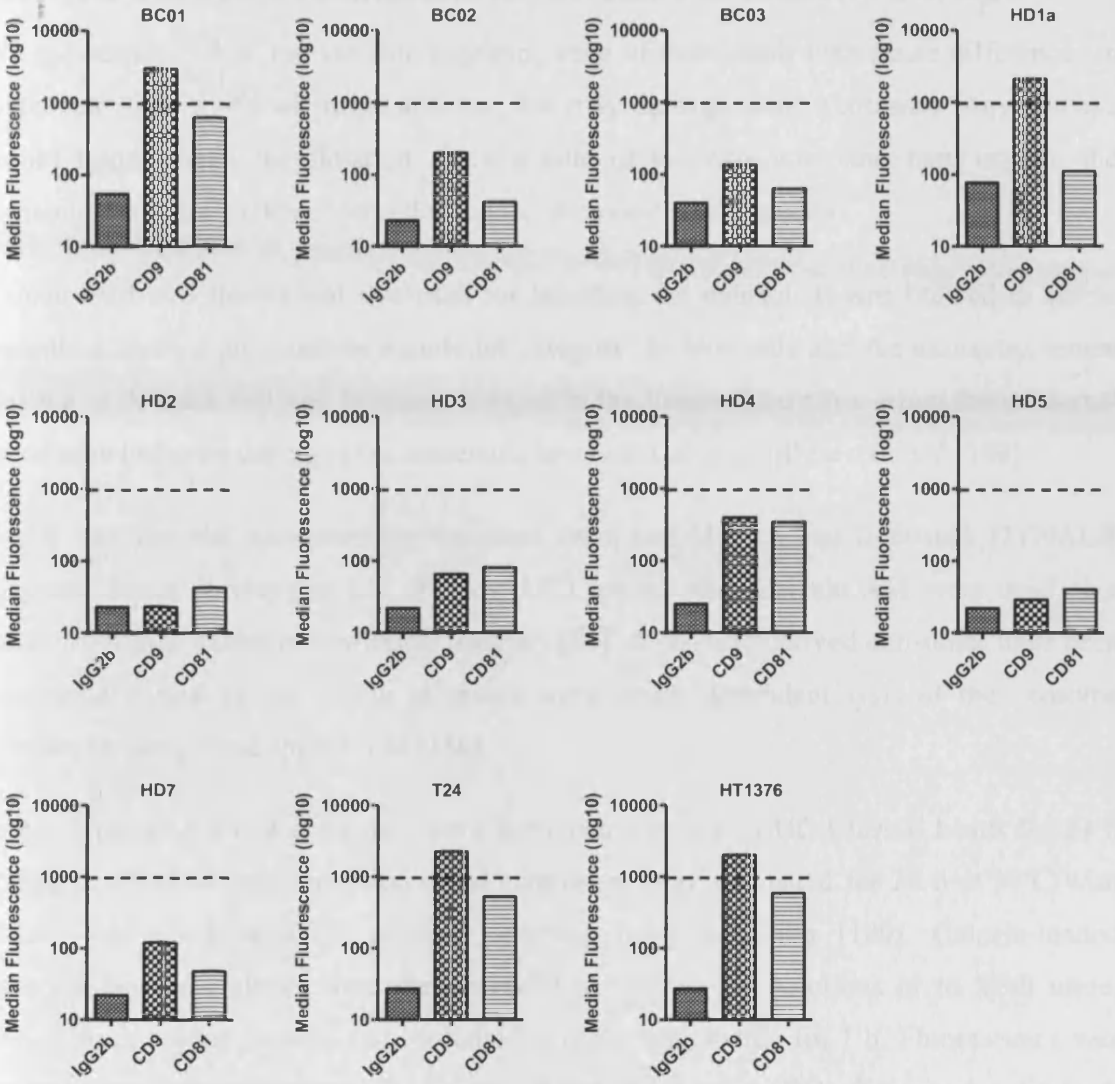


Figure 4.5: Exosome quality assurance of urine derived exosomes assessed by flow cytometry

Purified exosomes from fresh spot urine samples from three bladder cancer patients (BC), and six healthy donors (HD), along with CM derived exosome controls (T24 and HT1376) were coupled to micro-beads and stained for CD9 or CD81, or matching isotype IgG2b. Flow cytometry was then performed and the median fluorescence recorded. The dashed line represents the arbitrary CD9 median fluorescence threshold of 1000 for a good quality sample.

4.8 *The exosome membrane is resistant to osmotic damage*

We hypothesised that, the variable hydration state of individuals may cause differences in water/salt content of their urine and that this may damage urine exosomes. Any damage would impact upon the flotation characteristic of the exosomes and may explain the variability and low yields observed using the sucrose cushion method.

Calcein-AM is a fluorescent dye used for labelling the cytosol. It was utilised to assess osmotic influence on exosome membrane integrity. In live cells and the exosomal lumen Calcein is de-esterified and becomes trapped in the lumen. Therefore when the exosomal membrane becomes damaged the detectable levels of Calcein will be reduced [189].

B-cell line derived exosomes immobilised onto anti-MHC Class-II coated DYNAL® magnetic beads (Invitrogen Ltd, Paisley, UK) loaded with Calcein-AM were used as a model system to examine membrane integrity [86]. B-cell line derived exosomes have been successfully used by our group to assess complement dependant lysis of the exosome membrane using these approaches [189].

In brief, purified B-cell exosomes were incubated with anti-MHC Class-II beads for 24 h rolling at RT. The exosome micro-bead complexes were incubated for 20 h at 37°C with Calcein-AM (Invitrogen) to achieve complete bead saturation [189]. Calcein-loaded exosome-bead complexes were then exposed to various salt-solutions or to fresh urine, pooled from several donors, and incubated at room temperature for 1 h. Fluorescence was analysed by flow cytometry ((FACScan; Becton Dickinson (BD) Biosciences, Oxford, UK), running Cell Quest software (BD Biosciences)). Calcein-fluorescence was compared with fluorescence of anti-Class-I (RPE) (DAKO) stained exosome-beads in parallel tubes as a measure of whether exosomes remain attached to the bead surface.

The data are expressed as the ratio of Calcein:Class-I fluorescence. The results show that with decreasing salt concentration the ratio reduces indicating a loss of exosome-associated calcein, therefore increased exosome permeability (Figure 4.6a). When examining the effect of urine (Figure 4.6b) the Calcein:Class-I ratio remained stable.

These results suggest that exosomes are robust in osmotically hypotonic conditions and the signal remains at 70% of the control even in pure water. Urine from healthy donors has little or no affect on exosome membrane integrity, disproving the hypothesis that the differences in the hydration state of individuals may cause damage to urine exosomes.

4.9 *Exosomes are resistant to endogenous urinary proteases*

We also hypothesised that urine proteases may act to damage exosome constituents *in vivo* and/or *ex vivo* causing the apparent poor sample quality. To test this cell line (LnCAP) derived exosomes were incubated with fresh urine from three healthy donors, in the presence or absence of protease inhibitors (10 mM EDTA, 1 μg/ml Pepstatin-A, 1 μg/ml Leupeptin and 1 mM PMSF (Sigma Aldrich, Inc.)). Samples were incubated for 2 h or 18 h and then examined by immunoblotting for the expression of CD9 and TSG101. As a positive control for proteolysis exosomes were treated with the serine protease trypsin (Lonza Group Ltd). The results show there to be little/no urine mediated damage to exosome proteins TSG101 and CD9 in the presence or absence of protease inhibitors (Figure 4.6). The trypsin control however does show that exosomal proteins are subjected to damage in the presence of trypsin and that this damage can be prevented by the cocktail of protease inhibitors used.

In conclusion this shows that any proteolytic activity in fresh urine is unlikely to cause significant damage to exosomes. This suggests that while the exosomes are retained within the bladder, or are being handled for exosome purification, the endogenous protease activity is not sufficient to grossly impact the exosomally expressed proteins examined.

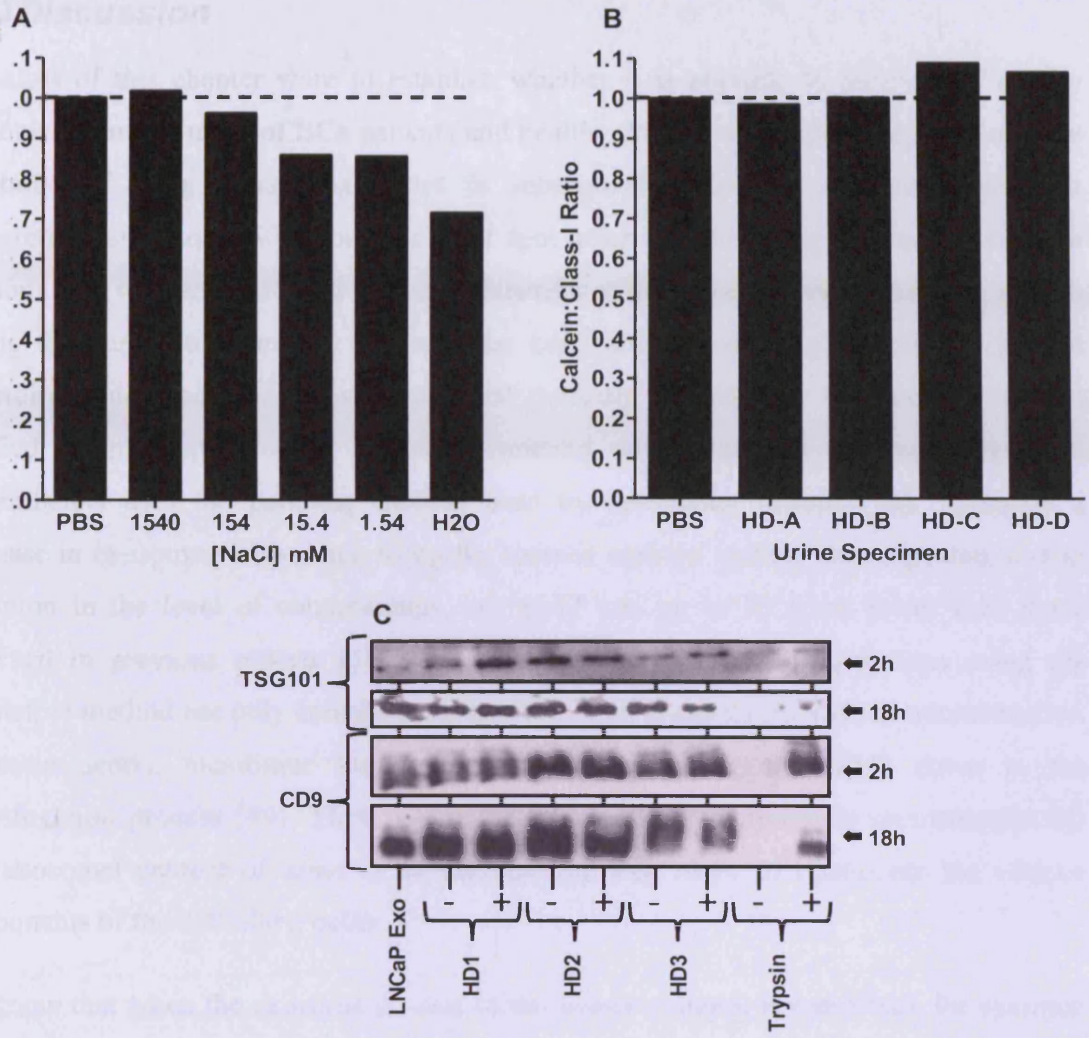


Figure 4.6: Examination of exosome membrane integrity and proteolytic damage

Immortalised B-cell line derived exosomes coupled to anti-MHC class II magnetic micro-beads were labelled with Calcein-AM, prior to incubation with various concentrations of NaCl (A) or with fresh urine specimens from four healthy donors (HD-A-D) (B). In parallel, identical beads were set up, in the absence of Calcein-AM dye, stained instead with anti-MHC Class-I (RPE) conjugated antibody. After 1h at room temperature, the fluorescence Calcein signal was compared with Class-I. Graphs A and B show the ratio of Calcein to Class I fluorescence (normalised to exosomes in PBS). To examine proteolytic damage to exosomes by urine, immunoblots (C) were performed for CD9 and TSG101 on LNCaP-derived exosomes; incubated for 2 or 18 h with fresh urine specimens (from three healthy donors), in the presence or absence of protease inhibitors. Trypsin was used as a positive control for proteolysis.

4.10 *Discussion*

The aims of this chapter were to establish whether it is possible to purify high quality exosomes from the urine of BCa patients and healthy donors, and importantly to assess the feasibility of using urinary exosomes in subsequent proteomics analysis. Initially it appeared possible to purify exosomes from spot urine samples using the sucrose cushion method. This was demonstrated by the elimination of the principal contaminating proteins during the purification process and also the concentration of a highly complex protein repertoire which included known exosomal proteins. In addition, our sucrose cushion purified samples appeared to be more enriched with exosomes and harboured less contaminants than the pelleting method used by competitor groups [34]. Although a decrease in exosome yields when using the sucrose cushion method was expected, due to reduction in the level of contaminants, our yield was up to 30 times lower than those observed in previous reports [143]. However, we suspect that preparations using the alternative method not only contain exosomes but other components such as microvesicles, apoptotic debris, membrane fragments, protein aggregates, all pulled down in the centrifugation process [99]. These reports therefore likely significantly overestimate the true exosomal content of urine as no attempt has been made to fractionate the various components of the 200,000 *g* pellet.

We know that when the exosome content of the source material is consistent, for example when using the CELLine™ flask system, the variation due to using the sucrose cushion method of preparation is less than 1% [163]. This suggests that the variability in the sample series is not introduced by the purification method used. The difficulty in using spot urine samples is that we do not know if serial collections from the same individual within hours of each other would give similar or different levels of urinary exosomes. However, we have shown that samples from the same individual do vary (up to four fold) from day to day. One potential way of creating a more consistent sample would be 24 h urine collection to average the sporadic variability that may occur during a 24 h period. Other modifications could include normalisation of the samples by comparing ratios of urine exosomes to urine creatinine instead of urine volume [143]. It is unknown whether this is a

fair comparison as there has been no documented correlation between urine exosomes and urine creatinine. Normalisation based on the level of THP contamination in the 200,000 *g* pellet of the pelleting method has also been suggested as levels of THP appeared to correlate with the levels of exosomal markers Alix and TSG101 [188]. However, this does mean that the exosome sample would be known to be contaminated with non-exosomal proteins and therefore interpreting any analysis performed on such preparations as an analysis of urinary exosomes is dubious.

The quality of the samples also appeared to be poor compared with CM as a source although the quality was still superior to the simple pelleting method. Even though the amount of protein was normalised for all of the samples, there were large variations in expression of expected exosomal proteins with only two samples considered as good quality based on the ExoQA assay.

The physiological parameters which influence the exosomal content of urine remains completely unknown. One may speculate that variations in hydration state, circadian rhythm, and renal function of the individual and/or the presence of protein or blood in the urine (a particular problem in BCa) may also have an influence on urine exosome content or the apparent urine exosome content. For example the presence of blood or protein in the urine may lead to an elevation in contaminants in the exosome sample. The results of the urine dipstick test for blood, protein, glucose and pH did not appear to correlate with the variation on quantity and quality. However, high blood levels were detected in the BCa patient samples. All of these had relatively low exosomes yields but HD and PCa patient samples containing blood did not show such low yields. Therefore blood may not have been the main influencing factor here.

One study examining urine microparticle proteomes in BCa discovered that contamination of urine samples with trace amounts (<0.002%) of blood led to noticeable differences in protein profiles [33]. Thus the samples were likely to be contaminated with non-exosomal proteins due to haematuria affecting both the quality and quantity of exosomes. However, similar variations were observed in the HD cohort where blood was not an influence.

Consequently the effect of haematuria on the exosome yield variation observed does not offer a full explanation for the data. Unfortunately there were also too few samples to perform any meaningful statistical analysis to investigate this further.

The potential effect of the hydration status of the donor on the integrity of the exosome membrane was investigated as a possible explanation for the low exosome yields we obtained. We speculated that hypotonic urine may damage exosome membrane integrity and this would in turn impact on their capacity to float on a sucrose cushion. The results showed that exosomes were not grossly affected by the hydration status of the donor. Pure water affected the membrane the most yet surprisingly exosomes still retained 70% of the control signal, demonstrating that they are structurally robust vesicles unlikely to be significantly damaged by osmotic variations of urine.

We hypothesised that endogenous protease activity in the fresh urine samples may have an effect on exosomal proteins. However this was deemed unlikely based on 18 h incubations with and without protease inhibitors where no/little affect on selected exosome proteins was observed. Therefore we would not expect to see significant exosome damage occurring *in vivo* or within the time frame of purification *ex vivo*. In contrast the effect of proteases on urine exosomes has previously been noted when using urine samples that have been frozen for prolonged periods of time (months). In this instance it is recommended that protease inhibitors are used [143].

In order to obtain a purer sample it may be necessary to utilise more labour intensive methods of exosome isolation. For example, utilising the linear sucrose method of purifying exosomes and only analysing the fractions of known exosomal density (1.12 and 1.19 g/ml) [47]. However, problems with co-localisation of common soluble proteins, such as albumin and immunoglobulins, with exosomes have been noted using this technique [68]. This may pose a problem if a urine sample is positive for blood or protein. Alternatively, exosomes could be isolated using an immunoaffinity capture method, utilising antibodies against known exosomal surface antibodies [133]. Immunoisolation has recently been used to isolate exosomes from CM for proteomics analysis [96]. Therefore, it

may be possible to isolate exosomes using an immunoaffinity based approach from more complex material such as biological fluids [64]. However, utilising such methods as linear sucrose gradients and immunoisolation does not alleviate the problems of low yields which seems a property inherent in the sample.

The poor exosome yields pose a problem when considering proteomics analysis of the samples. For two dimensional electrophoresis (2DE) a minimum of 50 μg of protein is usually required per gel to visualise the proteins and with protein yields as little as 6 μg from both healthy donors and BCa patients this is not feasible.

Another consideration would be to use the alternative simple pelleting method, to yield a greater amount of protein. However, proteomics analysis would not just identify exosomal proteins as other cell components, such as microvesicles, protein aggregates, membrane fragments, and apoptotic blebs in the pellet may also be co-purified. Abundant non-exosomal protein contaminants such as THP could mask less abundant exosomal proteins and it may be that such low abundance proteins that are of greater interest as biomarkers. However, the use of the reducing reagent DTT has been demonstrated to disrupt the polymeric network of THP from pelleted exosome preparations [188]. Nevertheless, this does not eliminate non polymeric THP as a contaminating protein and DTT may also significantly damage the exosome structure by attacking the extensive disulphide bridges that have been suggested as integral to stability of exosome structure [190].

When scrutinising studies utilising this pelleting method for proteomics analysis of urine exosomes [34, 59] and exosomes from other biological fluids [61] the characterisation of the exosome sample presented has been insufficient. This makes it difficult to determine how pure the sample is and therefore how many of the protein identifications are genuinely exosomal.

In conclusion it remains very challenging to isolate exosomes in high enough quantity and of good enough quality from fresh spot urine samples from BCa patients or healthy donors. It may be possible to modify the strategy for collection and purification, but this may be

very difficult and time consuming and is beyond the scope of this study. However, it may be possible to utilise a similar quantity of urinary exosomes obtained using the approaches described in this chapter to verify any candidate exosomal biomarkers. It may be that we have to use such an approach given the current limitations of urinary exosome purification.

Chapter 5:

Proteomics analysis of bladder cancer exosomes

5.1 *Introduction*

Proteomics analysis of exosomes may allow the identification of disease associated proteins as subcellular organelles offer a simpler proteome for analysis compared with the whole cell [191]. This proteome may be enriched in a subset of membrane and cytosolic proteins specifically incorporated in to the exosome. In addition unlike subcellular organelles such as the mitochondria, nucleus, and Golgi, exosomes are naturally secreted by the cell into the extracellular environment making exosomes an even more attractive non-invasive source for novel disease biomarkers. Exosome proteome studies have uncovered important proteins in exosome biology, such as MHC Class I [102] and II proteins [47], integrins [57, 95, 192] and annexins [34, 56, 61, 95]. With respect to our study, analysing a transitional cell carcinoma (TCC) exosome proteome presents an opportunity to identify cancer or bladder cancer specific proteins that may be novel biomarkers or potential targets for therapy.

This type of analysis is a developing field which has its inherent issues. Firstly, the diverse methods used to purify exosomes from simple ultracentrifugation to immunoisolation results in differences in the quality of the exosomes being analysed. It is therefore likely that a number of exosome proteomics studies report numerous false identifications with proteins present in the sample yet not in fact exosomally expressed. Exosomes from both biological fluids [34, 36, 59, 61, 62] and cell line sources [41, 47, 52, 100] have been isolated and analysed using proteomics. Biological fluid derived exosomes are nonetheless unavoidably complicated by the heterogeneous exosome sources, donor-related variability, sample contaminants such as Tamm-Horsfall protein (THP), and other factors [36, 188]. These issues therefore make biological fluid-derived exosomes a very challenging source of sample for exosome analysis.

An alternative approach using a homogenous cell line as an exosome source minimises variability considerably. We utilised a key biophysical property of exosomes, their ability to float on sucrose [42, 47], to isolate exosomes while further minimising contamination with other cellular components. This method was confirmed to produce very high quality

exosome samples (Chapter 3) from the cell conditioned medium (CM) of the different cell lines including the well characterised TCC cell line HT1376, which will be used in the subsequent analyses in this chapter [157].

The second issue complicating this research field are the mass spectrometry (MS) approaches used to identify proteins. Many early exosome proteome studies relied upon peptide mass fingerprinting (PMF), which lacks robust protein sequencing database comparisons [87, 99]. The use of PMF has been superseded by tandem mass spectrometry (MS/MS), which enable sequence data to be generated to facilitate unequivocal protein identification. However, stricter search criteria that are generally recommended for MS-derived sequence data have not been specified in all studies [59] and hence confidence in some reported identifications is not always high across all such studies.

Our study used a liquid chromatography-matrix assisted laser desorption/ionisation (LC-MALDI) MS workflow. Only peptides with good quality MS/MS data and proteins identifications from two or more peptides were included in the final results. It would nevertheless have been equally valid to use an electrospray ionisation (ESI) approach. MALDI and ESI are methods of soft ionisation for analysing proteomes and the most common mass analyser for a MALDI approach is a time of flight (TOF) analyser. This type of analyser offers good mass accuracy, high resolving power and sensitivity. Details of the different approaches for protein analysis using mass spectrometry will not be discussed further here. However, there are several good reviews on the subject [142, 193].

The proteomes of exosomes from different sources have been examined using various proteomics workflows including two dimensional electrophoresis (2DE) [40, 154], difference gel electrophoresis (DiGE) [95], and 1DE coupled with liquid chromatography-tandem mass spectrometry (1DE, LC-MS/MS) [34] as discussed in detail in the main thesis introduction. The LC-MALDI TOF/TOF-MS approach used in our study is a further high throughput technique that provides global proteomics data from the exosome source sample.

The proteins must be solubilised from a sample prior to any proteomic analysis. In our system the exosomes were purified and then stored in phosphate buffered saline (PBS). The exosomes cannot be solubilised in PBS as it will dilute the lysis buffer making solubilisation inefficient. In addition PBS contains salt which is incompatible with the 2D salt plug LC method used. Therefore the exosomes were repelleted and resuspended in Triethylammonium bicarbonate (TEAB) lysis buffer containing 20mM dithiothreitol (DTT) and 1% sodium dodecyl sulphate (SDS).

A typical biological membrane is made up of around 50% proteins by mass [194]. Exosomes on the other hand are known to be enriched in membrane proteins [87]. This high level of hydrophobic membrane protein which are poorly soluble in aqueous solutions necessitates the use of strong solubilising solutions to prevent aggregation and precipitation of the proteins [195]. Exosomes are also made of a partially detergent resistant membrane similar to lipid rafts which are known to be particularly difficult to solubilise by standard Triton X-100 or NP40 solubilisation [87, 193]. Furthermore, exosomes contain disulphide linked proteins that may be difficult to solubilise [196]. Due to the potential importance of exosomal membrane protein enrichment in disease and in the function of exosomes it was imperative that these membrane proteins are extracted efficiently.

5.2 *Aims*

The main aims of this chapter were to:

- Use a 2DE gel based approach to separate exosomal proteins and identify these proteins by MS
- Develop an LC-MALDI TOF/TOF MS workflow to enable high quality proteomics data to be achieved
- Identify large numbers of proteins from exosomes using the LC-MALDI workflow
- Compare the 2DE-MS and LC-MALDI workflows for protein identification
- Examine the protein identifications manually to identify any interesting or unexpected exosomal proteins

5.3 *Using 2DE-MS to separate and identify exosomal proteins*

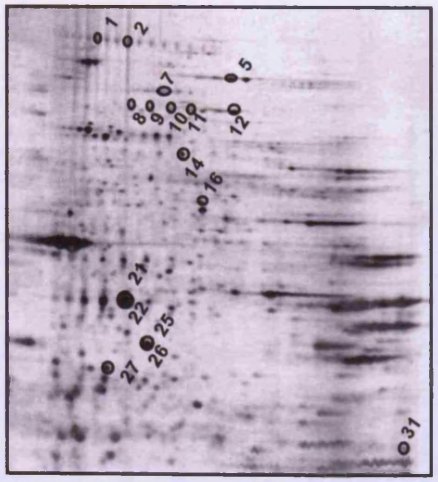
Prior to analysing exosomes using an LC-MALDI approach a two dimensional electrophoresis (2DE) workflow was used. We found it problematic to identify proteins from preparative gels loaded with 100 μg of proteins. The spots picked contained too little material to yield confident protein identifications by MS. Consequently the amount of exosomal protein loaded onto the gel was increased to 500 μg. Thirty two spots from a highly loaded gel (Figure 5.1a) were picked and peptides recovered by in gel trypsin digestion prior to MS identification. Overall proteins from 17 spots of intermediate level silver staining were successfully identified with publication quality data (high quality tandem MS data for 2 or more peptides) (Figure 5.1a-c).

The protein identifications included integrins α3 and α6, gelsolin, cytosolic enzymes lactate dehydrogenase and glyceraldehydes-3-phophate dehydrogenase (GAP DH), cytoskeletal proteins actin and cytokeratins, ezrin, and others (Figure 5.1b). Overall the identification hit rate for the 2DE approach was approximately 50%. This was unsatisfactory considering that 500 μg of protein was apparently loaded on to the gel. Furthermore, the usual level of positive identifications for human samples from 2D silver stained gels are approximately 70-80% for spots of the intensity picked (Ian Brewis, personal communication).

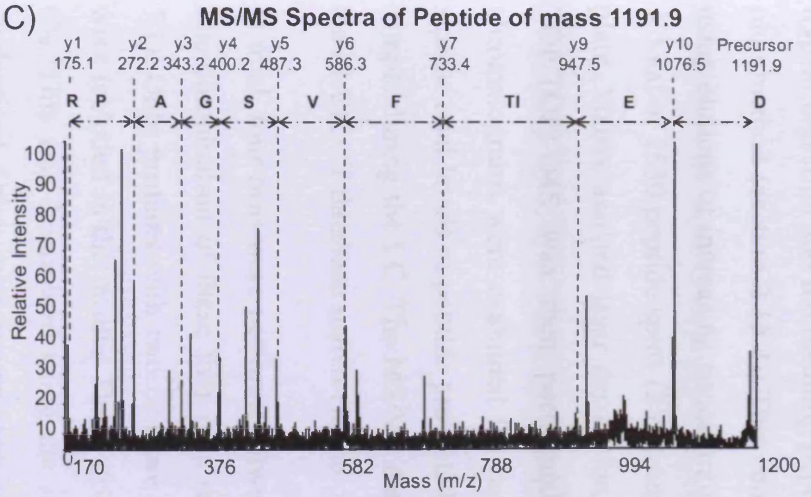
A number of factors relating to 2DE and membrane proteins may account for this relatively low identification rate. For example, membrane proteins are often insoluble in isoelectric focussing (IEF) compatible detergents such as urea, thiourea and CHAPS. SDS is the most effective detergent for hydrophobic proteins but it is incompatible with 2DE due to its negative charge [195]. Therefore, it is likely that certain membrane proteins were either not solubilised or were precipitated during IEF or SDS-PAGE resulting in either much reduced presence or absence on the final 2D gel. Identification of membrane proteins by MS can also be difficult due to problems with under-representation of transmembrane domains. This can be caused by issues with trypsin digestion where there is a lack of trypsin cleavage sites in these transmembrane domains. Poor protease accessibility to these

domains can also be a problem. Difficulties with protein solubility may also create difficulties as hydrophobic domains may aggregate and precipitate on removal of SDS. Extracting the peptides from the gel has also been documented as an issue [195].

A)



C)



B)

Spot Number	Protein Name	Accession Number	Sequence 1	Expect Value 1	Sequence 2	Expect Value 2	Sequence 3	Expect Value 3
21*	Actin, cytoplasmic 1	ACTB_HUMAN	QEYDESGPSIVHR	2.00E-07	VAPEEHPVLLTEAPLNPK	1.00E-06	SYELPDGQVITIGNER	4.90E-06
22*	Actin, cytoplasmic 1	ACTB_HUMAN	SYELPDGQVITIGNER	7.00E-10	VAPEEHPVLLTEAPLNPK	7.90E-10	QEYDESGPSIVHR	3.80E-08
27	Actin, cytoplasmic	ACTG_HUMAN	QEYDESGPSIVHR	5.10E-05	SYELPDGQVITIGNER	0.0042		
26**	Annexin A4	ANXA4_HUMAN	GAGTDEGCLIEILASR	1.60E-05	AEIDMLDIR	0.0048		
25**	Annexin A4	ANXA4_HUMAN	GLGTDEDAIISVLAYR	4.10E-12	GAGTDEGCLIEILASR	8.70E-12	NHLLHVFDEYKR	8.00E-11
12	Ezrin	EZRI_HUMAN	KAPDFVFYAPR	1.00E-08	APDFVFYAPR	1.90E-06	QLFDQVVK	0.00012
7	Gelsolin	GELS_HUMAN	EVQGFESATFLGYFK	0.0022	HVVPNEVVVQR	0.0035		
31	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	LVINGNPITIFQER	0.018	AGAHLQGGAK	0.03		
1	Integrin alpha-3	ITA3_HUMAN	EAGNPGSLFGYSVALHR	0.0004	YLLLAGAPR	0.0021	ARPVINIVHK	0.0022
2	Integrin alpha-3	ITA3_HUMAN	EAGNPGSLFGYSVALHR	3.00E-07	YTQVLWSGSEDQR	7.00E-05	YLLLAGAPR	0.00019
8	Integrin alpha-6	ITA6_HUMAN	NSYPDVAVGSLSDSVTIFR	7.10E-09	DGEVGGAVYVYMNQQGR	5.90E-07	DGWQDIVIGAPQYFDR	8.60E-07
9	Integrin alpha-6	ITA6_HUMAN	GIVSKDEITFVSGAPR	1.90E-09	DEITFVSGAPR	3.30E-09	NSYPDVAVGSLSDSVTIFR	6.60E-09
10	Integrin alpha-6	ITA6_HUMAN	DGEVGGAVYVYMNQQGR	9.70E-10	DEITFVSGAPR	2.60E-08	NSYPDVAVGSLSDSVTIFR	1.20E-07
11	Integrin alpha-6	ITA6_HUMAN	DEITFVSGAPR	5.10E-06	DGEVGGAVYVYMNQQGR	1.30E-05	NSYPDVAVGSLSDSVTIFR	0.0016
5	Programmed cell death 6-interacting protein	PDC6I_HUMAN	FYNELTEILVR	0.002	FLTALAQDGVINEEALSVTELDR	0.019	ELPELLQR	0.038
16	Rho GTPase-activating protein 1	RHG01_HUMAN	NPEQEPIPIVLR	7.30E-05	FLLDHQGELFPSPDPSGL	0.0074	LEQLGIPR	0.048
14	T-complex protein 1 subunit alpha	TCPA_HUMAN	AFHNEAQVNPER	4.40E-06	EQLAIAEFAR	0.0046		

* excised from the same spot

** excised from the same spot

Figure 5.1: Analysis of HT1376-derived exosomes using 2DE and MS

Protein extracts from HT1376 derived exosomes were resolved by 2DE on a pH 3-10 non linear gradient. 32 spots were chosen at random and excised and peptides recovered following trypsin digestion (A). 17 of which, annotated in A, yielded protein identifications (B). A representative MS/MS analysis from the data set is shown in C. The peptide is from integrin α6 (spot 10) Sequence 2. The peptide has a precursor mass of 1191.9 and is annotated to show the derived peptide sequence.

5.4 *Identification of exosomal proteins by LC-MALDI MS*

Due to the challenges in identifying exosomal proteins from 2DE gels a gel free approach (LC-MALDI) was used. Once the proteins had been solubilised they were subjected to trypsin digestion and the resulting peptides were then separated by a two-dimensional salt plug method (section 2.15.2). This comprised of a strong cation exchange (SCX) column using elutions of increasing concentrations of NaCl and a desalting reverse phase column. A total of 1530 peptide spots (255 from each of six salt plugs) were spotted onto a sample plate. Matrix assisted laser desorption/ionisation- time of flight/ time of flight (MALDI TOF/TOF) MS was then performed. Base peak chromatograms and extracted ion chromatograms were examined for each MS/MS run to confirm that there were broadly similar total levels of peptide present. This established that there was no significant loss of sample during the LC. The MS/MS data was used to search the Swiss-Prot database using the MASCOT database search engine.

In total, four runs were performed (two biological replicates and two technical replicates). The combination of these four runs resulted in the identification of 353 proteins (Table 5.1). Only proteins with two or more peptides and an expect (e) value of less than 0.05 were included in the results. These strict criteria produced a false discovery rate (FDR) of 0%. This was determined using the same Swiss-Prot database with the entire sequence randomised. Other exosome proteomics studies do not use such strict criteria and include one peptide data in their results [34, 59, 61]. If single peptide protein identifications were included here with an expect value of less than 0.0025, there would be an additional 261 proteins but the FDR would increase to 2.6% (approximately 16 false identifications). However, these extra 261 proteins will inevitably include some valid assignments.

We compared the LC-MALDI results with the 2DE-MS data and this revealed that 10 of the 11 unique proteins identified from the gel based approach (Figure 5.1c) were also identified by the LC-MALDI approach (Table 5.1). The only unique protein identified using the 2DE approach was Actin, cytoplasmic (ACTG_HUMAN). This comparison of proteomics approaches shows there is high agreement between these two methods for

resolving exosomal proteins or peptides. The LC-MALDI approach was nonetheless superior in identifying large numbers of high quality identifications.

Table 5.1: LC-MALDI MS-based protein identifications of HT1376-derived exosomes

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	1433B_HUMAN	14-3-3 protein beta/alpha	6	AVTEQGHELSNEER	0.00089	KEMQPTHPIR	0.00033	EMQPTHPIR	0.0057
	1433E_HUMAN	14-3-3 protein epsilon	2	NLLSVAYK	0.033	DSTLIMQLLR	7.90E-09		
	1433G_HUMAN	14-3-3 protein gamma	4	EHMQPTHPIR	0.00018	NLLSVAYK	0.033	NVTELNEPLSNEER	8.60E-06
	1433S_HUMAN	14-3-3 protein sigma	6	YEDMAAFMK	3.20E-06	GAVEKGEELSCEER	4.80E-06	SAYQEAMDISK	1.40E-07
	1433T_HUMAN	14-3-3 protein theta	7	KEMQPTHPIR	0.00033	QTIDNSQGAYQEAFDISK	3.10E-17	EMQPTHPIR	0.0057
	1433Z_HUMAN	14-3-3 protein zeta/delta	6	KEMQPTHPIR	0.00033	SVTEQGAELSNEER	1.40E-05	YLAEVAAGDDKK	0.0047
✓	PRS6B_HUMAN	26S protease regulatory subunit 6B	2	GVLMYGPPGCGK	5.00E-05	AVAHHTTAAFIR	1.00E-08		
✓	PRS7_HUMAN	26S protease regulatory subunit 7	2	QTLQSEQPLQVAR	1.90E-09	GVLLFGPPGTGK	0.024		
✓	PSD13_HUMAN	26S proteasome non-ATPase regulatory subunit 13	2	VHMTWVQPR	3.70E-05	YYQTIGNHASYYK	6.80E-06		
	PSMD2_HUMAN	26S proteasome non-ATPase regulatory subunit 2	2	AVPLALALISVSNPR	0.0034	FLRPHYGK	0.0035		
	RS16_HUMAN	40S ribosomal protein S16	2	GPLQSVQVFGR	9.60E-07	GGGHVAQIYAIR	2.00E-07		
	RS4X_HUMAN	40S ribosomal protein S4, X isoform	4	HPGSFDVVHVK	7.10E-05	GNKPWISLPR	8.40E-05	GIPHLVTHDAR	3.10E-06
	RSSA_HUMAN	40S ribosomal protein SA	2	FTPGTFTNQIQAAFR	2.70E-07	FAAATGATPIAGR	0.049		
	4F2_HUMAN	4F2 cell-surface antigen heavy chain	11	VAGSPGWVR	0.0077	LKLEPHEGLLLR	7.60E-09	GLVLGPHK	5.60E-06
	5NTD_HUMAN	5'-nucleotidase	6	YPFIVTSDDGR	0.0065	KAFEHSVHR	5.70E-05	GAEVAHFMNALR	2.00E-10
✓	RL10_HUMAN	60S ribosomal protein L10	3	GAFGKPQGTVAR	0.0068	VHGQVIMSIR	0.0023	LHPRHVIR	0.0013
✓	RL15_HUMAN	60S ribosomal protein L15	2	GATYGKPVHHGVNQLK	9.10E-09	RNPDTQWITKPVHK	0.0034		
✓	K6PP_HUMAN	6-phosphofructokinase type C	2	KFLEHLSGAGK	1.20E-06	VTLGHVQR	0.00019		
	GRP78_HUMAN	78 kDa glucose-regulated protein	12	IINEPTAAAIAYGLDK	5.40E-11	TWNDPSVQQDIK	1.80E-06	SDIDEVLVGGSTR	0.025
✓	THIC_HUMAN	Acetyl-CoA acetyltransferase, cytosolic	2	ILVTLLHTLER	0.00037	APHLAYLR	9.00E-06		
	ACTC_HUMAN[a]	Actin, alpha cardiac muscle 1	8	EITALAPSTMK	7.10E-05	HQGVMVGMGQK	2.50E-09	IWHHTFYNELR	7.50E-09
	ACTB_HUMAN[b]	Actin, cytoplasmic 1	14	IWHHTFYNELR	7.50E-09	EITALAPSTMK	7.10E-05	VAPEEHPVLLTEAPLNPK	1.00E-12
✓	ACL6A_HUMAN	Actin-like protein 6A	2	SPLAGDFTMQCR	0.00096	QGGPTYYIDTNALR	3.70E-06		
	ARP2_HUMAN	Actin-related protein 2	2	HIVLSGGSTMYPGLPSR	5.20E-14	GYAFNHSADFETVR	0.00064		
	LYPA2_HUMAN	Acyl-protein thioesterase 2	2	TYPGVMHSSCPQEMAAVK	2.60E-09	YICPHAPR	0.0014		
	ADK_HUMAN	Adenosine kinase	4	FKVEYHAGGSTQNSIK	0.0067	VIFTQGR	0.0012	VAQWMQQPHK	7.30E-07
	SAHH_HUMAN	Adenosylhomocysteinase	3	VADIGLAAWGR	0.00062	YPQLLPGIR	4.70E-06	ALDIAENEMPGLMR	1.60E-05
	ARF1_HUMAN[c]	ADP-ribosylation factor 1	2	MLAEDELR	0.013	QDLPNAMNAAEITDK	8.00E-12		
	AGRIN_HUMAN	Agrin	5	RPLQEHVR	0.005	SFLAFPTLR	0.0017	AAAVSSGFDGAIQLVSLGGR	0.011
	AK1A1_HUMAN	Alcohol dehydrogenase [NADP+]	3	HHPEDVEPALR	0.042	MPLIGLGTWK	0.0011	YALSVGYR	0.018
	PPB1_HUMAN	Alkaline phosphatase, placental type	2	GFFLFVEGGR	0.022	VQHASPAGTYAHTVNR	1.20E-08		
	ACTN1_HUMAN	Alpha-actinin-1	6	LASDLLEWIR	0.0004	KHEAFESDLAAHQDR	2.90E-06	HTNYTMEHIR	3.30E-05
	ACTN4_HUMAN	Alpha-actinin-4	9	MAPYQGPDAVPGALDYK	0.039	LSNRPAFMPSEGK	0.0012	LASDLLEWIR	0.0004
	ACTZ_HUMAN	Alpha-centractin	4	TLFSNIVLSGGSTLFK	0.00058	IWQYVYSK	0.00096	AQYYLPDGSTIEIGPSR	0.00013
	ENOA_HUMAN	Alpha-enolase	7	GNPTVEVDLFTSK	1.30E-06	YISPDQLADLYK	2.40E-07	IGAEVYHNLK	1.10E-07
	AMPB_HUMAN	Aminopeptidase B	2	KKPFVYTQGQAVLNR	7.40E-07	LFGPYVWGR	0.00027		
	ANXA1_HUMAN	Annexin A1	14	AAYLQETGKPLDETLK	3.30E-11	GGPGSAVSPYPTFNPSSDVAALHK	5.60E-10	CATSKPAFFAEK	6.20E-07
	ANX11_HUMAN	Annexin A11	5	DESTNVDMSLAQR	0.007	AHLVAVFNEYQR	6.10E-07	GTITDAPGFDPLR	0.011
	ANXA2_HUMAN	Annexin A2	16	AEDGSVIDYELIDQDAR	2.80E-09	AYTNFDAER	9.70E-06	TPAQYDASELK	4.70E-08
	ANXA3_HUMAN	Annexin A3	5	HYGYSLYSAIK	0.0018	LTFDEYR	0.014	GIGTDEFTLNR	0.018
	ANXA4_HUMAN	Annexin A4	3	ISQTYQQQYGR	1.20E-09	FLTVLCSR	0.00047	AASGFNAMEDAQTLR	0.026
	ANXA5_HUMAN	Annexin A5	8	QVYEEEYGSSLEDDVVGDTSGYYQR	6.80E-12	GTVTDFPGFDER	1.30E-05	SEIDLFNIR	2.20E-06
	ANXA6_HUMAN	Annexin A6	2	SLEDALSSDTSGHFR	0.0019	GFGSDKEAILDITSR	0.0023		
	ANXA7_HUMAN	Annexin A7	3	QMFAQMYQK	0.0031	GFGTDEQAIVDVVANR	0.036	VLELCTR	0.037
	ANXA8_HUMAN[d]	Annexin A8	4	FITLCTR	0.0043	LIVALMYPPYR	5.20E-07	ILVCLLQGSR	1.50E-05
	ASSY_HUMAN	Argininosuccinate synthase	3	GQVYILGR	0.0064	MPEFYNR	0.0011	QHGIPIPVTPK	2.90E-07
	SYRC_HUMAN	Arginyl-tRNA synthetase, cytoplasmic	3	GNTAAYLLYAFTR	3.00E-08	LFEFAGYDVLR	0.023	GFDILGIKPVQR	0.0016
	ARRD1_HUMAN	Arrestin domain-containing protein 1	2	VVYSPGEPLAGTVR	2.00E-05	VQLFESLSHGR	6.40E-06		

Protein accession number, name, peptide count, and top three peptides sequences with e-values are detailed. Novel identifications of proteins previously not identified as exosomal are marked and highlighted.

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
✓	SYDC_HUMAN	Aspartyl-tRNA synthetase, cytoplasmic	5	VTMLFLGLHNVR	0.0017	FGAPPHAGGGIGLER	1.10E-05	QSNSYDMFMR	0.043
	ATPB_HUMAN	ATP synthase subunit beta, mitochondrial	2	VALVYGQMNEPPGAR	0.031	AHGGYSVFAGVGER	3.30E-07		
	ACLY_HUMAN	ATP-citrate synthase	2	AKPAMPQDSVPSPR	8.00E-06	TIAIAEGIPEALTR	9.10E-05		
✓	KU70_HUMAN	ATP-dependent DNA helicase 2 subunit 1	2	SDSFENPVLQQHFR	1.10E-05	KPGGFDISLFYR	4.70E-05		
✓	KU86_HUMAN	ATP-dependent DNA helicase 2 subunit 2	3	LGGHGPSFPLK	8.10E-06	HSHWPCR	0.02	ANPQVGVAFPHK	8.60E-09
✓	DHX9_HUMAN	ATP-dependent RNA helicase A	2	YQILPLHSQFR	3.10E-05	HLEMNRHFGSHR	1.80E-05		
	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	7	SPAYTLVWTR	0.0058	CVVPGQAHAQVTWHK	0.015	SVPQGGSHSLR	0.0013
	BASI_HUMAN	Basigin	5	SELHIENLNMEADPGQYR	2.00E-06	SESVPPVTDWAWYK	0.0051	FPVSSSQGR	1.10E-05
	B2MG_HUMAN	Beta-2-microglobulin	2	VEHSDLSFSK	7.20E-07	VNHVTLSQPK	2.80E-06		
	ACTBL_HUMAN	Beta-actin-like protein 2	5	VAPDEHPILLTEAPLNPK	2.20E-06	HQGVMVGMGQK	2.50E-09	SYELPDGQVITIGNER	4.90E-13
✓	ACTY_HUMAN	Beta-centractin	2	MVQYVYSK	0.00096	YCFPNYVGRPK	3.70E-05		
	BGAL_HUMAN	Beta-galactosidase	3	TEAVASSLYDILAR	0.008	QHYGFVLYR	0.0025	YISGSHYSR	2.40E-05
✓	SYEP_HUMAN	Bifunctional aminoacyl-tRNA synthetase	3	QFIAAQGSSR	3.80E-06	AIQGGTSHHLGQNFSK	1.70E-12	KPYMVEYSR	0.0021
	PUR9_HUMAN	Bifunctional purine biosynthesis protein PURH	2	YGMNPHQTPAQLYTLQPK	6.60E-06	TLFGLHLSQK	0.00011		
	CALR_HUMAN	Calreticulin	3	KVHVIFNYK	7.10E-06	VHVIFNYK	0.0017	IKDPDASKPEDWDER	4.30E-06
	CTNA1_HUMAN	Catenin alpha-1	4	KHVNPVQALSEPK	4.40E-09	IAEQVASFQEEK	0.012	TSVQTEDDQLIAGQSAR	3.70E-12
	CTNB1_HUMAN	Catenin beta-1	5	NEGVATYAAAVLFR	0.00069	LVQNCLWTLR	0.00013	LHYGLPVVVK	2.70E-07
	CTND1_HUMAN	Catenin delta-1	7	FHPEPYGLEDDQR	0.00011	ALSAIADLLTNEHER	0.00011	SNAAAYLQHLCYR	2.90E-05
	CATD_HUMAN	Cathepsin D	10	QPGITFIAAK	7.20E-08	YYTVFDR	0.00045	EGCEAIVDTGTSLMVGPVDEVR	1.60E-07
	CD44_HUMAN	CD44 antigen	5	YGFIEGHVVIPR	1.60E-07	SQEMVHLVNK	2.20E-06	FAGVFHVEK	2.70E-06
✓	CD70_HUMAN	CD70 antigen	3	LYWQGGPALGR	3.00E-07	SFLHGPELDKGQLR	2.80E-05	LSRHQGCTIASQR	1.90E-05
	CD9_HUMAN	CD9 antigen	2	EVQEFYKDTYNK	1.40E-07	KDVLETFTVK	3.40E-08		
	CDC42_HUMAN	Cell division control protein 42 homolog	2	TPFLLVGTQIDLR	1.80E-12	WVPETHHCPK	5.30E-06		
	CLIC1_HUMAN	Chloride intracellular channel protein 1	5	LHIVQVVCK	1.90E-07	YLSNAYAR	0.042	LAALNPESNTAGLDIFAK	5.50E-05
	CTL2_HUMAN	Choline transporter-like protein 2	8	DGDCPAVLIPSKPLAR	0.0044	CQFAFYGGESGYHR	1.00E-07	CFPAIHAYK	1.60E-06
	CISY_HUMAN	Citrate synthase, mitochondrial	3	VVPGYGHAVLR	1.70E-08	ALGFPLERPK	0.0028	GLVYETSVLDPDEGIR	0.00063
	CLH1_HUMAN	Clathrin heavy chain 1	24	TSIDAYDNFDNISLAQR	3.20E-09	VGEQAQVVIIDMNIDPSNPIR	0.013	RPISADSAIMNPASK	0.00029
	CLH2_HUMAN	Clathrin heavy chain 2	2	IVLDNSVFSEHR	0.0013	NLQNLLILTAIK	1.20E-06		
	COPA_HUMAN	Coatomer subunit alpha	2	QLFLQTYAR	0.043	QQPLFVSGGDDYK	1.10E-08		
	COF1_HUMAN	Cofilin-1	3	YALYDATYETK	1.50E-05	EILVGDVGQTVDDPYATFVK	0.027	HELQANCYEEVKDR	0.00063
	COCA1_HUMAN	Collagen alpha-1(XII) chain	3	QYLVTYTPVAGGETQEVTVR	2.00E-10	ITYQPSTGEGNEQTTTIGGR	1.40E-11	NLQPDTSYTVTVVPVYTEGDGGR	0.0098
	CPNE3_HUMAN	Copine-3	2	DIVQFVPFR	0.0014	NNLNPVWRPPK	0.0057		
✓	H2AY_HUMAN	Core histone macro-H2A.1	5	AGVIFPVGR	1.10E-05	GKLEAITPPPAK	0.00028	EFVEAVLELR	0.021
	H2AW_HUMAN	Core histone macro-H2A.2	2	AGVIFPVGR	1.10E-05	HLLAVANDEELNQLLK	0.0032		
	CAND1_HUMAN	Cullin-associated NEDD8-dissociated protein 1	3	ADVFHAYLSLLK	0.029	MLTGPVYSQSTALTHK	6.30E-06	MLTFLMLVR	0.039
	DYHC1_HUMAN	Cytoplasmic dynein 1 heavy chain 1	4	FTQDTQPHYYSPR	0.00069	VAAPDVVVPTLDTVR	0.012	QYASYEFVQR	0.00064
	CYFP1_HUMAN	Cytoplasmic FMR1-interacting protein 1	4	ALNLAYSSIYGSYR	6.60E-06	YAPLHLVPLIER	0.0031	LMNFMYFQR	0.0012
	AMPL_HUMAN	Cytosol aminopeptidase	3	MPLFEHYTR	0.041	QLMETPANEMTPTR	3.20E-05	TLIEFLLR	0.0056
	SERA_HUMAN	D-3-phosphoglycerate dehydrogenase	2	QIPQATASMK	0.00029	DLPLLLFR	0.00019		
	DPYL2_HUMAN	Dihydropyrimidinase-related protein 2	3	VFNLYPR	0.013	QIGENLIVPGGVK	0.049	MVIPGGIDVHTR	0.002
	DNJA1_HUMAN	DnaJ homolog subfamily A member 1	2	TIVITSHPGQVK	3.30E-06	QISQAYEVLSDAK	1.20E-05		
	EHD1_HUMAN	EH domain-containing protein 1	8	KLNAFGNAFLNR	2.30E-09	EHQISPGDFPSLR	3.00E-09	QEESLMPSQVVK	5.20E-08
	EHD2_HUMAN	EH domain-containing protein 2	8	VYGALMWALGK	0.0013	MQELLMAHDFTK	4.10E-08	KLNPFGNTFLNR	2.40E-06
	EHD3_HUMAN	EH domain-containing protein 3	5	KLNAFGNAFLNR	2.30E-09	YLLEQDFPGMR	0.033	VHAYISSLK	2.90E-07
	EHD4_HUMAN	EH domain-containing protein 4	6	LPEIYIQLQR	3.70E-06	LFEAEAQDLFR	0.0028	VHAYISYLK	3.10E-08
	EF1A1_HUMAN	Elongation factor 1-alpha 1	6	THINVVVIGHVDSGK	9.70E-14	YYVTIIDAPGHR	1.10E-05	EHALLAYTLGVK	3.50E-08
✓	EF1D_HUMAN	Elongation factor 1-delta	2	ATAPQTQHVSPMR	0.0043	LVPVGYGIR	0.0042		
	EF1G_HUMAN	Elongation factor 1-gamma	5	ALIAAQYSGAQVR	1.30E-06	KLDPGSEETQTLVR	0.0055	AKDIFFAHLPK	0.0011
	EF2_HUMAN	Elongation factor 2	11	KEDLYLKPQR	7.20E-07	GPLMMYISK	0.00035	CLYASVLTAQPR	6.90E-06

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	ENPL_HUMAN	Endoplasmin	6	FAFQAEVNR	0.00077	FQSSHHPTDITSLDQYVER	0.00065	GVVDSDDLPLNVSR	2.20E-05
	EPHA2_HUMAN	Ephrin type-A receptor 2	2	YLANMNYVHR	0.0026	QSPEDVYFSK	2.30E-06		
	EGFR_HUMAN	Epidermal growth factor receptor	4	IPLENLQIR	7.10E-06	NLQELHGAVR	0.0012	ELVEPLTPSGEAPNQALLR	8.10E-05
	ES8L2_HUMAN	Epidermal growth factor receptor kinase substrate 8-like protein 2	2	SQPVSQPLTYESGPDEVR	1.30E-10	YWGPASPTHK	0.0017		
	EPIPL_HUMAN	Epiplakin	2	QPLQATFR	0.0013	LLEAQIATGGVIDPVHSHR	6.80E-05		
	DDR1_HUMAN	Epithelial discoidin domain-containing receptor 1	5	LHLVALVGTQGR	5.70E-06	NLYAGDYYR	0.049	LLLATYARPPR	0.041
	ERO1A_HUMAN	ERO1-like protein alpha	3	LIANMPESGPSYEFHLTR	4.80E-06	FDGLTEGEGPR	0.00061	QEVSLFNAFGR	0.0011
	IF4A1_HUMAN	Eukaryotic initiation factor 4A-I	3	GIYAYGFEKPSAIQQR	7.00E-11	LQMEAPHIVVGTPGR	8.40E-07	MFVLDEADEMLSR	6.90E-06
	IF4A2_HUMAN	Eukaryotic initiation factor 4A-II	3	GIYAYGFEKPSAIQQR	7.00E-11	LQAEAPHIVVGTPGR	0.0014	MFVLDEADEMLSR	6.90E-06
✓	IF4A3_HUMAN	Eukaryotic initiation factor 4A-III	2	GIYAYGFEKPSAIQQR	7.00E-11	KLDYGQHVVAGTPGR	9.40E-11		
	EIF3A_HUMAN	Eukaryotic translation initiation factor 3 subunit A	2	MHLSQIQR	0.00036	SGNALFHASTLHR	2.10E-06		
✓	EIF3B_HUMAN	Eukaryotic translation initiation factor 3 subunit B	3	ISVSFYHVK	0.0026	GTYLATFHQR	0.0016	FAVLHGEAPR	0.022
✓	EIF3C_HUMAN	Eukaryotic translation initiation factor 3 subunit C	3	SEQDQAENEGEDSAVLMER	0.0092	QGTYGGYFR	0.003	QPLLGPPESMR	4.90E-06
✓	EIF3L_HUMAN	Eukaryotic translation initiation factor 3 subunit L	2	QLEVYTSGGDPESVAGEYGR	1.80E-15	VFSDEVQQQAQLSTR	0.009		
✓	XPO1_HUMAN	Exportin-1	2	AVGHPFVIQLGR	1.70E-08	AIIASNMMYNGQYPR	0.0047		
	XPO2_HUMAN	Exportin-2	2	AADEEAFEDNSEEYIR	7.10E-09	IHLAQSLHK	1.70E-05		
	EZRI_HUMAN	Ezrin	11	IGFPWSEIR	8.70E-07	APDFVFYAPR	8.60E-09	FVKPDKK	4.70E-05
	FPPS_HUMAN	Farnesyl pyrophosphate synthetase	2	VLTEDEMGHPEIGDAIAR	1.20E-08	GLTVVVAFR	0.0075		
	FSCN1_HUMAN	Fascin	7	GEHGFIGCR	6.70E-05	YLKGDHAGVLK	0.0017	LVARPEPATGYTLEFR	0.046
	FAS_HUMAN	Fatty acid synthase	19	DNLEFFLAGIGR	0.0097	VTAIHIDPATHR	3.70E-07	FPQLDSTSFANSR	0.017
✓	FCRLA_HUMAN	Fc receptor-like A	2	LLFSFYK	0.00086	QSPQLEIR	8.00E-05		
	FRIH_HUMAN	Ferritin heavy chain	2	YFLHQSHEER	7.50E-07	ELGDHVTNLR	0.00025		
	FINC_HUMAN	Fibronectin	9	GDSPASSKPSINYR	0.0079	WLPSSSPVTGYR	0.026	YEKPGSPPR	0.023
	FLNA_HUMAN	Filamin-A	13	TGVELGKPTHFTVNAK	2.40E-08	YAPSEAGLHEVDIR	0.00088	YGGQPVPNFPSK	7.50E-05
	FLNB_HUMAN	Filamin-B	11	APLNVQFNSPLPGDAVK	0.00019	VVPCLVTPVTGR	0.00036	YGGELVPHFPAR	4.60E-05
✓	FLNC_HUMAN	Filamin-C	3	LIALLEVLSQK	3.20E-06	LIALLEVLSQKR	0.021	YGGDEIPYSPFR	0.022
	FLOT1_HUMAN	Flotillin-1	2	LTGVSISQVNHKPLR	2.50E-05	AQQVAVQEQEAR	1.40E-09		
	ALDOA_HUMAN	Fructose-bisphosphate aldolase A	6	IGEHTPSALAIMENANVLAR	0.022	LQSIGTENTEENRR	0.001	ADDGRPFPQVIK	1.50E-06
✓	GGEE2_HUMAN	G antigen family E member 2	2	IEDEPGDGPDVR	0.00039	GNDQESSQPVGSVIVQEPTEEK	0.0039		
✓	LEG1_HUMAN	Galectin-1	2	DSNNLCLHFNPR	4.70E-07	LPDGYEFK	8.30E-07		
	LEG3_HUMAN	Galectin-3	3	GNDVAFHFNPR	6.80E-06	IQVLVEPDHFK	1.10E-05	VAVNDAHLLQYNHR	6.20E-11
	LG3BP_HUMAN	Galectin-3-binding protein	4	ELSEALGQIFDSQR	2.00E-06	YSSDYFQAPSDYR	2.90E-05	AVDTWSWGER	4.30E-06
✓	OFUT2_HUMAN	GDP-fucose protein O-fucosyltransferase 2	2	QDVPSLEGAVR	0.022	LYHWQSPDHQVR	0.0013		
	GELS_HUMAN	Gelsolin	3	HVVPNEVVVQR	2.50E-06	QTQVSVLPEGGETPLFK	0.00044	AGKEPGLQIWR	0.00017
✓	GFPT1_HUMAN	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1	3	SVHFPGQAVGTR	6.40E-06	WATHGEPSPVNSHPQR	0.00092	VIFLEDDDVAAVVDGR	0.0069
	G6PD_HUMAN	Glucose-6-phosphate 1-dehydrogenase	3	NSYVAGQYDDAASYQR	0.0014	LSNHSSLFR	0.0002	IFGPWNR	0.00014
	GSTP1_HUMAN	Glutathione S-transferase P	5	DQQEAALVDMVNDGVEDLR	0.0037	ASCLYGQLPK	0.00013	PPYTVVYFPVR	8.10E-09
	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	9	VPTANVSVVDLTCR	4.40E-14	LEKPAKYDDIK	0.00052	AGAHLQGGAK	1.20E-06
	PYGB_HUMAN	Glycogen phosphorylase, brain form	5	LLPLVSDEVFIR	0.00047	QAVDQISSGFFSPK	3.70E-09	HLEIYAINQR	0.012
✓	PYGL_HUMAN	Glycogen phosphorylase, liver form	3	DYYFALAHTVR	7.20E-05	LHSRLGDDVFLR	2.10E-05	VIFLENYR	0.00099
✓	GT251_HUMAN	Glycosyltransferase 25 family member 1	2	NAAHALPTTLGALER	0.00012	RTPAYIPR	0.032		
	SYG_HUMAN	Glycyl-tRNA synthetase	5	TFFSFPAVVAPPK	0.00054	LPFAAAQIGNSFR	1.10E-06	IMYTVFEHTFHVR	0.0042
	GPC5C_HUMAN	G-protein coupled receptor family C group 5 member C	2	SSPEQSYQGDMYPTR	9.50E-09	VPSEGAYDILPR	4.50E-05		
	RAN_HUMAN	GTP-binding nuclear protein Ran	4	SNYNFEKPFLWLAR	0.00015	NLQYYDISAK	0.0016	FNVWDTAGQEK	5.40E-08
	GNAI2_HUMAN	Guanine nucleotide-binding protein G(i), alpha-2 subunit	2	TTGIVETHFTFK	0.00018	IAQSDYIPTQQDVLR	3.20E-08		
	GNAS1_HUMAN	Guanine nucleotide-binding protein G(s) subunit alpha isoforms Xlas	3	YTTPEDATPEPGEDPR	7.10E-06	QADYVPSDQDLLR	2.40E-11	TSVILFLNK	0.0013
	GBLP_HUMAN	Guanine nucleotide-binding protein subunit beta-2-like 1	2	VWQVTIGTR	0.0014	YWLCAATGPSIK	0.0023		
	HSP71_HUMAN	Heat shock 70 kDa protein 1	15	IINEPTAAAIAYGLDR	4.40E-15	DAGVIAGLNVLR	5.40E-10	AFYPEEISSMVLTK	0.00034
	HS71L_HUMAN	Heat shock 70 kDa protein 1L	9	DAGVIAGLNVLR	5.40E-10	IINEPTAAAIAYGLDK	5.40E-11	TTPSYVAFTDTER	5.40E-11

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	HSP76_HUMAN	Heat shock 70 kDa protein 6	5	IINEPTAAAIAYGLDR	4.40E-15	TTPSYVAFTDTER	5.40E-11	FEELCSDLFR	1.80E-07
	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	18	NQVAMNPTNTVFDAK	0.0001	IINEPTAAAIAYGLDK	5.40E-11	DAGTIAGLNVLR	4.60E-08
	HSPB1_HUMAN	Heat shock protein beta-1	7	LATQSNEITIPVTFESR	5.10E-13	VSLDVNHFAPDELTVK	5.20E-09	GPSWDPFR	3.20E-05
	HS90A_HUMAN	Heat shock protein HSP 90-alpha	14	HFSVEGQLEFR	1.30E-09	KHLEINPDHSIIETLR	2.90E-07	GVVDSEDLPLNISR	4.20E-09
	HS90B_HUMAN	Heat shock protein HSP 90-beta	15	HFSVEGQLEFR	1.30E-09	KHSQFIGYPITLYLEK	2.40E-09	GVVDSEDLPLNISR	4.20E-09
	HBA_HUMAN	Hemoglobin subunit alpha	2	TYFPHFDLSHGSAQVK	1.10E-09	VGAHAGEYGAEALER	6.50E-05		
	HBD_HUMAN	Hemoglobin subunit delta	2	LHVDPENFR	0.00032	LLVVYPWTQR	4.50E-06		
	HGS_HUMAN	Hepatocyte growth factor-regulated tyrosine kinase substrate	2	QEYLEVQR	0.0008	ALQNAVTTFVNR	0.00043		
✓	HNRPF_HUMAN	Heterogeneous nuclear ribonucleoprotein F	2	VHIEGPDGR	0.044	QSGEAFVELGSEDDVK	0.00015		
	HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	3	LLIHQSLAGGIIGVK	4.60E-05	TDYNASVSVPDSSGPER	4.80E-09	GSDFDCELR	0.0023
	H15_HUMAN	Histone H1.5	2	KATGPPVSELITK	3.30E-07	ALAAGGYDVEK	2.20E-05		
	H2A1B_HUMAN	Histone H2A type 1-B/E	2	AGLQFPVGR	6.70E-07	VTIAQGGVLPNIQAVLLPK	9.70E-10		
✓	H2AV_HUMAN	Histone H2A.V	2	AGLQFPVGR	6.70E-07	ATIAGGGVIPHIHK	5.60E-10		
	H2B1A_HUMAN	Histone H2B type 1-A	3	VLKQVHPDTGISSK	0.012	QVHPDTGISSK	2.70E-09	LLLPGELAK	1.80E-08
✓	H2B1B_HUMAN	Histone H2B type 1-B	6	VLKQVHPDTGISSK	0.012	KESYSIYVYK	5.50E-08	QVHPDTGISSK	2.70E-09
✓	H2B1C_HUMAN	Histone H2B type 1-C/E/F/G/I	6	VLKQVHPDTGISSK	0.012	QVHPDTGISSK	2.70E-09	AMGIMNSFVNDIFER	5.70E-05
	H33_HUMAN	Histone H3.3	2	EIAQDFKTDLR	9.50E-06	YRPGTVALR	0.00059		
	H4_HUMAN	Histone H4	9	VFLENVIR	9.40E-07	ISGLIYEETR	1.10E-09	TVTAMDVVYALKR	1.00E-09
✓	H4G_HUMAN	Histone H4-like protein type G	3	TVTAMAVVYVLK	0.03	KTVTAMAVVYVLK	1.30E-06	ILGLIYEETR	3.60E-05
	1A24_HUMAN	HLA class I histocompatibility antigen, A-24 alpha chain	13	FIAVGYVDDTQFVR	3.90E-14	GYHQYAYDGKDYIALK	1.60E-12	GYHQYAYDGK	6.30E-06
	1A80_HUMAN	HLA class I histocompatibility antigen, A-80 alpha chain	4	KGGSYSQAASSDSAQGSDVSLTACK	0.0043	DGEDQTQDTELVETRPAGDGTFQK	0.014	SWTAADMAAQITK	2.40E-07
	HLAG_HUMAN	HLA class I histocompatibility antigen, alpha chain G	4	APWVEQEGPEYWEETR	0.0099	WAAVVVPSGEEQR	3.60E-11	FIAMGYVDDTQFVR	0.0027
	1B15_HUMAN	HLA class I histocompatibility antigen, B-15 alpha chain	10	FIAVGYVDDTQFVR	3.90E-14	DGEDQTQDTELVETRPAGDR	0.0083	APWIEQEGPEYWDR	6.20E-11
	1B52_HUMAN	HLA class I histocompatibility antigen, B-52 alpha chain	9	FIAVGYVDDTQFVR	3.90E-14	DGEDQTQDTELVETRPAGDR	0.0083	THVTHHPVSDHEATLR	1.00E-08
	1B54_HUMAN	HLA class I histocompatibility antigen, B-54 alpha chain	8	FIAVGYVDDTQFVR	3.90E-14	WAAVVVPSGEEQR	3.60E-11	APWVEQEGPEYWDR	0.015
	1B59_HUMAN	HLA class I histocompatibility antigen, B-59 alpha chain	8	FIAVGYVDDTQFVR	3.90E-14	DGEDQTQDTELVETRPAGDR	0.0083	APWIEQEGPEYWDR	6.20E-11
	1B08_HUMAN	HLA class I histocompatibility antigen, B-8 alpha chain	5	APWIEQEGPEYWDR	6.20E-11	WAAVVVPSGEEQR	3.60E-11	DGEDQTQDTELVETRPAGDR	0.0083
	1C01_HUMAN	HLA class I histocompatibility antigen, Cw-1 alpha chain	3	APWVEQEGPEYWDR	0.015	THVTHHPVSDHEATLR	1.00E-08	FDSDAASPR	0.0044
	1C12_HUMAN	HLA class I histocompatibility antigen, Cw-12 alpha chain	7	FIAVGYVDDTQFVR	3.90E-14	WAAVVVPSGEEQR	3.60E-11	APWVEQEGPEYWDR	0.015
	1C14_HUMAN	HLA class I histocompatibility antigen, Cw-14 alpha chain	6	FIAVGYVDDTQFVR	3.90E-14	WAAVVVPSGEEQR	3.60E-11	APWVEQEGPEYWDR	0.015
	1C17_HUMAN	HLA class I histocompatibility antigen, Cw-17 alpha chain	6	FIAVGYVDDTQFVR	3.90E-14	APWVEQEGPEYWDR	0.015	YFYTAVSRPGR	0.0013
	1C03_HUMAN	HLA class I histocompatibility antigen, Cw-3 alpha chain	8	GEPHFIAVGYVDDTQFVR	0.00082	MYGCDVGPDGR	2.10E-06	WAAVVVPSGEEQR	3.60E-11
	IGSF8_HUMAN	Immunoglobulin superfamily member 8	8	SRPLPVHVR	0.05	LVAQLDTEGVGSLGPGYEGR	4.90E-13	HAAYSVGWEMAPAGAPGPGR	1.50E-10
	ITA2_HUMAN	Integrin alpha-2	10	VFSIPFHK	0.00082	IPLLYDAEHLTR	5.90E-11	TQVGLIQYANNPR	2.60E-06
	ITA3_HUMAN	Integrin alpha-3	12	ARPVNIVHK	2.20E-05	HMGAVFLLSQEAGGDLR	4.40E-08	VNGWATLFLR	5.70E-05
	ITA6_HUMAN	Integrin alpha-6	10	WNNVKPIR	5.90E-05	AFIDVTAAAENIR	4.40E-06	TAHIDVHFLK	1.90E-06
	ITAV_HUMAN	Integrin alpha-V	5	SHQWFGASVR	1.10E-09	LQEVGQVSVSLQR	7.10E-06	AMLHLQWPYK	0.0014
	ITB1_HUMAN	Integrin beta-1	10	GEVFNELVGK	0.00052	LLVFSTDAGFHFAGDGK	8.30E-13	DKLPQPVQPDPVSHCK	0.0022
	ITB4_HUMAN	Integrin beta-4	25	NVISLTEDVDEFR	4.90E-07	LVFSALGPTSLR	7.80E-09	LCTENLLKPDTR	7.30E-05
	ICAM1_HUMAN	Intercellular adhesion molecule 1	3	VELAPLPSWQPVGK	7.00E-05	DGTFPLPIGESVTVTR	0.00013	TFLTVYWTPER	0.0044
✓	MX1_HUMAN	Interferon-induced GTP-binding protein Mx1	7	DVPDLTLIDLPGITR	0.0076	IIFENHPYFR	0.00062	ALEEPAVDMLHTVTDMVR	0.0095
	IFM1_HUMAN	Interferon-induced transmembrane protein 1	2	MVGDVTGAQAYASTAK	2.90E-17	KMVGDVTGAQAYASTAK	2.50E-13		
✓	SYIC_HUMAN	Isoleucyl-tRNA synthetase, cytoplasmic	2	QLSSEELEQFQK	1.50E-07	YAHQSGFHVDR	0.0045		
	PLAK_HUMAN	Junction plakoglobin	4	LVQNCLWTLR	0.00013	HVAAGTQQPYTDGVR	7.40E-09	TTTYTQGVPPSQGDLEYQMSTTAR	0.0002
	JAM1_HUMAN	Junctional adhesion molecule A	3	LSCAYSGFSSPR	0.00021	VTFLPTGITFK	3.80E-07	KVIYSQPSAR	1.30E-05
	K1C13_HUMAN	Keratin, type I cytoskeletal 13	2	TRLEQEIATYR	0.0032	LEQEIATYR	0.00036		
	K1C14_HUMAN	Keratin, type I cytoskeletal 14	9	TKYETELNLR	0.0017	VLDELTLAR	3.50E-05	TRLEQEIATYR	0.0032
	K1C16_HUMAN	Keratin, type I cytoskeletal 16	5	VLDELTLAR	3.50E-05	TRLEQEIATYR	0.0032	LEQEIATYR	0.00036
	K1C17_HUMAN	Keratin, type I cytoskeletal 17	10	GQVGGENVEMDAAPGVDLSR	0.00017	ILTATVDNANILLQIDNAR	0.0003	VLDELTLAR	3.50E-05

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	K1C18_HUMAN	Keratin, type I cytoskeletal 18	4	IVLQIDNAR	0.011	VKLEAEIATYR	0.01	DWSHYFK	0.0065
	K1C19_HUMAN	Keratin, type I cytoskeletal 19	5	VLDELTLAR	3.50E-05	IVLQIDNAR	0.011	DYSHYYTTIQDLR	3.10E-05
	K2C1_HUMAN	Keratin, type II cytoskeletal 1	3	YEELQITAGR	0.0038	QISNLQQSISDAEQR	6.00E-14	GGGGGGYGSGGSSYGSGGGSYGSGGGGGGGR	4.90E-09
	K2C5_HUMAN	Keratin, type II cytoskeletal 5	6	NLDLDSIIAEVK	9.90E-06	LALDVEIATYR	0.00021	QNLEPLFEQYINNLR	0.011
	K2C6B_HUMAN	Keratin, type II cytoskeletal 6B	10	NLDLDSIIAEVK	9.90E-06	YEELQVTAGR	3.50E-05	ADTLTDEINFLR	1.10E-08
	K2C7_HUMAN	Keratin, type II cytoskeletal 7	3	AKQEELEAALQR	0.024	LPDIFEAQIAGLR	1.80E-07	QEELEAALQR	3.90E-07
	K2C8_HUMAN	Keratin, type II cytoskeletal 8	7	LSELEAALQR	0.012	WSLLQQQK	0.012	LEGLTDEINFLR	1.60E-06
	MFGM_HUMAN	Lactadherin	6	ILPVAWHNR	6.10E-07	IFPGNWDNHSHKK	0.00079	IFPGNWDNHSHK	1.10E-07
✓	LAMA3_HUMAN	Laminin subunit alpha-3	3	LPQELLKPR	0.0016	QISGTDGEGNNVFSGDFSR	2.90E-06	AIPVYLGSPPSGKPK	7.00E-06
✓	LAMB3_HUMAN	Laminin subunit beta-3	7	CQLHYFR	0.0034	VAEVQQVLRPAEK	0.00083	GYHPPSAYYAVSQLR	1.60E-05
✓	LAMC2_HUMAN	Laminin subunit gamma-2	4	HPSAHDVILEGAGLR	9.00E-12	AQGGDGVVPDTELEGR	3.00E-08	LAESHVESASNMEQLTR	0.0014
	LAT1_HUMAN	Large neutral amino acids transporter small subunit 1	2	SADGSAPAGEGEGVTLQR	5.20E-12	ALAAPAAEEKEEAR	3.80E-07		
	LSR_HUMAN	Lipolysis-stimulated lipoprotein receptor	2	AATSGVPSIYAPSTYAHLSPAK	1.40E-07	QGNAVTLGDYYQGR	5.00E-11		
	LDHA_HUMAN	L-lactate dehydrogenase A chain	8	LKGEMMDLQHGSLFLR	0.0027	VHPVSTMIK	0.00024	FIPNVVK	0.0027
	SCRB2_HUMAN	Lysosome membrane protein 2	2	VEEVGPYTYR	1.80E-05	KLDDFVETGDIR	0.0011		
	LAMP2_HUMAN	Lysosome-associated membrane glycoprotein 2	2	GILTVDELLAIR	1.50E-06	IPLNDLFR	1.00E-06		
	MIF_HUMAN	Macrophage migration inhibitory factor	2	PMFIVNTNVPR	1.10E-09	LLQGLLAER	1.50E-05		
	MVP_HUMAN	Major vault protein	16	VPHNAAVQVYDYR	4.90E-12	VSHQAGDHWLIR	1.40E-08	ELELVYAR	0.00083
	MDHM_HUMAN	Malate dehydrogenase, mitochondrial	6	TIIPLISQCTPK	6.80E-07	GYLGPEQLPDCLK	0.0018	GCDVVVIPAGVPR	3.40E-08
✓	SYMC_HUMAN	Methionyl-tRNA synthetase, cytoplasmic	2	QQGVLALRPYLQK	6.20E-05	ALTHIDHSLSR	0.027		
	MOES_HUMAN	Moesin	7	IGFPWSEIR	8.70E-07	APDFVFYAPR	8.60E-09	FVIKPIDKK	4.70E-05
	MOT1_HUMAN	Monocarboxylate transporter 1	3	KDLHDANTDLIGR	0.0031	DLHDANTDLIGR	1.80E-07	ESKEEETSIDVAGKPNEVTK	0.037
	MUC1_HUMAN	Mucin-1	3	DTYHPMSEYPTYHTHGR	2.10E-11	KNYGQLDIFPAR	0.00018	NYGQLDIFPAR	5.90E-05
✓	MPZL2_HUMAN	Myelin protein zero-like protein 2	3	NPPDVDGVIGER	9.60E-09	LSVVHTVR	0.00025	YDASILLWK	0.0001
	MYOF_HUMAN	Myoferlin	7	GKDGSNLPLPPQR	2.10E-06	IPANQLAELWLK	0.021	ILHQHLGAPEER	3.10E-07
	MYL6_HUMAN	Myosin light polypeptide 6	2	NKDQGTYEDYVEGLR	1.40E-05	EAFQLFDR	0.0011		
✓	MRLC2_HUMAN,	Myosin regulatory light chain MRLC2	2	GNFNYIEFTR	0.004	FTDEEVDELYR	6.70E-06		
	MYH10_HUMAN	Myosin-10	4	ADFCIIHYAGK	3.60E-05	LDPHLVLDQLR	0.00014	VKPLLQVTR	0.00083
	MYH11_HUMAN	Myosin-11	3	NWQWWR	0.0038	KEEELQAALAR	1.80E-05	VKPLLQVTR	0.00083
	MYH14_HUMAN	Myosin-14	2	KEEELQAALAR	1.80E-05	VKPLLQVTR	0.00083		
	MYH9_HUMAN	Myosin-9	22	QAQQERDELADEIANSSGK	8.90E-07	IMGIPEEEQMGLLR	1.20E-10	ADFCIIHYAGK	3.60E-05
	MYO1C_HUMAN	Myosin-Ic	3	KRPETVATQFK	0.0003	GEELLSPLNLEQAAYAR	0.015	TSFLLNLR	0.0061
	MARCS_HUMAN	Myristoylated alanine-rich C-kinase substrate	3	GEPAAAAAPEAGASPVEK	9.40E-10	EAPAEGEAAEPGSPTAAEGEAASAASSTSSPK	5.00E-06	GEAAAERPGEAAVASSPSK	5.70E-05
	NHRF1_HUMAN	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	2	AQEAPGQAEPPAAAEVQGAGNENEPR	9.80E-07	KGPSGYGFNLHSDK	0.0069		
	NQO1_HUMAN	NAD(P)H dehydrogenase [quinone] 1	3	FGLSVGHHLGK	1.80E-08	EGHLSPDVAEQK	5.10E-06	ALVLAHSER	0.00032
	NEP_HUMAN	Neprilysin	3	FIMDLVSSLSR	1.70E-06	YACGGWLK	0.006	NSVNHVIHDQPR	6.90E-06
	GANAB_HUMAN	Neutral alpha-glucosidase AB	4	GLLEFEHQR	0.021	MMDYLQGSGETPQTDVR	0.00074	VVIIGAGKPAAVVLQTK	0.0018
	AAAT_HUMAN	Neutral amino acid transporter B(0)	2	NIFPSNLVSAAFR	5.30E-11	EVLDSFLDLAR	2.00E-06		
	NBR1_HUMAN	Next to BRCA1 gene 1 protein	3	GAEGKPGVEAGQEPAEAGER	0.011	RPVVGSSEPFCHSK	0.018	KPLAHYSSLVR	4.00E-10
	NIBL1_HUMAN	Niban-like protein 1	3	VEGPAFTDAIR	0.025	FQELIFEDFAR	2.90E-07	VQQIVQPAMQAVIR	0.00023
	NICA_HUMAN	Nicastrin	2	SGAGVPAVILR	0.0028	LLYGFLIK	2.20E-05		
	NAMPT_HUMAN	Nicotinamide phosphoribosyltransferase	3	AVPEGFVIIR	1.00E-05	GVSSQETAGIGASAHLVNFK	1.10E-07	VYSYFEQR	0.015
	PNCB_HUMAN	Nicotinate phosphoribosyltransferase	2	AAFVAYALAFPR	3.50E-08	LDSGDLLQQAQEIR	0.0054		
	NADC_HUMAN	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	3	GAGWTGHVAGTR	0.00024	YGLLVGGAASHR	7.80E-06	GPAHCLLGER	2.80E-05
	NIT2_HUMAN	Nitrilase homolog 2	3	LALIQLQISSIK	6.10E-06	FAELAQIYAQR	4.20E-05	AVDNQVYVATASPAR	1.30E-07
	NDKA_HUMAN	Nucleoside diphosphate kinase A	3	TFIAIKPDGVQR	2.60E-06	DRPFFAGLVK	0.025	VMLGETNPADSKPGTIR	2.50E-07
	NDKB_HUMAN	Nucleoside diphosphate kinase B	3	TFIAIKPDGVQR	2.60E-06	DRPFFPGLVK	0.00077	VMLGETNPADSKPGTIR	2.50E-07
✓	OPTN_HUMAN	Optineurin	2	QEEDLETMTLR	8.00E-07	QSLMEMQSR	0.0026		
	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	8	EGMNIVEAMER	4.40E-08	FEDENFILK	6.40E-06	VSFELFADK	5.30E-08

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	PRDX1_HUMAN	Peroxiredoxin-1	7	LVQAFQFTDK	9.50E-08	TIAQDYGVLK	0.00081	QITVNDLPVGR	9.20E-08
	PRDX5_HUMAN	Peroxiredoxin-5, mitochondrial	4	LLADPTGAFGK	0.0002	GVLFGVPGAFTPGCSK	0.00039	VNLAELFK	5.50E-06
	PRDX6_HUMAN	Peroxiredoxin-6	2	LSILYPATTGR	0.0011	LPFPIDDR	5.60E-07		
	PGK1_HUMAN	Phosphoglycerate kinase 1	9	VSHVSTGGGASLELLEGK	1.10E-08	ALESPERPFLAILGGAK	3.10E-08	AHSSMVGVNLPQK	2.60E-11
	PGAM1_HUMAN	Phosphoglycerate mutase 1	3	HYGGLTGLNK	0.00015	HGESAWNLENR	0.00039	VLIAAHGNSLR	4.30E-05
✓	PKP3_HUMAN	Plakophilin-3	2	GQYHTLQAGFSSR	9.30E-09	LFNHANQEVQR	0.0056		
✓	AT2B1_HUMAN	Plasma membrane calcium-transporting ATPase 1	2	SSHNFMTHPEFR	0.00076	QVVAVTGDGTNDGPALK	2.20E-07		
✓	AT2B4_HUMAN	Plasma membrane calcium-transporting ATPase 4	2	QVVAVTGDGTNDGPALK	2.20E-07	AFHSSLHESIQKPYNQK	0.0014		
	IC1_HUMAN	Plasma protease C1 inhibitor	2	FQPTLLTLPR	0.0029	GVTSVSQIFHSPDLAIR	0.00071		
	PLEC1_HUMAN	Plectin-1	12	HKPLLIDMNK	4.80E-05	QEELYSELQAR	0.0001	LHVAILER	0.013
	PCBP1_HUMAN	Poly(rC)-binding protein 1	2	ESTGAQVQVAGDMLPNSTER	6.50E-07	IITLTGPTNAIFK	9.00E-05		
✓	PTRF_HUMAN	Polymerase I and transcript release factor	3	QAEMEGAVQSIQGELSK	0.0053	VPRFTFHVK	0.00013	KSFTPDHVVYAR	1.30E-05
	AT1A_HUMAN	Potassium-transporting ATPase alpha chain 1	2	LIFDNLKK	0.00025	VDNSSLTGESEPQTR	0.00061		
	SAP_HUMAN	Proactivator polypeptide	2	KLVGYLDR	0.0045	GCSFLPDPYQK	0.0036		
	PLOD1_HUMAN	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	2	LQLNYLGNYIPR	7.90E-05	LTHYHEGLPTTR	0.0014		
	PROF1_HUMAN	Profilin-1	5	DSLLQDGEFSMDLR	8.80E-08	CYEMASHLR	0.015	TFVNITPAEVGVLVGK	1.40E-09
	PDC6I_HUMAN	Programmed cell death 6-interacting protein	13	HCIMQANAEYHQSLAK	1.60E-08	LANQAADYFGDAFK	6.20E-06	FIQQTYPSGGEEQAQYCR	2.30E-07
	PDCD6_HUMAN	Programmed cell death protein 6	5	LSDQFHDLIR	3.10E-09	QALSGFGYR	1.10E-07	SIISMFDR	4.60E-05
	PROM2_HUMAN	Prominin-2	3	APGLLDSLYGTVR	0.02	RDLEALQSSGLQR	0.036	FLGPAEHLTFTPAAR	0.0033
	PRP4_HUMAN	Prostaglandin F2 receptor negative regulator	11	FTVSWYYR	0.00018	CSTPSTDATVQGNYEDTVQVK	8.80E-05	EHTDTFNFR	8.20E-06
✓	PSB4_HUMAN	Proteasome subunit beta type-4	2	QPVLSQTEAR	0.00028	AIHSWLTR	0.0047		
	PDIA1_HUMAN	Protein disulfide-isomerase	3	VDATEESDLAQQYGVR	9.10E-10	THLLFLPK	6.30E-05	ILEFFGLK	4.00E-05
	PDIA3_HUMAN	Protein disulfide-isomerase A3	6	MDATANDVPSPYEVR	4.00E-05	FVMQEEFSR	0.00014	ELSDFISYLQR	3.10E-07
	PDIA4_HUMAN	Protein disulfide-isomerase A4	3	RSPPPLAK	0.0072	YALPLVGHR	0.0031	GESDPAYQQYQDAANNLR	0.027
	PACN3_HUMAN	Protein kinase C and casein kinase substrate in neurons protein 3	5	AQYEQTLAELHR	0.0022	DLHQGIEAASDEEDLR	2.10E-07	LSALHLEVR	0.00069
	NDRG1_HUMAN	Protein NDRG1	4	GNRPVILTYHDIGMNHK	0.0001	TASGSSVTSLDGTR	4.20E-08	SIIGMGTGAGAYILTR	1.40E-15
	PP1R7_HUMAN	Protein phosphatase 1 regulatory subunit 7	2	QIDATFVR	0.0011	KVMLALPSVR	0.0071		
✓	RCC2_HUMAN	Protein RCC2	5	TKDGQILPVPNVVVR	0.00016	IEYDCELVPR	0.00078	VPSWGFGGYGR	2.10E-08
	S10AA_HUMAN	Protein S100-A10	2	EFPGFLENQKDPLAVDK	3.90E-06	PSQMEHAMETMMFTFHK	7.30E-12		
	S10AE_HUMAN	Protein S100-A14	4	SFWELIGEAAK	7.60E-05	SANAEDAQEFSDVER	3.90E-13	NFHQYSVEGGK	0.0027
	S10AG_HUMAN	Protein S100-A16	2	LIQNLDANHDGR	1.10E-05	AVIVLVENFYK	1.20E-06		
	S10A8_HUMAN	Protein S100-A8	2	GNFHAVYR	3.40E-05	LLETECPQYIR	0.018		
	S10A9_HUMAN	Protein S100-A9	2	NIETINTFHQYSVK	3.20E-09	LGHPDTLNQGEFK	3.20E-08		
✓	H2B2C_HUMAN_m	Putative histone H2B type 2-C	2	KESYSIYVYK	5.50E-08	ESYSIYVYK	2.40E-07		
	TBA4B_HUMAN	Putative tubulin-like protein alpha-4B	2	QIFHPEQLITGK	7.20E-10	LISQIVSSITASLR	1.60E-10		
	KPYM_HUMAN	Pyruvate kinase isozymes M1/M2	15	EAEAAIYHLQLFEELR	7.00E-11	CDENILWLDYK	0.0033	NTGICTIGPASR	2.20E-08
	GDIB_HUMAN	Rab GDP dissociation inhibitor beta	2	FKIPGSPPESMGR	3.60E-08	DLGTESQIFISR	0.012		
	RADI_HUMAN	Radixin	9	IGFPWSEIR	8.70E-07	APDFVFYAPR	8.60E-09	FVIKPIDKK	4.70E-05
	RAC1_HUMAN	Ras-related C3 botulinum toxin substrate 1	4	KLTPTYPQGLAMAK	8.50E-11	TVFDEAIR	2.80E-05	LTPTYPQGLAMAK	0.0054
	RAB10_HUMAN	Ras-related protein Rab-10	3	LQIWDTAGQER	1.30E-06	FHTITTSYYR	9.50E-08	AFLTLAEDLR	2.80E-06
	RAB1B_HUMAN	Ras-related protein Rab-1B	4	MGPGAASGGERPNLK	0.042	QWLQEDR	3.90E-05	LQIWDTAGQER	1.30E-06
	RAB5A_HUMAN	Ras-related protein Rab-5A	2	YHSLAPMYYR	7.50E-08	GAQAAIVVYDITNEESFAR	0.0024		
	RAB5C_HUMAN	Ras-related protein Rab-5C	3	YHSLAPMYYR	7.50E-08	GVDLQENNPASR	0.028	GAQAAIVVYDITNTDTFAR	0.017
	RAB6A_HUMAN	Ras-related protein Rab-6A	2	SLIPSYIR	0.036	LQLWDTAGQER	1.30E-06		
	RAB7A_HUMAN	Ras-related protein Rab-7a	8	QETEVELYNEFPEPIK	7.30E-05	DPENFPFVVLGNK	3.00E-07	DEFLIQASPR	0.045
	RRAS_HUMAN	Ras-related protein R-Ras	3	YQEQELPPSPPSAPR	0.012	LNVDEAFEQLVR	0.0057	KYQEQELPPSPPSAPR	0.0024
	PTPRF_HUMAN	Receptor-type tyrosine-protein phosphatase F	3	LVNIMPYELTR	0.016	TQQGVPAQPADFQAEVESDTR	0.00035	CHQYWPAER	0.02
✓	RTN4_HUMAN	Reticulon-4	2	HQAQIDHYLGLANK	1.00E-09	GPLPAAPPVAPER	1.30E-05		
	RAI3_HUMAN	Retinoic acid-induced protein 3	3	TNVNVFSELSAPR	0.00061	AHAWPSPYK	6.00E-05	AHAWPSPYKDYEVK	7.10E-08

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
	RHG01_HUMAN	Rho GTPase-activating protein 1	3	NPEQEPIPIVLR	0.00059	MPPSHQLDHSK	0.0054	FLTAFLVQISAHSDQNK	6.20E-07
	RHOG_HUMAN	Rho-related GTP-binding protein RhoG	3	LKEQGQAPITPQQGQALAK	0.00011	AVLNPTPIKR	0.0014	EQGQAPITPQQGQALAK	0.015
✓	RNAS7_HUMAN	Ribonuclease 7	2	DSQQFHLVPVHLDR	0.00012	SYVVACKPPQK	0.00047		
	SQSTM_HUMAN	Sequestosome-1	2	DHRPPCAQEAPR	0.0088	EAALYPHLPPEADPR	0.00011		
	MRCKA_HUMAN	Serine/threonine-protein kinase MRCK alpha	2	DFDGEDSDSPR	0.049	EEIEKLR	3.50E-05		
	PP1B_HUMAN	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	4	IYGFYDECK	0.0076	IKYPENFFLLR	4.60E-06	YPENFFLLR	0.0066
	ALBU_HUMAN	Serum albumin	2	YLYEIAR	0.0034	KVPQVSTPTLVEVSR	3.20E-11		
	SPRR3_HUMAN	Small proline-rich protein 3	2	QTFTPPPQLQQQQVK	6.90E-06	QPSQPPPQEIFVPTTK	9.40E-05		
	AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1	13	LNIPVSQVNPR	2.10E-07	AVFQANQENLPLK	4.70E-07	TSATWLALSR	1.10E-05
	AT1B1_HUMAN	Sodium/potassium-transporting ATPase subunit beta-1	2	SYEAYVLNIVR	2.40E-05	VAPPGLTQIPQIQK	6.60E-05		
✓	AT1B3_HUMAN	Sodium/potassium-transporting ATPase subunit beta-3	3	YFPVYGK	0.00017	IGLKPEGVPR	5.20E-07	LFIYNPTTGEFLGR	3.20E-05
✓	SC5A6_HUMAN	Sodium-dependent multivitamin transporter	2	SYGQDHLDTGLFPEKPR	2.00E-05	LLSLLPLSCQK	0.017		
	GTR1_HUMAN	Solute carrier family 2, facilitated glucose transporter member 1	6	VTILELFR	7.90E-06	GTADVTHDLQEMK	7.00E-07	KVTILELFR	2.30E-07
✓	SNX33_HUMAN	Sorting nexin-33	2	QQILFYQR	0.00051	LTPTHAASPVYR	2.10E-07		
✓	UAP56_HUMAN	Spliceosome RNA helicase BAT1	6	GSYVSIHSSGFR	7.30E-07	DFLLKPELLR	0.0024	VAVFFGGLSIK	2.50E-06
	SRC8_HUMAN	Src substrate cortactin	3	TQTPPVSPAPQPTEER	1.00E-05	GPVSGTEPEPVYSMEAADYR	0.0096	TVQGSGHQEHNIHK	8.50E-06
	STIP1_HUMAN	Stress-induced-phosphoprotein 1	3	LMDVGLIAIR	0.0032	LDPHNHVLYSNR	2.60E-07	YKDAIHFYNK	0.00019
	VAT1_HUMAN	Synaptic vesicle membrane protein VAT-1 homolog	2	VVTYGMANLLTGPK	0.00071	GYNLLKPMGK	0.0084		
	SDC1_HUMAN	Syndecan-1	2	NQSPVDQGATGASQGLLDR	0.02	EGEAVVLPEVEPGLTAR	1.00E-06		
✓	STX4_HUMAN	Syntaxin-4	2	VALVVHPGTAR	0.00031	QALNEISAR	0.02		
	SDCB2_HUMAN	Syntenin-2	2	FGDQLLQIDGR	1.70E-06	RAEIKPGVR	0.0061		
	TCPA_HUMAN	T-complex protein 1 subunit alpha	2	AFHNEAQVNPER	3.00E-08	IHPTSVISGYR	2.40E-06		
	TCPB_HUMAN	T-complex protein 1 subunit beta	5	KIHPQTIIAGWR	4.00E-07	IHPQTIIAGWR	1.20E-06	GATQQILDEAER	0.0017
	TCPD_HUMAN	T-complex protein 1 subunit delta	4	ALIAGGGAPEIELALR	0.00043	GIHPTIISESFQK	0.0002	QMQVLHPAAR	0.00051
	TCPE_HUMAN	T-complex protein 1 subunit epsilon	4	GVIVDKDFSHPQMPK	5.40E-05	QQISLATQMVR	2.90E-05	SLHDALCVIR	0.00089
	TCPH_HUMAN	T-complex protein 1 subunit eta	3	QQLLIGAYAK	1.90E-07	QVKPYVEEGLHPQIIR	0.0029	GGAEQFMEETER	0.0003
	TCPG_HUMAN	T-complex protein 1 subunit gamma	5	IPGGIEDSCVLR	0.0019	AVAQALEVIPR	2.70E-06	GISDLAQHYLMR	1.30E-05
	TCPQ_HUMAN	T-complex protein 1 subunit theta	2	QYGNEVFLAK	0.00013	FAEAFEAIPR	0.0021		
	THIO_HUMAN	Thioredoxin	3	MKPFFHSLSEK	2.80E-08	TAFQEALDAAGDK	0.0001	CMPTFQFFK	2.20E-06
	ERP44_HUMAN	Endoplasmic reticulum resident protein ERp44	2	TPADCPVIAIDSFR	0.0082	HPLLHIQK	0.00024		
	SYTC_HUMAN	Threonyl-tRNA synthetase, cytoplasmic	2	QLENSLNEFGEK	0.0016	LADFGVLHR	0.00033		
	TSP1_HUMAN	Thrombospondin-1	3	FVFGTTPEDLR	0.00047	NALWHTGNTPGQVR	1.40E-05	GFLLLASLR	0.0033
	TOLIP_HUMAN	Toll-interacting protein	2	AIQDMFPNMDQEVIR	0.0017	GPVYIGELPQDFLR	1.70E-06		
	RHOA_HUMAN	Transforming protein RhoA	3	TCLLIVFSK	0.0029	EVFEMATR	0.0027	QVELALWDTAGQEDYDR	3.30E-17
	TAGL2_HUMAN	Transgelin-2	3	QMEQISQFLQAAER	3.90E-05	GASQAGMTGYGMPR	0.013	TLMNLGGLAVAR	6.00E-08
	TERA_HUMAN	Transitional endoplasmic reticulum ATPase	3	QTNPSAMEVEEDDPVPEIR	3.30E-11	GVLFYGPPGCGK	5.00E-05	WALSQSNPSALR	6.30E-08
	TKT_HUMAN	Transketolase	3	ESWHGKPLPK	0.0026	VLDPFTKPLDR	0.0034	GITGVEDKESWHGKPLPK	2.40E-07
	TMBI1_HUMAN	Transmembrane BAX inhibitor motif-containing protein 1	2	AVSDSFGPGEWDDRK	0.029	AVSDSFGPGEWDDR	3.80E-10		
	TPIS_HUMAN	Triosephosphate isomerase	8	RHVFGESDELIGQK	0.0064	KFFVGGNWK	3.20E-06	SNVSDAVAQSTR	2.00E-09
	TPP1_HUMAN	Tripeptidyl-peptidase 1	2	LFGGNFAHQASVAR	2.60E-08	LYQQHGAGLFDVTR	3.00E-10		
✓	TPBG_HUMAN	Trophoblast glycoprotein	2	AGAFEHLPSLR	0.0025	DVLAQLPSLR	0.0022		
	TBA1B_HUMAN	Tubulin alpha-1B chain	13	AFVHWYVGEGMEEGEFSEAR	1.30E-05	QLFHPEQLITGK	7.20E-10	AVFVDLEPTVIDEVR	1.90E-12
	TBA1C_HUMAN	Tubulin alpha-1C chain	13	AFVHWYVGEGMEEGEFSEAR	1.30E-05	QLFHPEQLITGK	7.20E-10	AVCMLSNTTAVAEAWAR	0.012
	TBA4A_HUMAN	Tubulin alpha-4A chain	12	AFVHWYVGEGMEEGEFSEAR	1.30E-05	QLFHPEQLITGK	7.20E-10	AVFVDLEPTVIDEIR	0.00011
	TBB5_HUMAN	Tubulin beta chain	14	AILVDLEPGTMDSVR	9.40E-10	ISVYYNEATGGK	2.00E-10	ISEQFTAMFR	1.00E-05
	TBB2A_HUMAN	Tubulin beta-2A chain	10	AILVDLEPGTMDSVR	9.40E-10	ISEQFTAMFR	1.00E-05	EVHIQAGQCGNQIGAK	3.00E-15
	TBB2C_HUMAN	Tubulin beta-2C chain	14	ALTVPELTQQMFDAK	2.20E-07	ISEQFTAMFR	1.00E-05	YLTVAAVFR	3.30E-07
	TBB3_HUMAN	Tubulin beta-3 chain	11	ALTVPELTQQMFDAK	2.20E-07	AILVDLEPGTMDSVR	9.40E-10	ISEQFTAMFR	1.00E-05
	TACD2_HUMAN	Tumor-associated calcium signal transducer 2	8	FVAAVHYEQPTIQIELR	9.60E-10	THHLIDLR	1.50E-07	GEPLQVER	0.00033

Table 5.1: continued

Novel ID	Accession Number	Protein Name	Peptide Count	Peptide 1	E-value 1	Peptide 2	E-value 2	Peptide 3	E-value 3
✓	U520_HUMAN	U5 small nuclear ribonucleoprotein 200 kDa helicase	5	QLPHFTSEHK	0.0042	TNLLLQAHLSR	0.0036	MTQNPNYYNLQGISHR	0.0026
	UBIQ_HUMAN	Ubiquitin	5	TLSDYNIQK	9.80E-05	EGIPPDQQR	3.70E-05	TITLEVEPSDTIENVK	3.40E-12
	UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme 1	7	LAGTQPLEVLEAVQR	9.50E-10	QPAENVNQYLTDPK	6.30E-10	LQTSSVLVSGLR	0.0088
✓	UROK_HUMAN	Urokinase-type plasminogen activator	2	VSHPLPWR	6.40E-06	MTLTGVSWGR	1.30E-05		
	VPS28_HUMAN	Vacuolar protein sorting-associated protein 28 homolog	2	QVQGSESSIDEFCR	5.20E-08	MSHLPPDFEGR	1.80E-05		
	VPS4B_HUMAN	Vacuolar protein sorting-associated protein 4B	3	IYIPLPEPHAR	0.043	GILLFGPPGTGK	0.0085	FPHLFTGK	0.0013
	VASP_HUMAN	Vasodilator-stimulated phosphoprotein	2	YNQATPNFHQWR	3.60E-05	VQIYHNPTANSFR	0.019		
✓	VAPA_HUMAN	Vesicle-associated membrane protein-associated protein A	2	VAHSDKPGSTSTASFR	5.30E-05	KVAHSDKPGSTSTASFR	0.0046		
	VATA_HUMAN	V-type proton ATPase catalytic subunit A	5	HFTEFVPLR	0.00012	VGHSELVGEIR	0.0017	FTMVQVWPVR	0.00016
	VATG1_HUMAN	V-type proton ATPase subunit G 1	2	EEAQAEEQYR	0.00071	MTLQTYFR	0.0034		
✓	WBP2_HUMAN	WW domain-binding protein 2	2	QPVFGANYIK	0.0031	KGTVYLTPYR	3.70E-06		

Some of the proteins identified could equally have been different isoforms, based on the set of peptides identified, these are indicated below

[a] - ACTS_HUMAN
[b] - ACTG_HUMAN
[c] - ARF3_HUMAN
[d] - AXA82_HUMAN
[e] - EF1A3_HUMAN
[f] - GGEE3_HUMAN
[g] - GNAS2_HUMAN
[h] - H2A1C_HUMAN; H2A1D_HUMAN; H2A1H_HUMAN; H2A1J_HUMAN; H2A1_HUMAN; H2A2A_HUMAN; H2AC_HUMAN; H2A3_HUMAN; H2AJ_HUMAN
[i] - H2A2_HUMAN
[j] - H2B1J_HUMAN; H2B1O_HUMAN; H2B2E_HUMAN; H2B3B_HUMAN
[k] - H2B1D_HUMAN; H2B1H_HUMAN; H2B1K_HUMAN; H2B1L_HUMAN; H2B1M_HUMAN; H2B1N_HUMAN; H2B2F_HUMAN; H2BFS_HUMAN
[l] - MRLC3_HUMAN
[m] - H2B2D_HUMAN

5.5 *DTT is required for effective exosome solubilisation*

In order to obtain exosome-derived trypsin digest peptides for nano-LC we first used a protocol encompassing a 1% (w/v) SDS extraction, normally sufficient to solubilise membrane proteins [195]. Unfortunately initial attempts with this standard protocol yielded very low numbers of protein identifications. Only three proteins with multiple peptide assignments were identified even though we performed the experiment several times. Cultured cell lysates commonly processed in the laboratory generally yield 300-500 protein identifications using this method and this has been achieved for a number of different samples (I.A. Brewis, personal communication) and see [169] for an example. This highlighted significant issues with the exosome protein solubilisation possibly caused by their partially detergent resistant membrane or due to the high levels of disulphide cross-links, which are a property of exosomes [87, 196]. Comparisons of the effectiveness of different solubilising buffers using 1DE have been made by members of our group and demonstrated that using SDS alone produced poorer solubilisation than using SDS and DTT combined. The protocol (section 2.15.1) was therefore modified to include 20mM DTT, 1% SDS and heating at 95°C to achieve greater solubilisation efficiency of the exosomes and yielded more than 100 times more protein identifications.

5.6 *Preliminary observations regarding the identified proteome*

Manual assessment of the 353 protein identifications revealed a number of proteins consistent with exosome biosynthesis. For example the list included members of the endosomal sorting complex required for transport (ESCRT) family, including vacuolar protein sorting-associated protein 28 homolog (vps-28), vacuolar protein sorting-associated protein (vps-4B), ubiquitin-like modifier-activating enzyme, and ubiquitin. This indicated that the sample analysed was of multivesicular body (MVB) origin.

Membrane trafficking and proteins involved in fusion processes were also identified (Clathrin heavy chain 1, Rab-11B, Rab-5A, Rab-6a, Rab-7a, Rab GDP dissociation

inhibitor beta, Annexin A1, A2, A3, A4, A5, A6, A7, Annexin A8-like protein and Annexin A11).

Markers of endosomes and lysosomes were also present (EH domain-containing protein 1 and 2, Lysosome membrane protein 2, Lysosome associated membrane protein-2, tripeptidylpeptidase 1, Cathepsin-D, Sequestosome-1). Furthermore several proteins with chaperone functions were identified (HSP70, hsc70, HSP90, stress-induced-phosphoprotein 1, T-complex protein 1, endoplasmin).

Due to the way in which the exosomes are formed it is expected that the exosome lumen contains components of the cytosol. An assortment of cytosolic enzymes (Glyceraldehyde-3-phosphate dehydrogenase, cytosol aminopeptidase, cytosolic acetyl-CoA acetyltransferase, nicotinate phosphoribosyltransferase) and cytoskeletal constituents (actin, Alpha-actinin-4, cytokeratins, ezrin, tubulin, myosin) were identified.

Diverse transmembrane proteins were also abundant in the list, including multiple integrins (β1, β4, α3, α6, αV), MHC molecules, tetraspanins, EGF-R, Mucin-1, CD44, syndecan-1 and various membrane transporters such as solute carrier family 2 and 3, 4F2 cell-surface antigen heavy chain, Choline transporter-like protein, Sodium/potassium-transporting ATPase subunit beta-3. The proteome identified here is broadly consistent with that expected for exosomes and comparable to proteomic identifications made by other exosome researchers [141].

The dataset was manually compared with the exosome proteome database ExoCarta identifying 63 proteins (highlighted in Table 5.1) not previously identified in exosome proteome studies, present in the ExoCarta database (accessed 15th February 2010). The comparisons were made using corresponding EntrezGene identifications for associated protein encoding genes. Amongst the unique proteins identified were three subunits of the complex glycoprotein laminin (Laminin subunit α-3, β-3 and γ-2), which is thought to be involved in cell attachment. Various membrane proteins were also newly identified as exosomal, including CD70, which is involved in T-cell activation. A known tumour

associated protein trophoblast glycoprotein (5T4) was also identified. 5T4 has not been previously identified by proteomics means, but has been confirmed to be expressed by prostate cancer patient urine exosomes by immunoblotting by our group [36]. Two specifically vesicle related proteins were also identified, Syntaxin-4 and Vesicle-associated membrane protein-associated protein A (VAMP-A). Syntaxin proteins are thought to play a role in the docking of vesicles and VAMP-A is thought to be involved in vesicle trafficking and fusion.

To further evaluate the dataset to identify any potential bladder cancer markers, PubMed searches were performed for each of the proteins in the dataset plus the term "cancer" and/or "bladder cancer". This helped identify numerous proteins that may be involved in the pathology of bladder cancer including β-catenin, galectin-3 and -1, cathepsin D, CD44, CD70, epidermal growth factor receptor, hnRNP K, cytokeratins (CK) 17, 18, and 19, Nicotinamide phosphoribosyltransferase, peroxiredoxin V, neprilysin, basigin, urokinase-type plasminogen activator and more. It is evident that many of the proteins identified are associated with cancer and therefore may be potential tumour markers requiring further investigation.

Overall the results demonstrate a high quality dataset in both sample and MS data quality and amongst the highest number of protein identifications from an exosome sample. The dataset is broadly consistent with that of other exosome proteome studies and furthermore shows the potential to help identify novel BCa markers and proteins of importance in exosome biogenesis and function.

5.7 *Anomalous MHC class I identifications*

During manual inspection of the proteomics dataset some unexpected MS identifications occurred, it was therefore important to question the validity of these anomalies. In the current study the LC-MALDI MS data contained multiple identifications for HLA molecules. These identifications all passed our quality criteria (Expect values <0.05 and identifications based on more than one peptide). However the number of HLA

identifications was higher than physiologically possible as they included five HLA-B alleles and five HLA-C alleles (Table 5.2) from one homogenous cell source.

This anomaly could have been caused by contamination of the source cell line with other cells from different donor(s), inadvertent contamination of the sample by researchers, or issues related to how MASCOT designated HLA haplotype nomenclature based on peptide sequences generated from MS. In order to investigate these possibilities PCR-based MHC haplotype analysis was carried out of the researcher and HT1376 cell line, by a clinical diagnostic service (Welsh Blood Service, Llantrisant, Wales, UK). The results of which showed the researcher to have no alleles that corresponded to those in the MS list, whereas the HT1376 cells were haplotyped as HLA-A*24; -B*15(62); Cw*03(9), confirming it as a homogenous cell line.

Subsequently the peptide sequences obtained by MS were examined in more detail to evaluate how they were assigned by MASCOT to give HLA nomenclature (Table 5.2). From this analysis it was clear to see several peptide sequences had been assigned to multiple HLA types. For example, sequence FDSDAASPR was designated to HLA-B15, -52, -54, and -59 and to HLA-C01, -C12, -C17, and –C03. However there were also some peptides that were only assigned to a single designation. These unique sequences were assigned to HLA-A24 (APWIEQEGPEYWDEETGK, AYLEGTCVDGLR, and WEAAHVAEQQR), HLA-G (APWVEQEGPEYWEEETR, FIAMGYVDDTQFVR, and THVTHHPVFDYEATLR) and HLA-C03 (GEPHFIAVGYVDDTQFVR). There were no unique peptides for any HLA-B allele, although of the HLA-B subtypes identified HLA-B15 was assigned the greatest number of peptides. This highlighted the need for manual analysis of peptides designated as MHC Class I identifications to clarify any potential confusion arising from such MASCOT results.

The confusion with the MHC class I peptide designations brought into question the designations of peptides to other groups of proteins. Therefore some of these groups were also manually examined including 14-3-3 proteins, CKs, heat shock proteins (HSP) and histones. Of the 14-3-3 proteins only one (14-3-3ε) out of six had no unique peptides

designated to it and hence casts some doubt on the validity of this identification. Again for the cytokeratins, only one (CK13) out of the 11 CK designations had no unique peptides. All seven of the HSP proteins had unique peptides designated to them whereas only eight out of the 12 histone designations (Table 5.3) had unique peptides assigned. This manual analysis of the data, to examine how MASCOT assigns peptide sequences to protein identifications, highlights potential issues with proteins that exhibit certain levels of homology. This is a known issue in the field of proteomics, but is normally not addressed. Manual analysis of homologous proteins in this manner is therefore recommended for gaining full confidence in the MS/MS-derived identifications made.

Table 5.2: Examination of MASCOT-designated MHC Class-I identifications, highlighting the assignment of peptide sequences to more than one protein identification

MASCOT Designated HLA-identifications													
A24[b]	A80	B15[b]	B52	B54	B59	B08	C01	C12	C14	C17	C03[b]	HLA-G[c]	Peptide Sequences assigned a HLA-designation
■		■		■	■			■	■	■			FIAVGYVDDTQFVR
				■			■			■			APWVEQEGPEYWDR
												■	**APWVEQEGPEYWEEETR[a]**
■													**APWIEQEGPEYWDEETGK[a]**
		■	■		■	■		■	■		■		APWVEQEGPEYWDR
■													**AYLEGTCVDGLR[a]**
		■	■								■		AYLEGLCVEWLR
		■	■	■	■	■							DGEDQTQDTELVETRPAGDR
■	■							■		■			DGEDQTQDTELVETRPAGDGTFQK
		■	■	■	■	■	■	■	■	■	■		FDSDAASPR
												■	**FIAMGYVDDTQFVR[a]**
											■		**GEPHFIAVGYVDDTQFVR[a]**
		■	■	■	■								GGSYSQAASSDSAQGSDVSLTA
■													GYHQYAYDGKDYIALK
■													GYHQYAYDGK
■	■												KGGSYSQAASSDSAQGSDVSLTACK
■													KWEAAHVAEQQR
		■									■		MYGCDVGPDGR
■	■												SWTAADMAAQITK
			■				■	■	■	■	■		THVTHHPVSDHEATLR
												■	**THVTHHPVFDYEATLR[a]**
		■		■	■	■							THVTHHPISDHEATLR
■	■												THMTHHPISDHEATLR
■		■	■	■	■	■		■	■		■	■	WAAVVVPSGEEQR
								■		■	■		YFYTAVSRPGR
■									■				YFSTSVSRPGR
		■	■	■	■								YFYTAMSRPGR
■													**WEAAHVAEQQR[a]**

a. Peptide sequences highlighted in bold, represent those assigned to a single HLA identification

b. Indicates HLA-Haplotype of HT1376 cells by PCR

c. Positive expression confirmed by immunoblotting

Table 5.3: Examination of MASCOT-designated Histone identifications, highlighting the assignment of peptide sequences to more than one protein identification

MASCOT Designated Histone identifications												
H15	H2A1B	H2AV	H2AW	H2AY	H2B1A	H2B1B	H2B1C	H2B2C	H33	H4	H4G	Peptide Sequences assigned a Histone designation
	■	■										AGLQFPVGR
			■	■								AGVIFPVGR
■												**ALAAGGYDVEK**[a]
						■	■					AMGIMNSFVNDIFER
		■										**ATIAGGGVIPHIHK**[a]
										■		**DNIQGITKPAIRR**[a]
										■		**DNIQGITKPAIR**[a]
										■		**DAVTYTEHAK**[a]
									■			**EIAQDFKTDLR**[a]
						■		■				ESYSIYVYK
							■					**ESYSVYVYK**[a]
				■								**EFVEAVLELR**[a]
				■								**GKLEAIITPPPAK**[a]
			■	■								HILLAVANDEELNQLLK
											■	**ILGLIYEETR**[a]
										■		**ISGLIYEETR**[a]
■												**KATGPPVSELITK**[a]
											■	**KTVTAMAVVYVLK**[a]
										■		**KTVTAMDVVYALK**[a]
							■					**KESYSVYVYK**[a]
						■		■				KESYSIYVYK
					■	■	■					LLLPGELAK
					■	■	■					QVHPDTGISSK
										■		**RISGLIYEETR**[a]
				■								**SIAFPSIGSGR**[a]
											■	**TVTAMAVVYVLK**[a]
										■		**TVTAMDVVYALKR**[a]
										■		**TVTAMDVVYALK**[a]
					■	■	■					VLKQVHPDTGISSK
										■		**VFLENVIR**[a]
	■											**VTIAQGGVLPNIQAVLLPK**[a]
									■			**YRPGTVALR**[a]

a. Peptide sequences highlighted in bold, represent those assigned to a single histone identification

5.8 *Discussion*

The aims of this chapter were to establish a proteomics workflow for obtaining high quality protein identifications from exosomes using an LC-MS/MS approach. In Chapter 3 it was established that exosome purification utilising a sucrose cushion was sufficient to yield good quantities of high quality exosomes. However, it was apparent that exosome samples could not be prepared for proteomic analysis by LC-MS/MS in the standard way used for whole cell lysates (1% NP40) [169]. Exosomes are difficult samples to work with as they are composed of a cholesterol and sphingolipid rich membrane which is partially detergent resistant. This is similar to that of plasma membrane microdomains lipid rafts and caveolae and unlike that of the overall plasma membrane [87, 193]. These require stronger conditions, either 1% NP40 or 1% SDS and heating, to disrupt the membrane. In fact common detergents such as Triton X-100 are used to specifically isolate these detergent resistant microdomains [193]. The use of the strong reducing agent DTT was required to disrupt the high levels of disulphide cross-links in exosomes. The DTT, SDS and boiling provided vastly more effective exosome solubilisation compared with SDS alone. SDS is however normally sufficient for membrane protein solubilisation [195].

The 2DE work performed also highlighted probable issues with exosomal membrane proteins. Even on a preparative gel loaded with 500 µg of protein only approximately 50% of the protein spots picked yielded high quality protein identifications. These unidentified proteins may be hydrophobic membrane proteins which are difficult to identify due to under-representation of transmembrane domains. Furthermore, the incompatibility of ionic detergents such as SDS with IEF prevents the effective solubilisation of hydrophobic membrane proteins [195]. The inefficiency of the 2DE workflow led us to explore gel-free methods of identifying exosomal proteins. The subsequent LC-MALDI MS workflow identified 353 proteins. This is amongst the highest number exosomal proteins identified in a single study [141]. Furthermore, the data presented are also of the highest quality in the exosome field.

Along with the numerous known exosomal proteins identified, membrane and cytosolic, other proteins not thought to be expressed by exosomes were also identified including proteins known to be associated with organelles such as the ER, mitochondria and nucleus. Although samples were checked for the degree of contamination by immunoblotting for known ER proteins gp96 and calnexin, it is possible that the levels of these and other unexpected proteins are below the detection limits of the immunoblotting technique.

Generally in the exosome field it is accepted that exosomes contain minimal proteins from these different cellular compartments but it is not entirely known to what degree this rule is true. Furthermore, our dataset is not the first to identify proteins from cellular compartments thought to be under represented in exosomes. For example nuclear proteins such as histones have been identified in numerous studies [56, 61, 99, 101, 106, 111] including studies using highly purified immunoisolated exosomes [96]. The mitochondrial/nuclear protein peroxiredoxin has also been identified in numerous studies [56, 62, 98, 99, 101]. It is not known whether these proteins are specifically loaded into the exosomes or alternatively taken up from the microenvironment into the endosomal system and packaged into exosomes [197]. Given the interrelationship between exosome production and the degradation machinery of the cell it may be that proteins from diverse cellular compartments do at some point encounter multivesicular body (MVB) compartments. As a consequence a proportion of these proteins may be secreted in exosomes instead of entering the lysosome. In addition, cancer cells have many poorly understood cellular alterations which may modify the normal trafficking of some proteins. For example, the protein hnRNPK identified in the current study usually located in the nucleus may locate to the cytoplasm in certain cancers changing its normal distribution [198, 199]. These unexpected proteins may therefore be genuinely expressed in exosomes and it would be of interest to clarify if exosomal expression of proteins such as hnRNP K is representative of abnormalities within neoplastic cells.

ExoCarta, a repository for exosome proteomics studies, was a useful tool for comparing data from our study with other exosome proteomics data deposited in ExoCarta [140]. It allowed us to check that our proteome was consistent with that of other exosomes and

furthermore demonstrated that we were not the only group identifying unexpected nuclear, ER and mitochondrial proteins [59, 61, 96, 101]. Through manual checking of our dataset we were also able to identify 63 previously unidentified exosomal proteins. BCa-exosomes have never been analysed by proteomics methods before therefore some of the 63 unique identifications may be bladder or bladder cancer specific. The potential of these proteins as BCa specific markers is something that requires further investigation.

The examination of the physiologically impossible number of HLA identifications in our exosome dataset has highlighted the inability of MASCOT to distinguish between correct and incorrect HLA identifications. It also shows potential problems with other protein groups exhibiting homologous sequences. Proteins which are particularly homologous, such as HLA proteins, may be particularly difficult for MASCOT to pin point. MASCOT will potentially designate peptides to more than one protein when in fact only one of the proteins is the true identification. This may be the case with some of the histone proteins identified but more investigations would be required to clarify this. Whilst this is beyond the scope of this study this problem is no doubt one that affects numerous proteomics studies and is a problem that is not to our knowledge widely discussed or raised.

The LC-MALDI MS workflow used in the current study has been shown to be effective in identifying large numbers of exosomal proteins of high quality and more than double that of the only other LC-MS/MS study [145]. Other exosome proteomics studies have identified more exosomal proteins but these utilise a 1DE LC-MS/MS approach allowing a further degree of protein separation [59, 61, 96, 101]. Fewer fractionation steps were performed for our analysis compared with 1DE LC-MS/MS therefore less processing time on the LC and MS was required likely reducing the cost, in time and money, of our study. Even though our proteins were not subjected to this high level of separation overall results are comparable. This suggests such intensive fractionation may not be required to obtain good resolution of exosomal peptides.

Although these 1DE LC-MS/MS studies appear to have many more protein identifications the study with the most rigorous purification procedure utilising an immunocapture method

only identified around 50 more proteins than the current study. Two 1DE LC-MS/MS studies used simple pelleting methods and as a consequence probably analysed samples containing cellular constituents other than exosomes, therefore identifying numerous non-exosomal proteins [59, 61]. Furthermore, these two studies also analysed samples from bodily fluids, urine and saliva, which are much more complex source materials for exosomes and likely contain many contaminants. THP has been shown to be a contaminant of urine derived exosomes prepared by the simple pelleting method, thus one could assume that this is unlikely to be the only contaminating protein of the samples analysed by Gonzales *et al.,* (2009) [59, 188]. Insufficient evidence is presented within several exosome LC-MS/MS studies to confirm that the sample analysed is that of pure exosomes [59, 61, 96, 101]. However, one of the studies does present some vesicle size data showing the majority of vesicles present to be consistent with exosomes, but there are a significant number of vesicles over 100 nm also present and non-vesicular fibrillar material was also observed in a similar study by the same group [34, 61].

The data presented in the urine exosome study that has the highest number of exosome protein identifications recorded (1132) included one peptide identifications in their results [59]. As a consequence the data will have a much higher FDR than our study (0%). Therefore many of the identifications may not be genuine. Looking at the exosome proteomics studies with larger datasets highlights the problems faced with comparing our dataset to others. There are clearly issues with sample quality and data analyses that need to be addressed within the field, which are discussed in the thesis introduction (section 1.3).

In conclusion our dataset is one of the highest quality in the field due to the strict protein identification criteria and well characterised high quality exosome samples used for analysis. This new dataset will provide a platform for future studies in the realm of exosome biogenesis and exosome biology (functions) and may contain protein identifications of interest in terms of biomarkers for BCa. Nevertheless, this established knowledge in handling this difficult sample for such a global proteomics workflow will provide enormous scope for follow up studies with exosomes from other sources.

Chapter 6:

Validation of the exosome proteomics dataset

6.1 *Introduction*

Having generated a large complex proteomics dataset from BCa derived exosomes it was essential to test the validity of the data. We believed that our dataset was amongst the highest quality in the field based on the quality of the prepared samples and the strict identification criteria used. Nevertheless, the dataset required interrogation using a number of approaches to help ascertain the true quality of the proteomics results. *In silico* analysis using database tools is one approach. Computational techniques can be used to allocate proteins to categories based on predefined gene ontology (GO) information. Although this is useful in many proteomics studies this categorisation may become biased by the researcher. For example, if a protein is present in more than one cell compartment the researcher may chose to allocate this protein to the compartment that most fits their requirements. With respect to the exosome proteomics field the method of allocating a protein to a specific category is not always indicated [34]. In addition, when database analysis is included it is not necessarily statistically based and this may potentially incorporate bias into a study [59, 61, 101, 200]. However, there are some exosome proteomics studies that use statistical analysis such as over representation analysis (ORA) or specific analysis of domain/motif enrichment [56, 96].

ORA enables the identification of statistically significant enrichment or depletion of gene set categories to which the proteins identified have been allocated. Comparisons of an experimental dataset with predefined databases can highlight associations with key biological themes. Furthermore, because this approach is statistically informed these associations are unlikely to occur by chance and no bias is introduced by the researcher. Conde-Vancells *et al.,* (2008) used ORA to analyse rat hepatocyte exosome proteomics data. A number of high associations were identified with functional activities and biological processes that may be associated with the physiological role of hepatocyte derived exosomes, such as lipid metabolism and cell signalling [56]. We believe that using an ORA approach will reveal any statistically significant associations within our dataset

with respect to key biological themes, such as molecular function and biological processes, as well as any significant associations with disease.

There are also other bioinformatics tools available which may provide useful insights into the potential of the dataset which include the building of theoretical protein interaction networks. These can be produced to show the theoretical interactions of identified proteins within a dataset. In addition, repositories such as BioGRID allow interactions with other proteins (not necessarily identified within a study) to be explored [201]. Graner *et al.,* (2009) utilised pathway analysis software (Ingenuity Systems Inc.) to identify significant interaction networks associated with proteins identified in brain tumour derived exosomes. Networks involved in immunological functions and cancer were identified [105]. Formulating theoretical protein interaction networks using the current dataset may help identify proteins of interest with respects to the function of exosomes in BCa. Network analysis could also help to elucidate the role of proteins within exosomes with currently unknown functions. Furthermore, interaction networks may help identify potential therapeutic targets for disease by highlighting pathways that feature greatly or abnormally in cancer cells.

By performing *in silico* analysis of a proteomics dataset the results can be examined as a whole making it possible to identify potentially important features particularly with respect to disease. Furthermore, statistical comparisons with other exosome proteome datasets should enable us to establish whether our dataset is consistent with what is expected of a high quality exosome proteomics dataset. For example, is the data consistent with a proteome of vesicle/endosomal origin? Also, are the molecular functions associated with the dataset in agreement with the known functions of exosomes?

It is also equally important to gather empirical evidence to confirm that protein identifications are of exosomally expressed proteins and that the dataset is therefore of high quality, and not replete with false identifications. However, it is unfeasible to verify all 353 protein identifications. Therefore, it would be useful to focus attention on proteins that

have associations with bladder cancer (BCa) as they may be of particular interest. It may also be valuable to validate proteins with potential specific exosomal significance.

Exosomal proteins identified by MS in other studies are not always extensively verified. Nevertheless, where evidence is presented verification is generally by means of immunoblotting [96, 202]. However, the verification of a protein is sometimes demonstrated as present in the purified sample but this is not necessarily demonstrating a protein as unequivocally exosomally expressed. One means of demonstrating genuine exosomal expression is by immunogold electron microscopy [34, 47, 58, 100]. Another approach is by continuous sucrose gradient purification of exosomes. Here the expression can be correlated with sample density to reveal if the protein is predominantly present within the accepted range of exosome density (1.12 – 1.2 g/ml) [47, 87, 96]. In order to verify any MS-identified proteins as exosomal it is therefore essential to utilise one or more of these techniques.

In order to evaluate the bladder specific/selective expression of some of the proteins identified it would also be useful to examine whether the verified HT1376-exosome MS-identified proteins are specific to this particular exosome population or specific to BCa-exosomes when compared with exosomes from other carcinoma sources. This preliminary analysis would not provide any definitive evidence of biomarker validation. It would nonetheless give an indication of the broad or restricted expression of these proteins within exosomes. It may also provide clues as to whether any proteins that may be indicative of BCa. The opportunity also presents itself to evaluate *ex vivo* exosomes, given that we had some urinary exosome preparations from both healthy donors and bladder cancer patients, for expression of some of the MS-identified proteins.

6.2 *Aims*

The aims of this chapter were to:

- Perform unbiased bioinformatics analysis of the MS dataset to reveal any biological themes within the dataset
- Verify proteins from the MS dataset are genuinely present in exosomes using a range of laboratory techniques
- Analyse the expression of verified MS-identified proteins in exosomes from a variety of sources

6.3 *The MS dataset is consistent with an exosome phenotype*

The exosome database ExoCarta is a repository for proteins and RNA identified in exosomes from a variety of sources and was developed in order to help researchers identify reliable exosome markers [140]. The database contains data from 75 exosome studies (accessed 19th June 2010) and provides an invaluable resource for researchers. This repository enabled us to compare our dataset with all other exosome proteomics datasets within the ExoCarta database.

We compared our dataset with ExoCarta submissions containing a minimum of 10 matching gene identifiers to our study (total of 44). Comparisons were also limited to human studies using MS-based approaches [40, 57, 87, 96, 100, 101, 150]. Our dataset was converted from Swiss-Prot accession numbers to EntrezGene identifications using BioMart. ORA using hypergeometric distribution was then applied in R against a background of all human genes with EntrezGene identifications. This was to determine whether there were more overlapping genes with ExoCarta gene sets than could be expected by chance. FDR correction was applied to control for multiple testing.

The results revealed significant associations with seven (out of the 44 examined) MS based exosome proteomics studies (Figure 6.1a). Of particular interest was that our data showed very significant overrepresentation of protein-encoding genes isolated from colorectal carcinoma cells [96, 101]. Furthermore, four of the studies were of exosomes derived from cancer cells (mesothelioma, melanoma and colorectal) and five were of exosomes of epithelial origin (intestinal, mesothelioma, tracheobronchial and colorectal). This indicated that the proteome characterised by our data is in agreement with other studies of similar cells, with particularly significant overlaps with high quality proteomics studies of colorectal cancer.

6.4 *Unbiased overrepresentation analysis of the MS dataset*

Utilising GeneGO MetaCore (Version 5.4), similar ORA analyses were performed comparing our dataset with gene sets from GO and proprietary GeneGO data (converted into SwissProt identifications using BioMart). Four gene set categories were analysed (disease biomarker, diseases in general, biological process and cellular compartment; Figure 6.1b-e respectively) in order to determine whether the dataset is consistent with a proteome of vesicle/endosomal origin, whether the overall functional associations identified are similar to known exosomal functions and also to assess associations with disease. The top ten results for each category are shown (Figure 6.1b-e) and ordered by ORA *p* value.

Where the disease biomarker category is concerned, the data indicated the most significant associations to be with bladder cancer (Figure 6.1b). This finding was reassuring and supported the premise that exosomes may be a useful tool for identifying sets of disease specific markers. Other carcinomas, of the colon and breast, also showed highly significant associations suggesting extensive representation of proteins common to various types of carcinoma.

Associations with cancer of the gastrointestinal tract were revealed when examining general disease association, as well as metastatic cancer, respiratory tract disease (including lung cancer), and carcinomas (Figure 6.1c). Although significant overrepresentation was not identified with diseases of the genitourinary tract (including BCa) within the top 10 associations significant associations were identified within the top 40. The association of BCa within the top 40 rather than the top 10 may be related to the accuracy of ORA as it is limited by the quality and size of the gene sets queried. Also, there may be a disproportionate amount of respiratory and gastrointestinal studies compared with bladder studies and this may have skewed the results. However, our statistically based analysis did suggest that HT1376-exosomes express proteins strongly related to neoplastic disease and in particular carcinomas (Figure 6.1b and c).

The biological process results revealed significant associations with cytoskeletal control, intercellular adhesion, matrix adhesion, antigen presentation and protein folding (Figure 6.1d). Examination of the cellular compartment associations demonstrated significant associations with membrane vesicles, the cytoplasm and cytoskeleton (Figure 6.1e). One would expect to see associations with membrane vesicles and cytoplasm based on the origin of exosomes and the cytoplasmic content of their lumen. However, associations with the cytoskeleton were surprising although a number of cytoskeletal proteins have been identified in other exosome proteome studies [56, 96, 145]. The most unexpected results were the predominant associations with melanosomes and pigment granules. These are specialised compartments of pigments cells of the eye and skin and hence one would not expect to find them in association with BCa-exosomes.

In summary, the statistically based unbiased analyses used here demonstrated that our BCa exosome proteome had statistically significant similarities to other exosome proteome studies and that the BCa exosome proteome identified showed phenotypic indicators suggestive of a proteome of carcinoma origin.

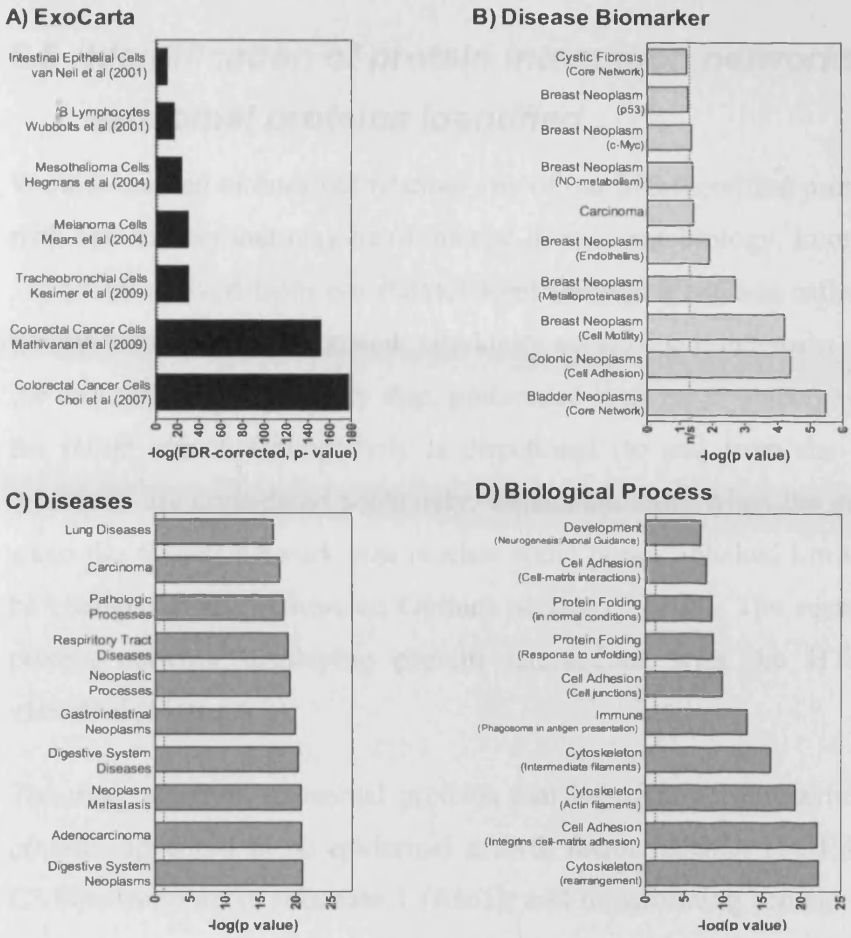


Figure 6.1: Summary of over-representation analysis of the nano-LC/MS-derived protein identifications against gene sets from ExoCarta and GeneGO

Our protein list was first converted to an EntrezGene-identified gene list before undertaking ORA using hypergeometric distribution. Results were filtered to include comparisons with MS-based studies only and those reporting 10 or more matching genes. How well our data compared with exosome protein profiles from specified cell types is displayed as the –log (p value) corrected for false detection rate A). ORA analysis utilising MetaCore used the Swiss-Prot identifications for the identified protein list. The top 10 overrepresented genes contained within each of the following group headings are shown: disease biomarker (B), diseases (C), biological process (D), and cellular compartment (E). The dotted line indicates p= 0.05; hence, columns to the left of this are not statistically significant.

6.5 *Identification of protein interaction networks from the exosomal proteins identified*

We also wanted to discover whether any of our MS-identified proteins had any interactions with one another that may be of interest in exosome biology. In order to examine this gene identifiers derived from our dataset went through a process called auto expand. GeneGO describes this as "Auto expand: Gradually expands sub-networks around every object from the root object list. At every step, preference is given to objects with more connectivity to the initial object. Connectivity is directional (to and from the initial object), and both directions are considered separately. Expansion halts when the sub-networks intersect, or when the overall network size reaches some pre-established limit (50 by default, but may be changed in the Advanced Options section)." [203]. The result of auto expand was a protein network displaying protein interactions with the HT1376-exosomal proteins identified (Figure 6.2).

The most apparent exosomal proteins that have known interactions with other exosomal proteins appeared to be epidermal growth factor receptor (EGFR), ubiquitin, Ras-related C3 botulinum toxin substrate 1 (Rac1), and transforming protein RhoA (RhoA). EGFR, a cell surface receptor for epidermal growth factors, was shown to have a positive effect on six proteins including guanine nucleotide-binding protein G(i), alpha-2 subunit (G-protein alpha-i2), glutathione S-transferase P (GSTP1), MUC1, β1 integrin (ITGB1), MHC Class I (HLA-Cw3). EGFR was also shown to have a negative influence on plakoglobin and α2 integrin and was negatively influenced by ubiquitin.

Ubiquitin is a regulatory protein which predominantly functions to label proteins for proteasomal degradation. However, ubiquitinylation of proteins is also known to be involved in the shuttling of some proteins to the multivesicular body (MVB) for inclusion in to exosomes. Here, ubiquitin was shown to have a negative effect on exosomally identified proteins filamin C, β-catenin, EGFR, RhoA, hnRNP K, and fibronectin. Ubiquitin was only shown to have a positive influence on one protein, namely 26S protease

regulatory subunit 6B (PSMC4) and to be positively influenced by Ubiquitin-like modifier-activating enzyme 1 (UBE1).

RhoA and Rac1 are small GTPases involved in numerous cellular events including cell-cell adhesion and cytoskeletal rearrangement. In this HT1376-exosome protein network they were mainly positively influenced by proteins including EGFR. Rac1 and ubiquitin nevertheless both have a negative effect on RhoA. Also of note were the links with integrin proteins which appeared to influence and be influenced by these small GTPases.

Other proteins that appeared to have significant interactions with numerous exosomal proteins identified in the current dataset included c-Myc and cytoskeletal actin. C-Myc is a transcription factor regulating the expression of 15% of all genes and is often over expressed in cancer [204]. This consequently causes altered expression of c-Myc regulated genes involved in cell proliferation leading to cancer. C-Myc within this network is the protein exerting the most influence over other proteins (23). Some of these proteins exert negative feedback on c-Myc. Actin is a cytoskeletal protein that is involved in cell structure, vesicle transport and cell motility and in this network cytoskeletal actin interacts with five proteins identified in HT1376 exosomes. Cytoskeletal actin was shown to exert positive effects on profilin 1, vasodilator-stimulated phosphoprotein (VASP), cytoplasmic FMR1-interacting protein 1 (CYFIP1), alpha-actinin 1, and moesin.

This protein network provided new insights into the potential protein interactions involved in exosome biogenesis and function offering future opportunities to manipulate these and modulate exosome biology. These protein interactions are derived from literature evidence nevertheless it is important to note that the exosomal interactions of these proteins are unknown and would therefore require experimental validation.

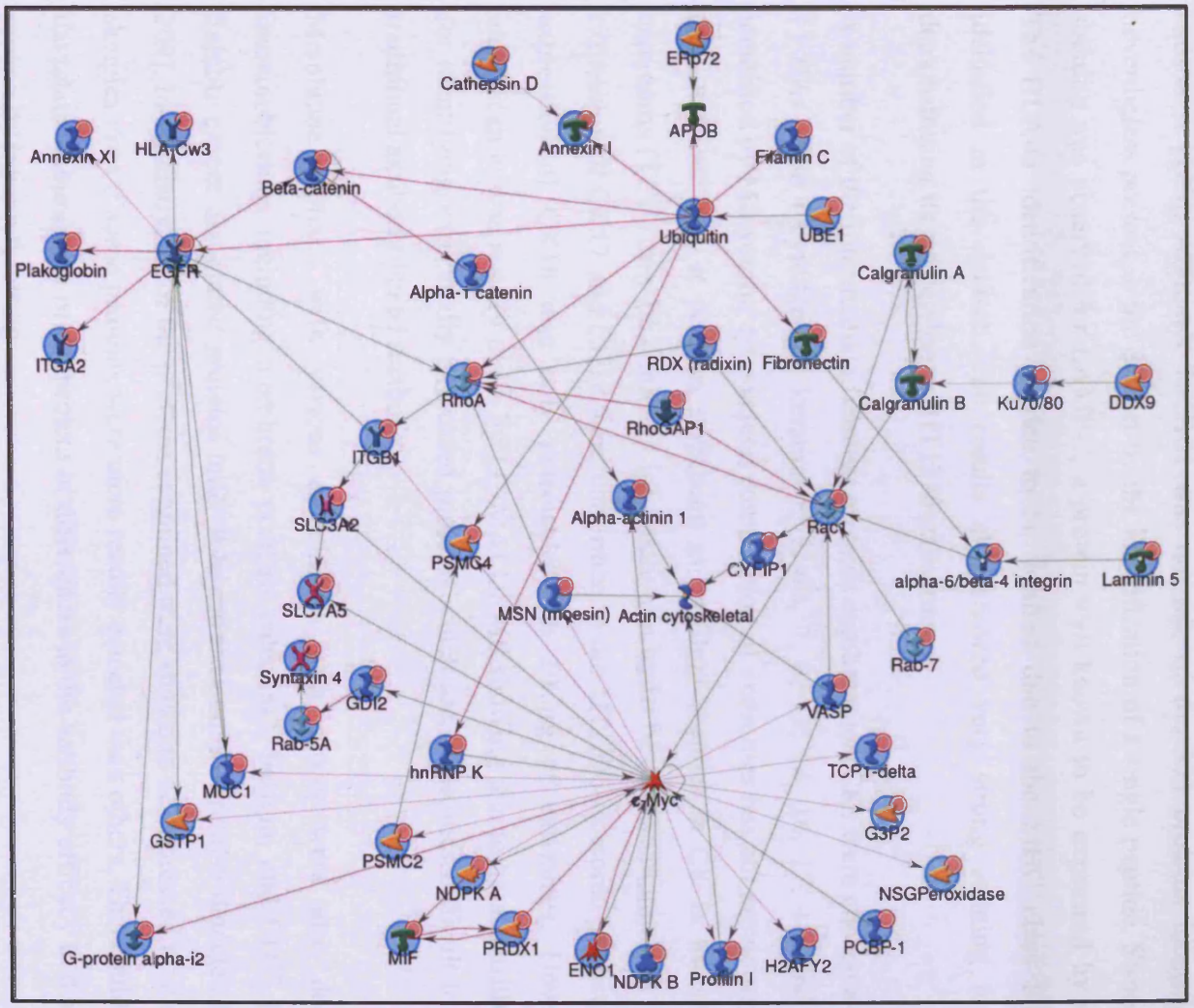


Figure 6.2: Protein networks associated with the HT1376-exosome MS dataset.

All proteins in a blue circle are proteins identified in HT1376 exosomes. Green lines indicate a positive effect, red lines indicate a negative effect, and blue/grey lines indicate an unspecified effect. The direction of the arrow indicates the direction of the proteins influence.

6.6 *Proteins identified by MS are present in HT1376-exosomes*

In order to confirm the presence of a number of proteins identified by MS proteins were validated firstly by immunoblotting. Up to 20 μg of HT1376-exosome protein was analysed using a panel of antibodies (Figure 6.3). The MVB marker protein TSG101, known to be enriched in exosomes, was labelled as an indicator for the presence of exosomes [108]. Although TSG101 was not one of the 353 proteins identified it was nevertheless present in the dataset by the identification of a single peptide. Strong positive staining was observed for LAMP-2, a protein well known to be expressed by exosomes. The HLA-G identification needed to be verified due to the MHC class I anomalies identified in the dataset. The results also showed very strong staining for HLA-G demonstrating its expression by HT1376-exosomes.

A number of the intermediate filament proteins cytokeratins (CK) were chosen as a total of 11 CKs (type I cytoskeletal keratins 1, 5, 6B, 7, 8, 13, 14, 16, 17, 18, and 19) were identified by MS yet the cytoskeletal composition of exosomes has not previously received any attention. This is perhaps surprising as profiling changes in CK in transitional cell carcinoma (TCC) may be clinically informative in terms of disease monitoring [164]. The expression of CK17 and CK18 were confirmed in our HT1376-exosome preparations. The expression of CK18 was only detectable with 20 μg of exosomes. However, this identification was readily made by LC-MALDI MS showing this to be a sensitive method for identifying exosomally expressed proteins which are sometimes difficult to detect by traditional antibody-based methods.

Membrane proteins with various associations with cancer were also detected by immunoblotting, including membrane proteins galectin-3, basigin, and CD73 [205-208]. Soluble cancer associated proteins hnRNP K and β-catenin were also detected [198, 199, 209]. In summary, all of the proteins examined were shown to be expressed in our exosome samples even if some proteins were more readily detected than others. This could be due to the relative abundance of the protein or differences in the antibody efficacy in detecting the protein by immunoblotting.

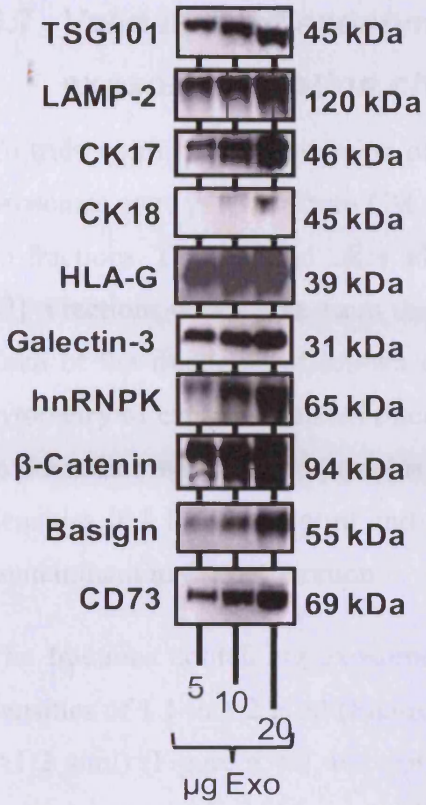


Figure 6.3: Detection of proteins of interest in HT1376-exosome samples by immunoblotting.

Three doses (5, 10 and 20 µg) of HT1376-exosome (Exo) protein were solubilised and then subjected to 1DE and immunoblotting with a panel of antibodies in order to determine whether the samples expressed a selection of proteins identified by LC-MALDI MS. The protein labelled and molecular weights are shown.

6.7 *Validation of exosomal expression of proteins based on exosome flotation characteristics*

To truly confirm the expression of these MS-identified proteins by HT1376-exosomes the exosomes were purified from CM using a continuous sucrose gradient which was split in to 15 fractions. This method takes advantage of the flotation characteristics of exosomes [46, 47]. Fractions were taken from the gradient and their density determined by refractometry. Each of the fractions, of known density, were analysed by immunoblotting and by flow cytometry of exosome-coated micro-beads (detailed in section 2.10). This made it possible to determine whether the proteins of interest, identified by MS, were present between the densities if 1.1 and 1.2 g/ml and therefore expressed by HT1376-exosomes rather than a contaminant in the preparation.

The fractions containing exosomes were identified by staining for TSG101 highlighting densities of 1.1 to 1.2 g/ml (Figure 6.4a-c). Some staining was observed in denser fractions (>1.2 g/ml) (Figure 6.4a), but this was generally weak and may be due to the presence of exosome or protein aggregates. The expression of several proteins of interest, such as 5T4, CD44 and β-catenin, all co-localised at the same density range which is consistent with their exosomal expression. The expression of HLA-G was also present at this density range (Figure 6.4c). Both CD73 and CK17 (Figure 6.4 a and b respectively) co-localised predominantly at the same density range. However, they both also appeared in the more dense regions from 1.17 and 1.28 g/ml. The only protein not to be found predominantly at classical exosomal densities was α tubulin (Figure 6.4c) which was found spanning the density range of 1.1 to 1.24 g/ml. There was no predominant staining at exosomal densities (highlighted by TSG101 staining). It is therefore not clear if α tubulin is genuinely exosomally expressed or if it is a soluble constituent.

Overall, out of the 11 proteins examined all but one appears to be genuinely exosomally expressed. This validation emphasises quality of the original preparations used for the proteomics analyses. It also demonstrates that the MS dataset is one of high quality containing genuine exosomal proteins.

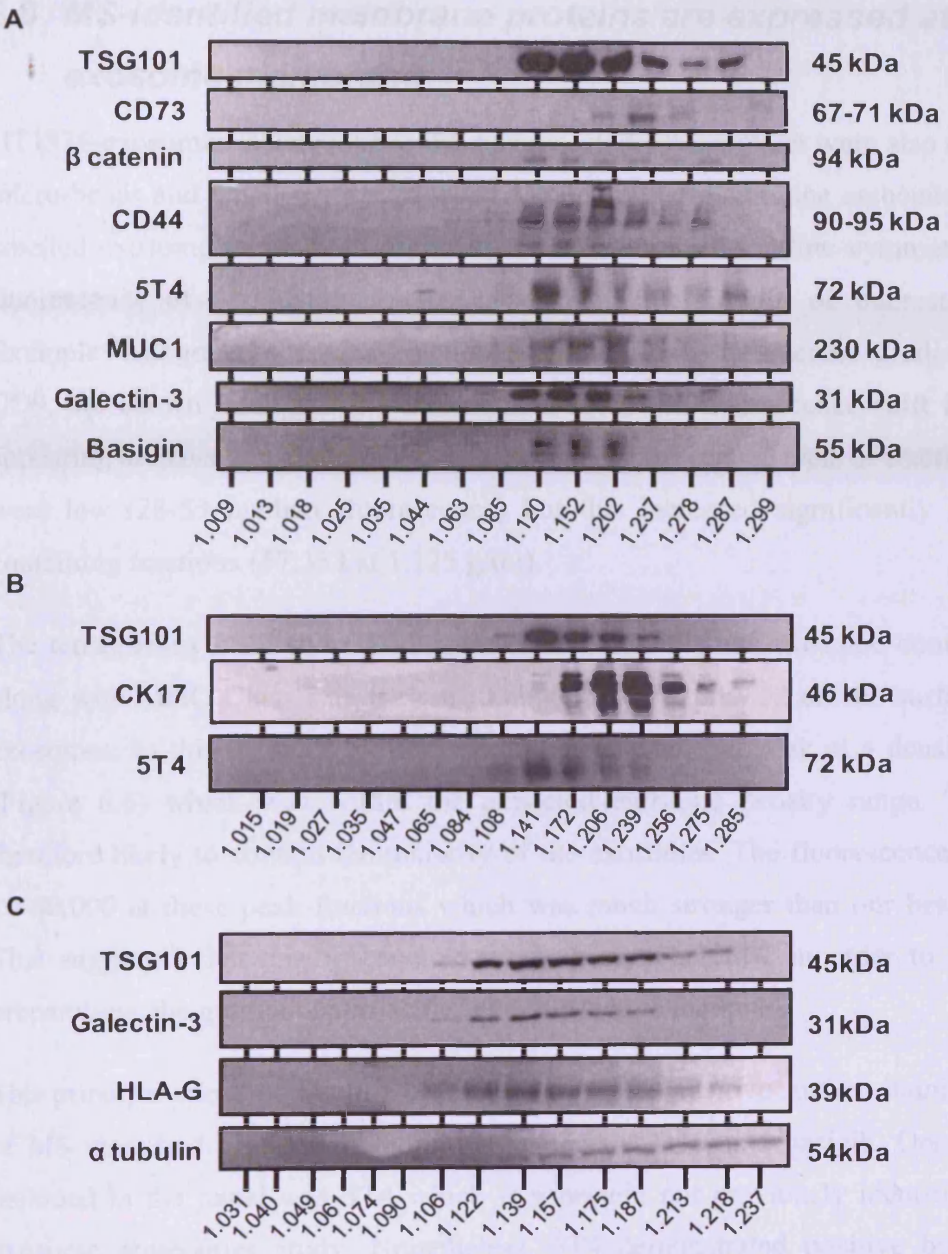


Figure 6.4: Expression of proteins of interest by immunoblotting of HT1376 exosomes separated by linear sucrose gradient.

HT1376-exosomes were purified by three different continuous sucrose gradients (a-c) and split in to 15 fractions. Two thirds of each fraction was then solubilised and subjected to 1DE and immunoblotting for a panel of antibodies to identify whether proteins identified by LC-MALDI MS are genuinely exosomally expressed. The protein labelled and its molecular weight is shown.

6.8 *MS-identified membrane proteins are expressed at the exosome membrane*

HT1376-exosomes isolated using the sucrose-gradient approach were also coupled to latex micro-beads and labelled for a panel of MS-identified membrane associated proteins. The labelled exosome micro-bead complexes were subjected to flow cytometry. The median fluorescence of the histogram for each membrane protein of interest was recorded. Example histograms for each fraction collected from the sucrose gradient, labelled for CD9, are shown in Figure 6.5 and demonstrated the fluorescence shift in CD9 staining appearing at exosome containing densities. The fluorescence levels of fractions one to three were low (28-53 median fluorescence) but this increased significantly in the exosome containing fractions (57,333 at 1.125 g/ml).

The tetraspanins CD9 and CD81 were used to identify the exosome containing fractions along with MHC Class I as they are known to be expressed on the surface of HT1376-exosomes. In this instance they revealed a clear principal peak at a density of 1.12 g/ml (Figure 6.6) which was within the expected exosome density range. This fraction is therefore likely to contain the majority of the exosomes. The fluorescence magnitude was 10-60,000 at these peak fractions which was much stronger than our best ExoQA assay. This suggested that this fraction was very pure. Therefore in order to get highly pure preparations the gradient approach is a very effective method.

This principal exosome fraction (1.12 g/ml) revealed positive surface staining for a number of MS-identified proteins including β1 integrin, CD36, and basigin. One of the proteins included in the panel was 5T4 which is a protein not previously identified in any other exosome proteomics study. Nonetheless, 5T4 demonstrated positive but weak surface staining on the exosomes (Figure 6.6).

We have also shown the technique to be reproducible demonstrating similar levels and patterns of expression for a number of membrane associated proteins (CD9, basigin, 5T4

and MHC Class I) (Figure 6.7). The peak fractions at around 1.1 g/ml (gradients A and B) were consistent with the expected exosome range with all the antibodies tested.

Fractions were also labelled with a calnexin-specific antibody (Figure 6.6) which revealed low levels of expression in the exosome containing region. Slightly elevated levels were observed in the denser fractions. This confirmed the absence of calnexin, an endoplasmic reticulum (ER) protein, as expected. Furthermore, it demonstrated the specificity of the staining for the other proteins tested and their correct membrane orientation.

Overall, the data presented demonstrated that a number of the proteins identified, using the LC-MALDI workflow, are expressed by HT1376 exosomes. Furthermore, we have demonstrated that using our modified solubilisation method (SDS and DTT) we have been successful in the identification of genuine membrane-associated exosomal proteins which are often difficult to solubilise. Further validation is however required with respect to the association of this panel of proteins with BCa or cancer.

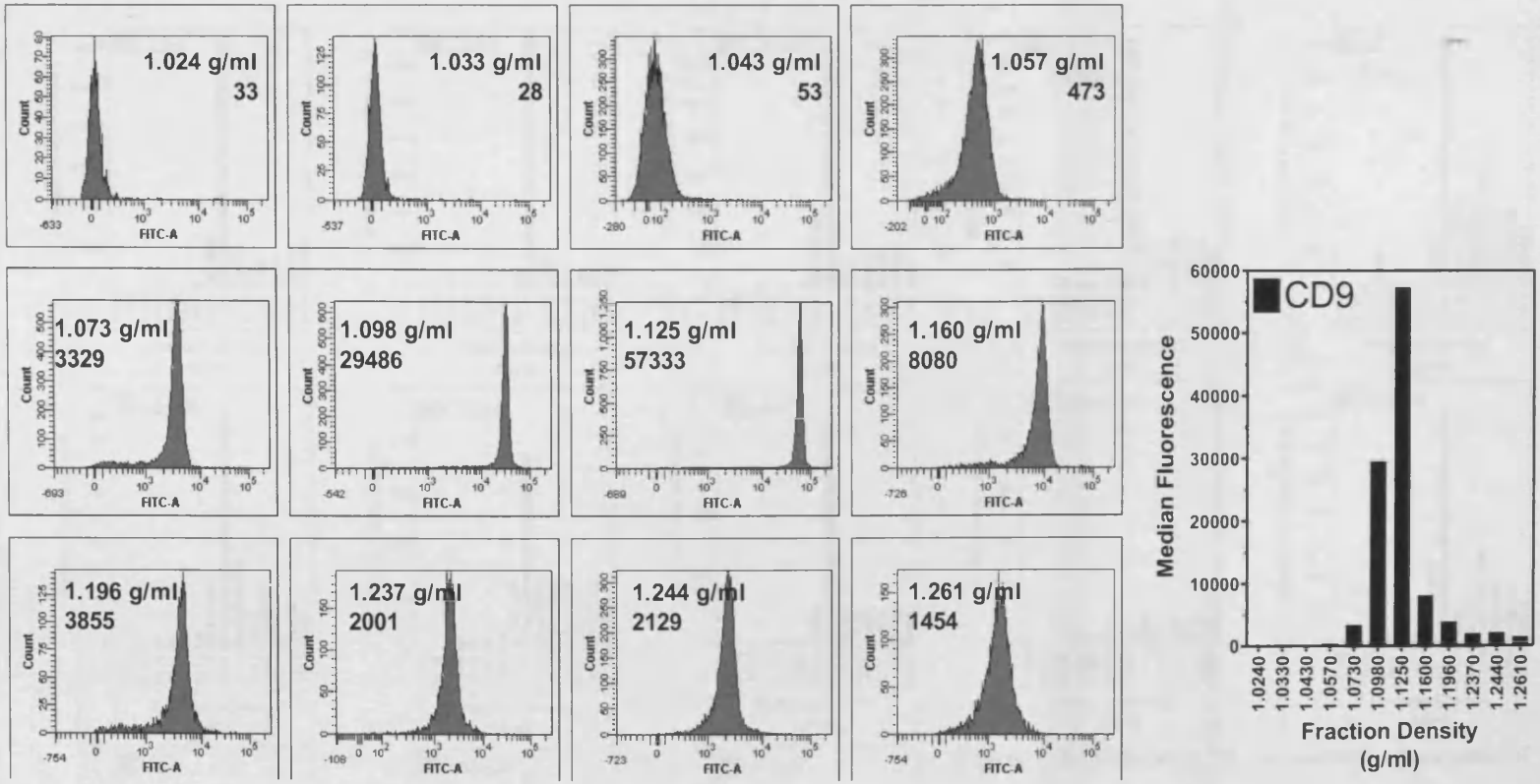


Figure 6.5: Flow cytometry histograms and corresponding graph for CD9 of linear sucrose gradient fractions of HT1376 derived exosomes.

One third of 12 fractions for a continuous sucrose gradient separation of HT1376-exosomes were coupled to latex micro-beads and subjected to flow cytometry. The median fluorescence for CD9 is indicated for each fraction along with the fraction density. On the right hand side the data has been plotted demonstrating the CD9 rich fractions within the exosome-containing densities.

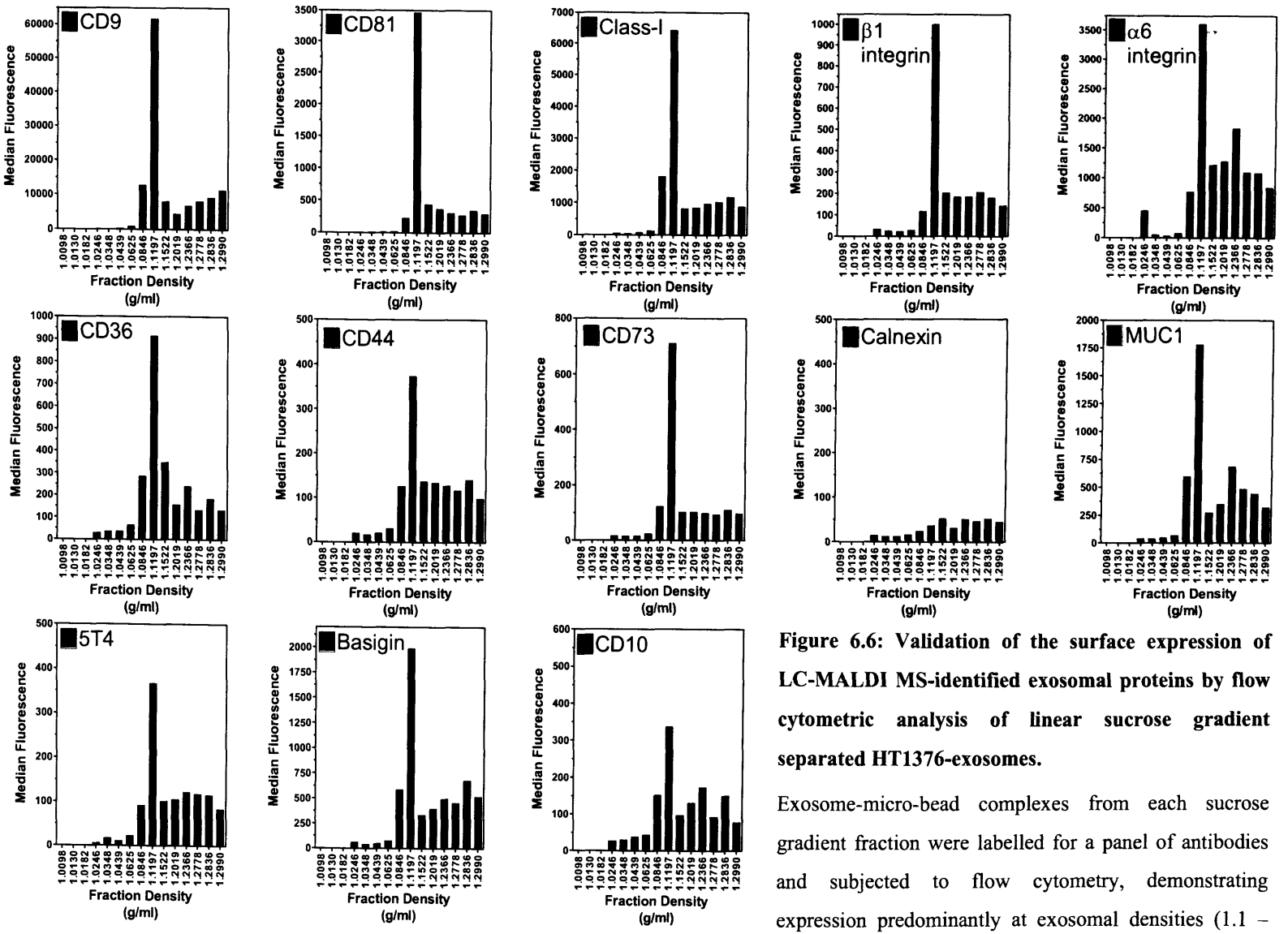


Figure 6.6: Validation of the surface expression of LC-MALDI MS-identified exosomal proteins by flow cytometric analysis of linear sucrose gradient separated HT1376-exosomes.

Exosome-micro-bead complexes from each sucrose gradient fraction were labelled for a panel of antibodies and subjected to flow cytometry, demonstrating expression predominantly at exosomal densities (1.1 – 1.2 g/ml)

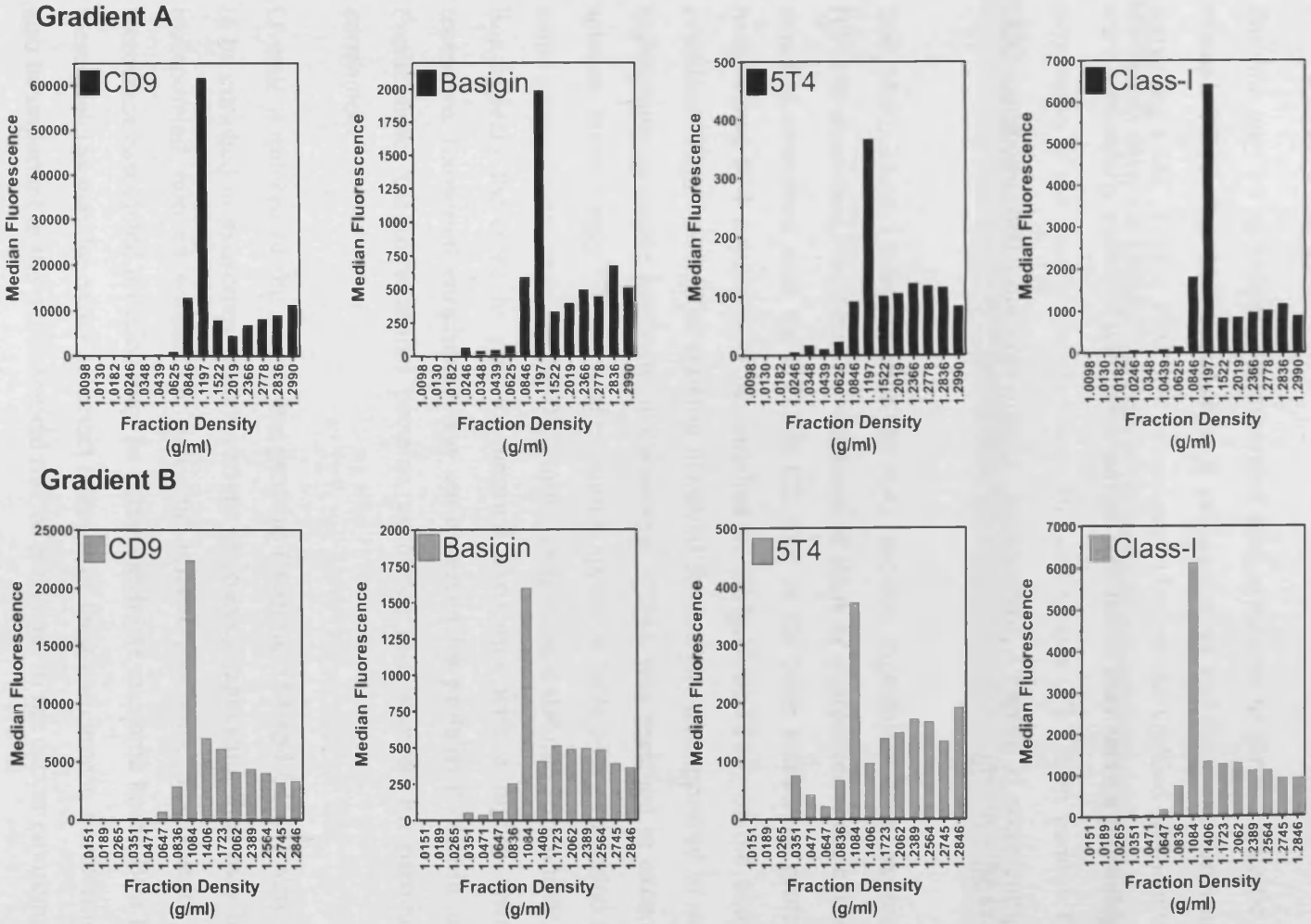


Figure 6.7: Reproducibility of protein verification using flow cytometry of linear sucrose gradient separated HT1376-exosomes.

Fractions from two continuous sucrose gradient separations (A and B) of HT1376-exosomes were coupled to latex micro-beads and labelled with a panel of antibodies then subjected to flow cytometry. The median fluorescence for each antibody and fraction for two experiments are shown demonstrating the reproducibility of the technique.

6.9 *Some MS-identified proteins are enriched in exosomes from other sources*

Proteins may be specifically incorporated into exosomes to serve a specific purpose. For example MHC class II is enriched in B cell exosomes and these exosomes are capable of activating $CD4^+$ T cells [47]. To investigate whether the verified MS-identified proteins were particularly enriched within exosomes, and hence may serve a physiological role, the expression of the proteins in exosomes and cell lysates (CL) from various TCC and non-TCC carcinoma cell lines (previously characterised, Chapter 3) were compared (Figure 6.8).

The MHC class I identification HLA-G showed significant high expression only in HT1376-exosomes. Furthermore, the level of HLA-G expression in the exosomes was not enriched compared with the HT1376 CL. Out of the other membrane proteins examined basigin and 5T4 appeared to be enriched in all but one (T24) of the exosome sources examined (Figure 6.8). The staining observed for basigin also appeared in multiple bands, highlighting probable isoforms of the protein. CD44 was enriched in exosomes from all sources. Interestingly T24-derived exosomes appear to have poor expression for almost the entire protein panel apart from CD44, in which it showed definite enrichment. The T24 cell line appears therefore to produce unusual exosomes with a highly restricted protein repertoire. Exosomal enrichment was not observed for proteins β-catenin and galectin 3. Furthermore, the intraluminal proteins examined also showed no particular exosomal enrichment.

Overall, a number of the membrane proteins (basigin, 5T4 and CD44) were demonstrated to be enriched in exosomes from a variety of sources suggesting they may be specifically incorporated into exosomes for exosome related functions. The results indicate that membrane associated proteins may be better candidate exosome based BCa biomarkers as these proteins may be easier to detect because of their enrichment. Furthermore, they may also be important in the physiological role of exosomes in the cancer environment.

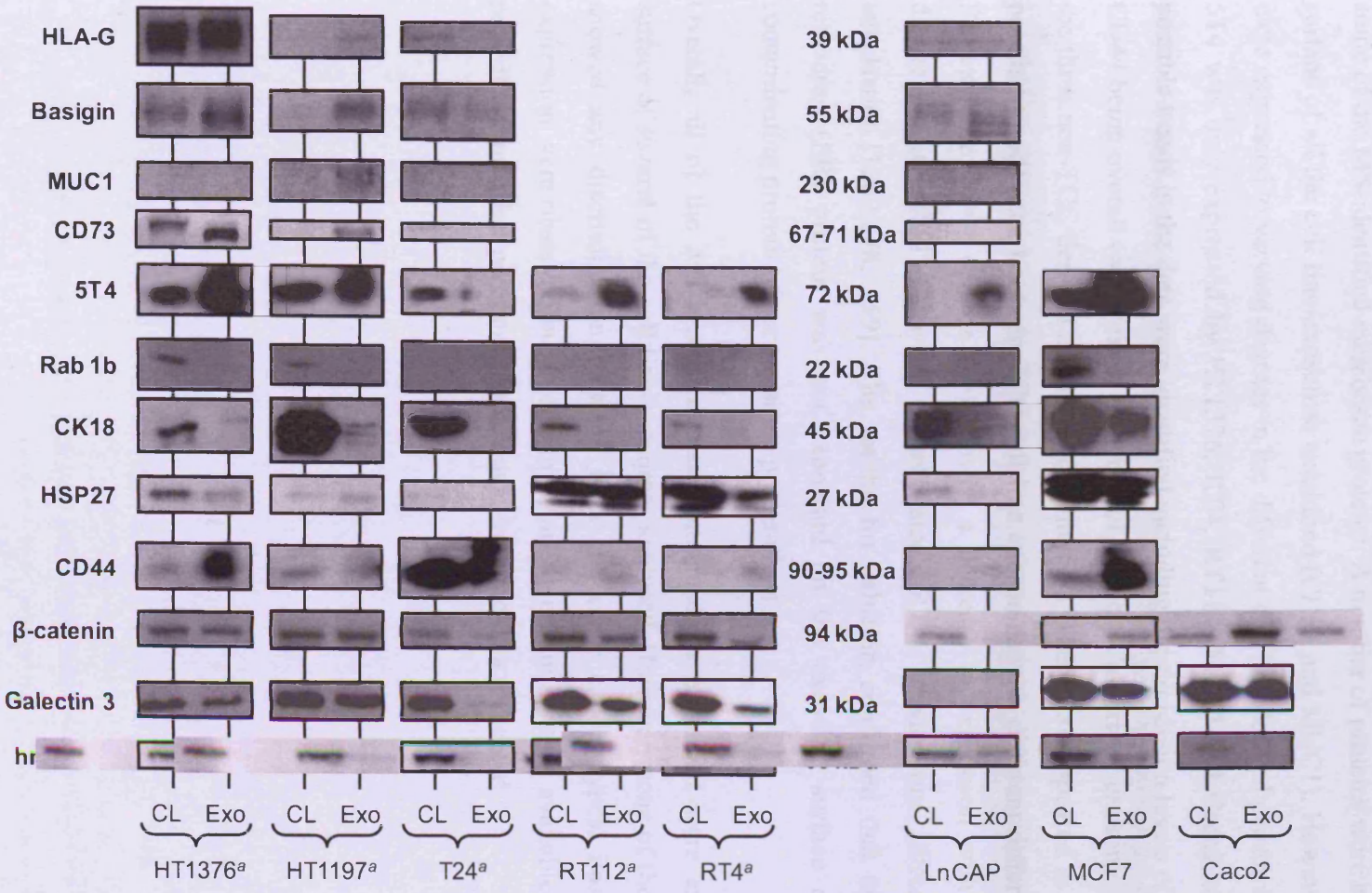


Figure 6.8: Comparison of cell lysates and exosomes from different sources by immunoblotting for proteins of interest.

Five μg of cell lysate (CL) or exosomal proteins (Exo) from each cell line was solubilised and subjected to 1DE and immunoblotting with a panel of antibodies in order to determine whether proteins identified by LC-MALDI MS are enriched in exosomes from different sources (section 2.2). The protein labelled and its molecular weight are shown. a = Transitional carcinoma cell line.

A flow cytometry based approach was also employed to analyse the surface expression of some of the MS-identified membrane proteins. A number of proteins were expressed on the surface of all the cell line-exosomes examined (CD81 and MUC1). However, the majority were expressed to varying degrees in the different cell line derived exosomes. For example 5T4 was only expressed by HT1376, RT4, RT112, MCF7 and Caco2 exosomes. Some possible trends in the data were identified including the expression levels of integrin α6 and CD44 being overall two or more time higher on the BCa-derived exosomes compared with the three non-TCC derived exosomes (Figure 6.9). There also appeared to be a number of possible trends seen between TCC cell line exosomes with different differentiation status. For example CD44 (Figure 6.9) showed a decrease in expression with an increase in differentiation (RT112- well differentiated, RT4- moderately differentiated, T24- anaplastic) [156, 158, 159]. The results for calnexin confirmed that this endoplasmic reticulum (ER) protein was not expressed on the exosome surface and was not a contaminating protein of our exosome preparations.

Overall, all of the MS-identified membrane proteins examined were expressed at the surface of several of the cell line-exosomes analysed. However, none of the proteins tested showed any discrimination between BCa and other cancer types. Possible trends in expression were observed but due to the limited preliminary data available no conclusions regarding biomarker potential can be made from the data presented.

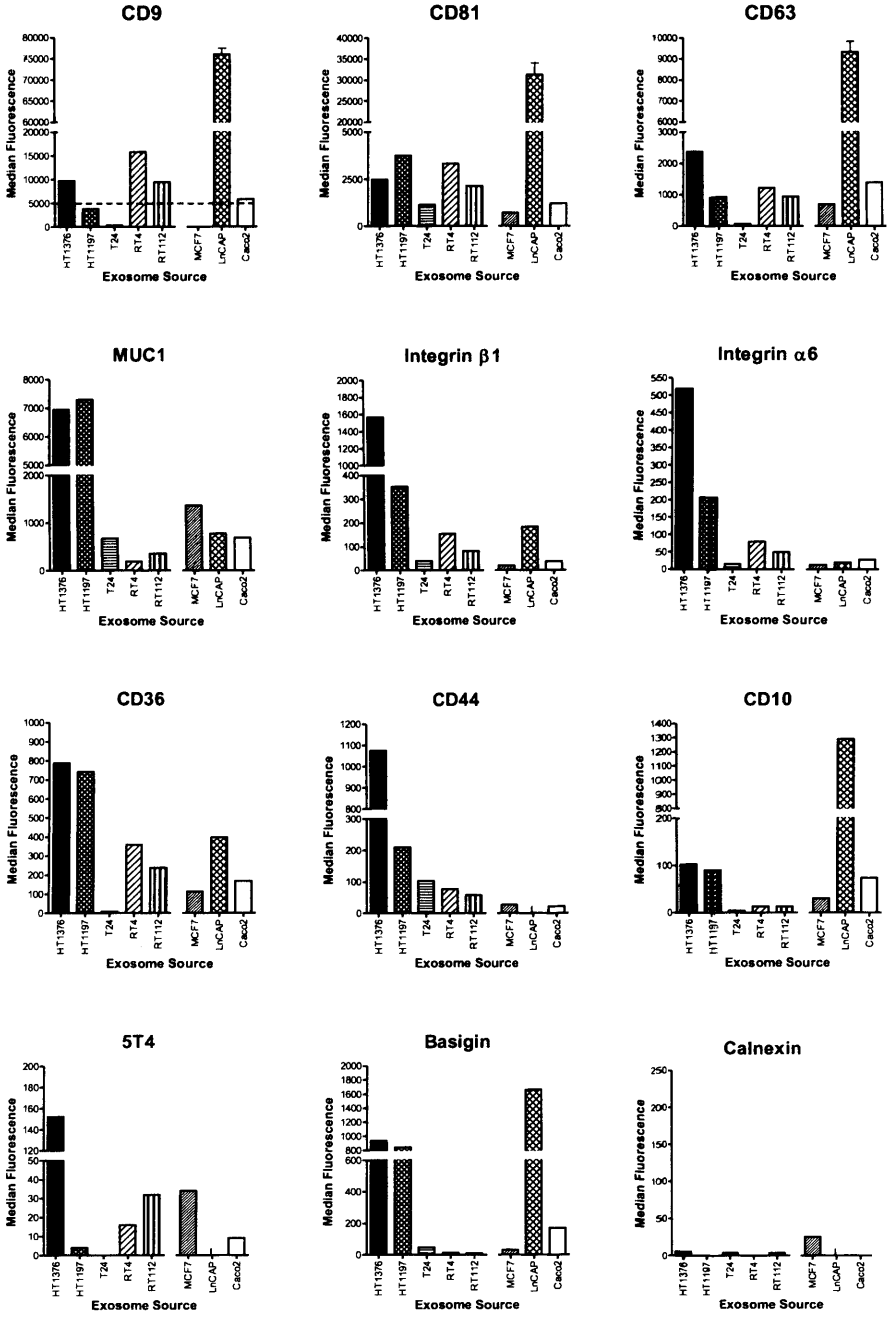


Figure 6.9: Comparison of exosomes from different cell line sources.

Latex micro-beads were coated with exosomes and then subjected to labelling with a panel of Abs and subsequently counterstained with an Alexa Fluor-488 conjugated secondary. The exosome coupled micro-beads were then analysed using flow cytometry. The dashed line represents an arbitrary cut off point of median fluorescence 5000 for CD9 as a threshold for high quality exosome preparations.

6.10 *Exosomal intra-luminal proteins can be semi-quantitatively measured by flow cytometry*

In order to assess the exosomal expression of intraluminal proteins of interest exosome bead complexes were subjected to a fixation and permeablisation (fix-perm) procedure using Beckman Coulters IntraPrep™ kit (see section 2.12). This enabled us to use a panel of antibodies to label intraluminal proteins of interest. Fix-perm had never been performed before on exosome micro-bead complexes and therefore preliminary investigations were performed using a number of cell line-derived exosomes.

Exosome micro-bead complexes from the same batch were subjected to either fixation alone or fix-perm and labelled for the membrane protein CD9, intraluminal protein Rab1b or stained with an isotype matched to the antibody. The data shown in Figure 6.10a is representative of three experiments on exosomes derived from three different sources. A minimum of a tenfold increase in the median fluorescence for Rab1b was measured in all fix-perm samples compared with fixation alone. CD9 and the isotype fluorescence levels did not significantly alter.

The levels of four CKs and other intraluminal proteins were measured using this fix-perm method and exosomes from multiple cell sources were evaluated. Two proteins, HSP90 and hnRNP K, demonstrated relatively low expression (median fluorescence <500) in all cell line exosomes suggesting these proteins are poorly represented in exosomes. Expression of Rab1b varied (2,200-30,000) with the highest expression in three of the TCC cell lines exosomes (HT1197, RT4 and RT112). The expression of the four CKs examined showed very different profiles. Only HT1376 and HT1197-exosomes demonstrated the presence of CK7 and CK19. Expression levels were highest for CK17 with particularly high median fluorescence in HT1197, RT4, and RT112. Overall, intraluminal proteins could be detected using the fix-perm technique. However, none of the MS-identified intraluminal proteins were able to distinguish between BCa and other carcinomas.

In summary, the data presented showed that the MS-identified proteins examined were broadly expressed by exosomes of diverse cellular origins. Overall, none of the proteins examined showed any BCa specificity.

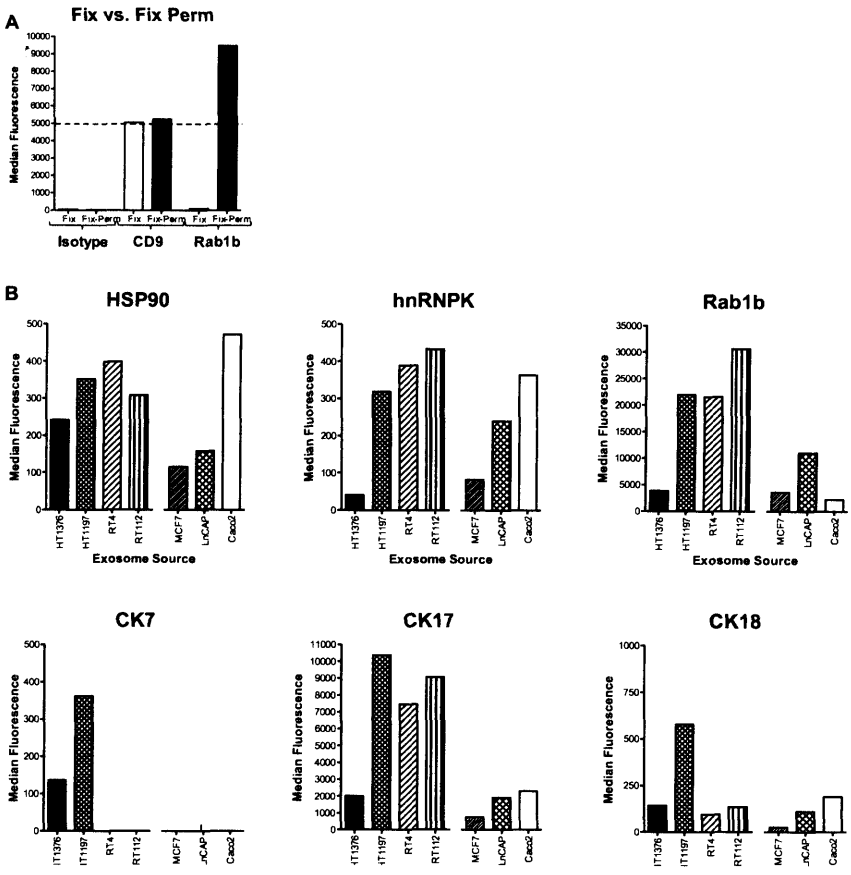


Figure 6.10: Comparison of intraluminal protein expression of fix permeabilised exosomes

Latex micro-beads were coated with exosomes and subjected to fix-perm and labelling with a panel of Abs, subsequently counterstained with an Alexa Fluor-488 conjugated secondary to measure the levels of intraluminal proteins in exosomes (A) Comparison of fix-perm and fixation only to demonstrate the increase in detectable Rab1b with permeablisation. (B) The exosome micro-bead complexes were analysed by flow cytometry. The dashed line represents an arbitrary cut off point of median fluorescence 5000 for CD9 as a threshold for high quality exosome preparations.

6.11 *Proteins of interest are found in urine exosomes from bladder cancer patients*

We also evaluated the expression of the protein panel in exosomes derived from BCa patient and healthy donor urine. This allowed us to examine the expression of these MS-identified proteins in exosomes derived from an *ex vivo* source. Furthermore, as we had urinary exosome samples, although very limited, from both healthy donors and BCa patients we were also able to take a preliminary look at the possible differences in protein expression profiles of BCa urinary exosomes compared with healthy donor urinary exosomes. The urinary exosomes were analysed by flow cytometry of exosome micro-bead complexes (by necessity due to some specimens of 2.5 μg protein).

The quality of each urinary exosome sample was determined by examining the CD9 expression level (fluorescence >5000 required to be considered high quality). Only three (HD04, HD11 and BC02) out of the seven samples analysed were considered high quality (Figure 6.11). The purest BCa sample, BC02, demonstrated the highest expression of all of the proteins in the BCa group apart from 5T4. However, the two purest healthy donor samples showed very different expression profiles from one another (Figure 6.11). The normal level of CD9 on urinary exosomes is not known. We also do not know if these levels fluctuate with time of day or with other physiological variables. The data shown suggests that there are intrinsic differences between urinary exosomes derived from different individuals.

All of the MS-identified membrane associated proteins examined such as integrin β1 and basigin were detected on urine exosomes but showed no significant differences between BCa and healthy donors (Figure 6.11). The expression levels of all the proteins varied greatly within the healthy donor and BCa group. The levels of the ER protein calnexin, not considered to be constitutively expressed by exosomes, also fluctuated greatly. In all cases levels were higher than that of the cell line-exosomes suggesting a degree of non-exosomal material to be present in these preparations (Figure 6.9).

We also used the fix-perm method on these samples. Significant expression of Rab1b was seen in two of the healthy donor samples but expression was poor throughout the BCa samples. Cytokeratin 17 expression was observed in four out of five healthy donors compared with no expression in the BCa urinary exosomes. Higher expression was seen for all three intraluminal proteins in the healthy donor urinary exosomes compared to BCa patient urinary exosomes (Figure 6.12). This indicated a possible loss of these proteins in urinary exosomes from patients with BCa or the presence of interfering substances in BCa that make the CK difficult to detect.

Overall, this preliminary examination of urinary exosomes showed it is possible to detect most of these proteins in urinary exosomes, albeit with considerable sample-sample heterogeneity. This variation may be caused by the difficulties in isolating pure exosomes from urine which is a complex source material (detailed in Chapter 4). Alternatively, donor variation in exosome protein constituents may also account for these differences. However, it should be noted that both the membrane protein and intraluminal protein results presented are preliminary and more samples would need to be analysed to determine the significance of these findings.

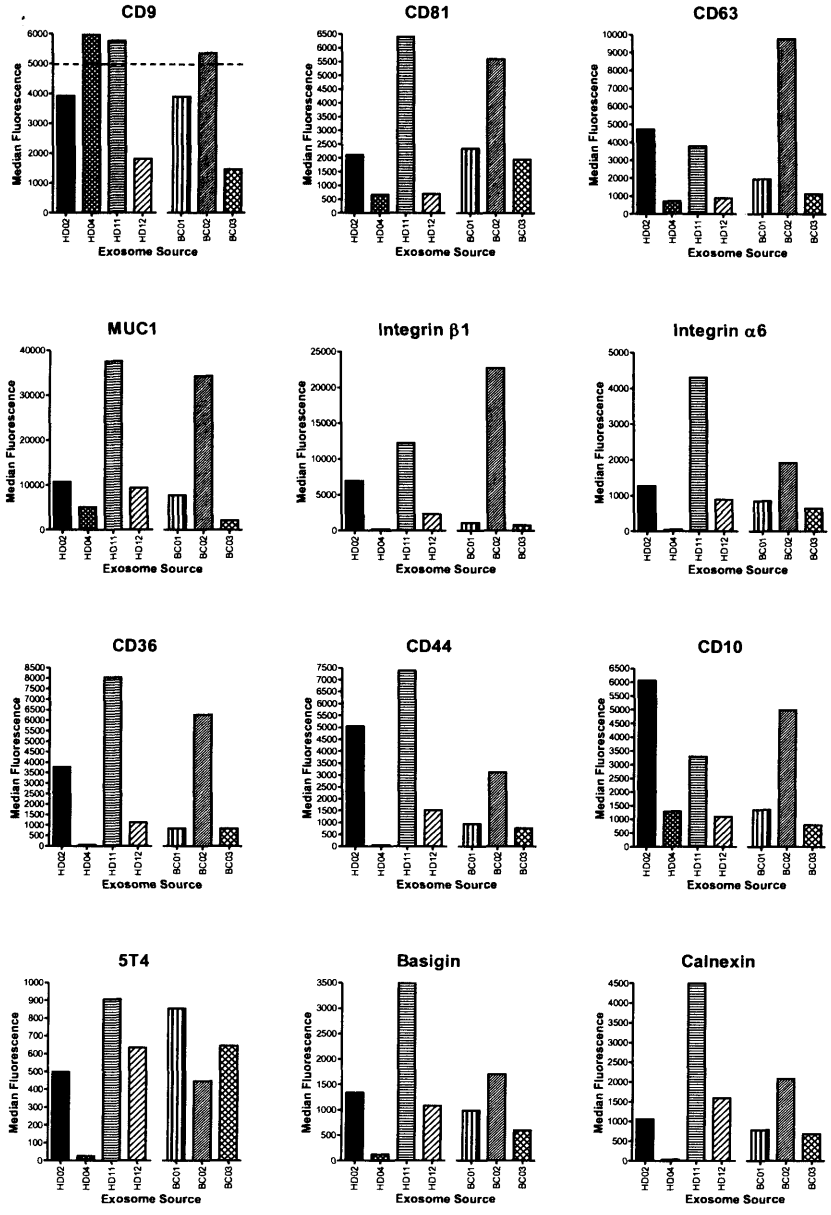


Figure 6.11: Examination of the expression of proteins of interest on the surface of urinary exosomes from healthy donors and bladder cancer patients.

Latex micro-beads were coated with urinary exosomes and labelled with a panel of Abs to identify any expression differences between healthy donors and BCa patients. Exosome-micro-bead complexes were subsequently counterstained with an Alexa Fluor-488 conjugated secondary and analysed by flow cytometry. The dashed line represents an arbitrary cut off point of median fluorescence 5000 for CD9 as a threshold for high quality exosome preparations.

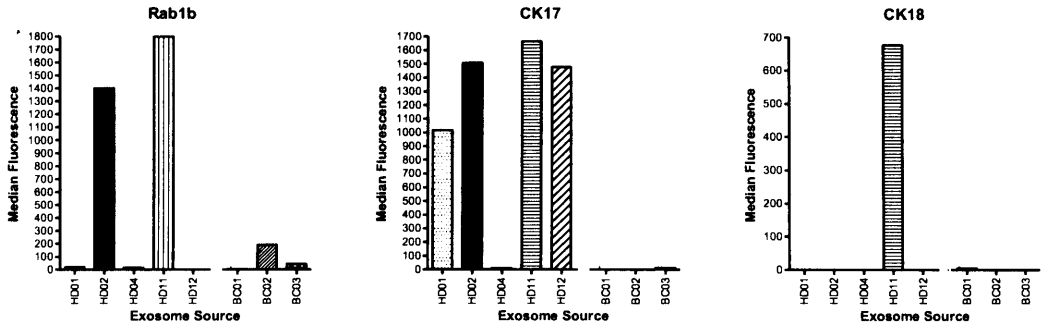


Figure 6.12: Examination of the expression of internal proteins of interest in urinary exosomes from healthy donors and bladder cancer patients.

Latex micro-beads were coated with urinary exosomes and subjected to fix-perm and labelling with a panel of Abs to identify any expression differences between healthy donors and BCa patients. Exosome-micro-bead complexes were subsequently counterstained with an Alexa Fluor-488 conjugated secondary and analysed by flow cytometry.

6.12 *Discussion*

The main aims of this chapter were to perform unbiased bioinformatic analysis of our HT1376-exosome proteome dataset, to verify that MS-identified exosomal proteins are present in exosome preparations and determine if these proteins are differentially expressed in exosomes from different sources.

The statistically based bioinformatics approach showed that our results are consistent with an exosome phenotype when compared with MS-identified exosomal protein datasets from other studies. The GeneGO MetaCore ORA demonstrated that the HT1376-exosome BCa proteome had the highest associations with bladder cancer in the disease biomarker category. This suggested that the exosome proteome may reflect the status of the originating cell very well and points to exosome analysis as a viable approach for discovering disease relevant proteins. Furthermore, other highly significant associations were observed with other carcinomas which indicated that the exosome proteome may contain a repertoire of proteins characteristic of a diverse range of carcinomas. The ORA also showed high associations with various vesicular compartments and the cytoplasm which is indicative of the origin of exosomes.

However, the analysis raised a number of issues with unexpected results such as predominant associations with the control of the cytoskeleton. Through manual inspections of the data this statistically discovered association appears to be valid. Cytoskeletal proteins identified included multiple cytokeratins (type I cytoskeletal keratins 1, 5, 6B, 7, 8, 13, 14, 16, 17, 18, and 19), actins (cytoplasmic 1, actin-like protein 6A, actin-related protein 2, and α cardiac muscle 1), myosin (myosin-1c, -10, and -14), tubulin (putative tubulin-like protein α-4B and tubulin β chain), and cytoskeletal linking proteins (filamin-A and –B, α-actinin-1 and -4, and plectin-1). Actin and myosin are both involved in vesicle transport, indicating that they may be involved in exosome formation or intracellular transport. Furthermore, some of these were confirmed by immunoblotting and/or flow cytometry of exosome coated micro-beads. The importance of cytoskeletal proteins in exosome biology is currently unknown.

There were also unexpected results in the cell compartment associations. Strong associations were identified with tissue specific organelles melanosomes and pigment granules. These are responsible for the storage of melanin in pigment cells of the eye and skin. This was a surprise as it is highly unlikely that BCa cells resemble pigment cells. Melanosomes are however a specialised endosomal compartment that strongly resembles multivesicular bodies in morphology and composition. Therefore, one explanation for this association is that the highly conserved molecular machinery involved in protein chaperone functions, membrane fusion and budding events, and trafficking of proteins to the melanosome may bear considerable resemblance to the machinery giving rise to exosomes [210].

A protein network was created from the dataset using GeneGO to give a theoretical indication of potential protein interactions that may influence formation, composition, and function of exosomes. For example, the oncogene c-Myc is often unregulated in cancer and this may increase the presence of proteins involved with cell proliferation, cell motility or adhesion [204]. It would be interesting to examine the effect of c-Myc inhibition on the protein composition of exosomes and subsequent function. However, this inhibition would likely be severely detrimental to the cell and cells that are dying/dead do not actively secrete exosomes. Here other targets within the network are probably better candidates for testing the validity of this network.

We also checked if some of the MS-identifications were in fact proteins expressed by exosomes. Each of the proteins chosen for verification was selected for its relevance/importance in cancer or exosome biology. All of the proteins examined were confirmed to be present in HT1376-exosomes. Only one protein we examined, α tubulin, was identified to be expressed equally in known exosome containing fractions (1.12 – 1.2 g/ml) and the hyperdense fractions of continuous sucrose gradient purified exosomes. This suggests that α tubulin may be a soluble constituent or is also present in denser vesicles, protein aggregates, or cell debris, as well as being exosomally expressed, having said that α tubulin has been identified by proteomics analysis of exosomes isolated by

immunocapture. This method eliminates soluble proteins which suggests that the presence of α tubulin in exosomes is genuine [96].

Other proteins (CD44 and CK17) were shown by immunoblotting to be expressed more strongly at denser regions of the exosome range. This could be due to protein or exosome aggregation or that these particular proteins may only be present in a dense sub-population of vesicles, but this needs to be confirmed. The presence of different proteins in different sized exosomes is something that has been explored. Kang *et al.,* (2008) discovered that different sized exosomes have different protein profiles but the functional/physiological relevance of such distinct subpopulations remains unclear [211].

Investigations into the expression of several MS-identified proteins in exosomes of other cell line origins identified four membrane proteins (basigin, CD44, CD73 and 5T4) that were enriched in exosomes compared to corresponding CLs. These enriched proteins may be specifically incorporated in to the exosomes and might therefore play an important role in their biogenesis or function. When comparing TCC to non-TCC derived exosomes few trends in expression were observed. This implies that these proteins are not a good disease selective choice. CD44 and α6 integrin showed a slight trend towards higher expression in TCC-exosomes. Some trends were also observed between anaplastic, moderately differentiated and well differentiated cell derived exosomes. However, these findings are not yet fully convincing and would require additional studies with clinically-derived materials. Another way to further analyse a proteins biomarker potential would be to analyse protein expression in normal and BCa tissue.

The development of a fix-perm assay for examining the intraluminal proteins of exosomes is not yet a fully validated and reproducible assay. Nevertheless, the assay has allowed us to demonstrate protein expression of cytokeratins by exosomes with scarce specimen quantity. Alterations in the cytokeratin profile of exosomes may be clinically useful because the CK profile can change with epithelial differentiation [164]. Exploring *ex vivo* exosomal CK profiles may therefore have the potential to help diagnose or act as a prognostic indicator for BCa. This is something which is worth investigating further.

However, the fix-perm assay would require further validation and many more samples would be needed to perform this.

The evaluation of the urinary exosome samples from BCa patients and healthy donors confirmed the presence of each protein in some if not all urinary exosomes examined. This confirmed that it is possible to detect potential biomarkers in exosomes derived from biological fluids, such as urine. Due to the limited number of samples available for analysis all of the data collected is preliminary. As a result any trends observed warrant further investigation. In addition, the few samples we did have were very variable in quality and quantity and this would need to be addressed if exosomes of consistently high quality were to be purified from urine samples.

This pilot *ex vivo* work highlighted the fact there is great variability between individuals and that each and every patient's cancer is different. All TCCs are not caused by the same genetic abnormality and therefore different patients may have different alterations in protein expression [212]. This heterogeneity needs to be taken in to consideration when looking for biomarkers. Some genetic alterations may be common to many subtypes of TCC leading to the altered expression of a common set of proteins. Therefore, it would be prudent to evaluate the biomarker potential of multiple proteins altered in cancer in order to form a biomarker panel. A biomarker panel is more likely to be able to take into account these individual differences compared with a single biomarker.

Overall, the MS-identified proteins chosen for verification in exosomes were present in some if not all exosome samples tested, including exosomes derived from BCa patient urine. The verification of these proteins using several different techniques helped to confirm the high sample and MS data quality used in this study. The examination of protein expression in exosomes and cell lysates has shown some proteins to be specifically enriched in exosomes. These proteins were membrane proteins suggesting that it is primarily membrane proteins that are incorporated into exosomes. This also implies that these enriched proteins could have specific functions in exosomes. The roles of most of these proteins in exosome biology remain unknown to date and therefore require more

attention. The proteins chosen for verification and evaluation were selected based on literature searches. This does not mean that other proteins within the dataset are not worthy of investigation or that the proteins examined were the most likely biomarkers.

In summary, the combination of the verification approaches used to assess the MS-dataset generated has shown that the exosome samples analysed were of high quality. We also demonstrated the genuine exosomal expression of a number of these identified proteins. The preliminary data presented here offers a platform for further investigations of the proteins identified. It may be particularly useful to evaluate the 63 unique exosomal protein identifications as these are the proteins which may only be present in BCa-exosomes and not in exosomes from other sources. Overall, the combination of protein verification, ORA, and protein networks has highlighted proteins potentially significant to exosome biology.

Chapter 7:

General discussion

7.1 *Summarising discussion*

Bladder cancer (BCa) is the fifth most common cancer in the UK and second only to prostate cancer as the most common cancer of the genitourinary tract [1]. The management of BCa involves a lot of careful staging and interpretation of clinical information. There are currently no sufficient non-invasive tests available to replace the invasive cystoscopic procedures required to further investigate suspected BCa and monitor its recurrence. The currently available non-invasive tests, such as BTA-stat, NMP22, and ImmunoCyt™ are unable to identify all BCa with high sensitivity and specificity and this is often be due to the influence of benign conditions. This highlights the need for new tools that can reduce the amount of invasive clinical work needed to diagnose and manage BCa. The research presented within this thesis has focussed on the identification and characterisation of proteins in purified exosomes. This work is important for improved biological understanding and the future potential for the development of novel BCa protein biomarker assays.

It has been proposed that molecular profiling or combining currently available diagnostic tests may improve the diagnosis and monitoring of BCa as opposed to using a single marker test [30, 31]. Whether multiple or single biomarkers are the way forward it is clear that exosomes are an ideal source of material for such assays. They are a subcellular fraction of the whole cell and known to be enriched in tumour antigens and membrane proteins [37-42]. These enriched proteins are thought to be specifically incorporated in to exosomes during their biogenesis in the endocytic tract [78, 79, 82]. As well as membrane proteins, some stress-related proteins can also be elevated in exosomes from cells undergoing forms of stress (hypoxia, heat and radiation). In this situation exosomes can represent the stress states of the parent cell. Overall, BCa derived exosomes may provide a complex panel of BCa associated protein markers that could be detected using an exosome based multiple biomarker assay.

There is no single accepted method for the purification of exosomes and this has unfortunately led the use of a variety of methods each providing samples with varying

degrees of purity. Methods utilising the intrinsic properties of exosomes [42, 47, 67, 133] rather than a simple pelleting method [47] should produce purer samples [133]. This was demonstrated by comparing our sucrose cushion method [67] to the simple pelleting method commonly used in other exosome studies [34, 59, 61, 99]. The sucrose cushion method purified exosomes showed higher expression of known exosomal proteins (for example CD9, CD81 and TSG101) and lower expression of the endoplasmic reticulum (ER) proteins compared with the simple pelleting method. Although more elaborate methods could improve the purity further (such as linear gradients) the sucrose cushion provided a good compromise approach satisfying purity, yield and preparation time.

The issues concerning the quality of exosome samples are something that needs to be addressed in the field and a consensus needs to be reached. Based on this study we take the view that at least one intrinsic property of exosomes is used for their isolation. Future studies should provide evidence demonstrating both enrichment of known exosome proteins, compared with the corresponding whole cell lysate, and the lack of known non-exosomal constituents. This evidence should also not be based on just a single technique but a combination such as immunoblotting, electron microscopy or flow cytometry analysis of exosomes micro-bead complexes. Overall the exosome sample analysed should be consistent with the exosome definition proposed in the thesis introduction (section 1.2.4). Most published studies do not reach these standards and this may call into question conclusions drawn from this work.

In the current study, high quality exosomes were consistently purified from the CM of several cell lines and when characterised were shown to be consistent with an exosome phenotype. The development of an exosome sample quality assurance assay (ExoQA) using minimal material to analyse the sample quality allowed us to measure the presence of tetraspanins in the samples as an indicator of quality. This flow cytometric assay of exosome micro-bead complexes also provided the basis for the verification of MS-identified proteins later in the study. The initial work optimising exosome purification and sample quality control was extremely important in ensuring that any further analysis of

these exosomes was of samples consistent with exosomes and not a complex mixture of vesicles, cell debris, and protein aggregates. Furthermore, it also established a bank of high quality cell line derived exosomes that could be used in future analyses. Overall, the sucrose cushion method for exosome purification was sufficient to achieve high sample quality which was determined using a minimal amount of sample.

When we purified exosomes from urine using the sucrose cushion method we were able to produce purer samples than the simple pelleting used by other groups [34]. We demonstrated that it was possible to purify exosomes from the urine of BCa patients and healthy donors. Unfortunately, the protein yield was 30 times lower than previously reported [143]. However, the pelleting method produced samples containing more non-exosomal contaminants giving an inaccurate overestimation of the true physiological concentration of urinary exosomes. We also observed variability in exosome yield which has been indicated in another urinary exosome study [188]. Differences in quantity and quality were observed between different donors as well as different samples from the same donor. It is not known what effect hydration state, proteinuria, haematuria or other variables have on the exosome content of urine. The potential effect of hydration status and endogenous protease activity was investigated in the current study, neither of which appeared to have any significant effect on exosome integrity and hence would not impact their capacity to be isolated using the sucrose cushion method. Whilst it was encouraging to have purified exosomes from urine the variability observed is a significant concern. We would need to address this before conducting meaningful proteomics analysis on such specimens. This was a major justification for examining a single homogenous TCC cell line as a more consistent source of BCa-exosomes for proteomics analysis.

Proteomics studies on exosomes have previously identified proteins of importance in exosome biology and also potentially significant proteins in disease [56, 57, 95, 144]. One major source of variation is the methods for preparing exosomes. Beyond this another significant problem lies in interpreting MS datasets in other studies. It is difficult to compare our dataset with other studies that have used peptide mass fingerprinting (PMF) or

MS/MS as it is common for the search criteria not to be reported [59, 87, 99]. Overall interpretation of nearly all exosome proteomics studies is difficult even with the development of the exosome proteome database ExoCarta [140].

In this study high quality exosomes, confirmed by ExoQA, derived from a well characterised human TCC cell line (HT1376) resulted in the identification of 353 proteins using LC-MALDI. Only proteins with good quality MS/MS data and two or more peptides were reported in the final results giving a false discovery rate (FDR) of 0%. If protein identifications based on one peptide had been included, as they are in some other exosome proteome studies [34, 59, 61], the FDR would have increased to 2.6%. This reaffirms the value of using a conservative approach to accept protein identifications.

The number of protein identifications made in the current study is amongst the highest in the exosome proteomics field [59, 61, 96, 101]. Furthermore, out of the 353 protein identifications 63 were unique to our study when compared with other studies in the ExoCarta database. The dataset presented is also arguably one of only two studies [96] with large numbers of identifications (>250) in which both strict exosome purification (utilising an inherent property of exosomes) and strict protein identification criteria (for example two or more peptides) are used. Our study and that of Mathivanan *et al.,* (2009) on colon carcinoma exosomes furthermore demonstrate similar numbers of protein identifications (353 and 394 respectively) [96]. The dataset presented in the current study also demonstrated a significant overlap with their exosome data. This suggests correlations with the protein phenotype of exosomes derived from carcinomas.

Unbiased statistical bioinformatics analysis (overrepresentation analysis (ORA)), comparing our dataset with the whole human genome showed our results to have high association with bladder and other carcinomas. Coupled with the comparisons with other human exosome proteomics studies which showed particularly high associations with exosomes from diverse carcinomas [96, 101], the bioinformatics analysis indicated that exosomes derived from BCa cells reflect shared features of carcinoma cells. The bioinformatics analysis has shown that using an unbiased method of analysis still

demonstrates that our BCa proteome is consistent with a typical/expected exosomal phenotype.

Manual inspection of our proteomics dataset revealed physiologically impossible numbers of MHC class I proteins. This issue appeared to be caused by homologous protein sequences within the MHC class I protein group. This is an important consideration when analysing proteomics data containing proteins with homologous sequences. It is an issue that will impact on most global proteomics studies and emphasises the importance of manually curating data.

Other unexpected results were also uncovered including the identification of a number of proteins from cellular compartments not considered to be represented in exosomes (nucleus, mitochondria and ER). However, many of these proteins were found in numerous other exosome proteome studies including those using rigorous purification techniques suggesting that they are indeed exosomally expressed [96]. Statistically high associations with the cytoskeleton were also uncovered and appeared to be true. A lot of the identified cytoskeletal proteins were cytokeratins. Interestingly the expression profile of cytokeratins is known to alter with differentiation of bladder epithelial cells. Therefore the cytokeratin profile of exosomes may be worth investigating further as they may reflect that of the parent cell. This may indicate the presence of a tumour and assist in tumour grading [164].

Several MS-identified proteins were verified as unequivocally present in HT1376 exosome samples using ultracentrifugation on continuous sucrose gradients. This approach identified 5T4, basigin, and others to be predominantly present at expected exosomal densities. Two of the proteins (CK17 and CD73) were expressed at a density slightly greater than expected. This indicated that exosomes of different densities may have different protein profiles which has also been shown by Kang *et al.,* (2008) [211]. Such details emphasise that even a homogenous cell source can produce an assortment of exosome subpopulations. Studies on the physiological importance of such populations, bearing distinct proteomes, have not yet been performed.

Overall, out of all the proteins verified the membrane proteins appeared to be of most interest. Only membrane proteins were found to be specifically enriched in cell line exosomes compared with their CLs. It appears that these proteins may be incorporated during exosome biogenesis to play specific roles in the extracellular functioning of exosomes. These enriched proteins may therefore be worth investigating further as biomarkers for cancer and with respect to their biological function.

Preliminary investigations regarding the expression of these MS-identified proteins in different BCa cell line and non-TCC cell line-exosomes uncovered no definite trends in protein expression. However, the expression of cell adhesion related proteins integrin α6 and CD44 were slightly elevated in the TCC-exosome compared with the non-TCC-exosomes indicating that these proteins require further investigation. Both of these proteins have incidentally also been shown to have altered expression in BCa [213, 214]. We also established that MS-identified proteins could be detected in urinary exosomes derived from BCa patient and healthy donor urine. Unfortunately, due to the very limited number of BCa samples we were unable to identify any specific trends in protein expression between health and disease. Further evaluation using a large number of high quality urinary exosome preparations and evaluation of their expression in healthy and BCa tissue is needed.

In summary, we have developed an LC-MALDI workflow for the analysis of BCa derived exosomes. This first published proteomics study on bladder cancer exosomes has yielded high quality protein identifications from high quality samples. The importance of using well characterised exosome specimens and strict MS criteria have been highlighted. The 63 previously unreported exosomal proteins could hold potential to be exosome BCa markers and may have the potential to be used in a multi-marker BCa test. Many of the proteins discovered may also have biological significance to exosome formation or function. The use of unbiased statistical bioinformatics analysis has demonstrated that our proteomics dataset reflects features of carcinoma cells further supporting the use of exosomes as a source of disease biomarkers. In addition, the proteins chosen for verification were present

in cell line derived and BCa urine exosomes. The study has opened the door to investigating the functions of exosomes with respect to BCa and in general. However, far more work is required to examine the proteins identified with respect to their biomarker potential and their exosomal function.

7.2 *Future directions*

The current study has generated a vast amount of data that may be significant in discovering BCa biomarkers. However, all of the potential marker proteins identified need substantial further evaluation ideally using clinically derived samples. Although such materials can be challenging in terms of exosome purification, as we demonstrated for urine, it remains important to pursue these aspects using such difficult samples. In addition to marker evaluation, the identifications summarise a complex molecular repertoire. The functions of most of these identifications in exosome biogenesis and function remain unexplored. There are many options moving forward and some of the most promising are discussed below.

7.2.1 Comparative proteomics analysis of urinary exosomes to identify BCa markers

Comparative proteomics of BCa patient and healthy donor urinary exosomes is the ultimate goal and this approach should highlight the differences between health and disease. However, in Chapter 4 of this thesis it was found that urine was a very challenging source of exosomes. Urinary exosomes purified by our sucrose cushion method were found to be very variable in quantity and quality. Therefore much work is required to optimise preparation methods to obtain purer exosomes and to reduce the variability observed. One way of reducing the irregularity in quantity in order to create a more consistent sample may be to do a 24 h urine collection with the intention of averaging out the periodic variability that occurs throughout the day. However, it is not known whether 24 h urine collection or improved purification procedures will actually increase the total amount of exosomes purified. If urine exosome levels are inherently low these methods cannot themselves elevate the yields significantly as this is a physiological issue.

To reduce the variability in sample quality one approach that could address this would be modifying the purification procedure possibly by using linear sucrose gradient purification. The fractions of known exosomal density (1.12 and 1.2 g/ml) would then be analysed [47]. However, co-localisation of common soluble proteins (albumin and immunoglobulins) with exosomes has been observed with this method of isolation [68]. This could be a particular problem if haematuria or proteinuria is present [68]. Alternatively or in combination with sucrose gradient isolation, urinary exosomes could also be isolated using an immunoaffinity capture method [133]. For example an anti-CD9 antibody may be the best choice of capture antibody as CD9 was shown to be expressed on the surface of all of the cell line and urine derived exosomes examined. The use of this common exosomal protein may improve the chances of capturing all of the exosomes compared with using a specific antibody which may only capture a subset of the exosomes. For example, one group has utilised the colon cancer antigen A33 to immunoisolate exosomes for proteomic analysis [96]. However, there is still a danger of missing any sub populations that are CD9 negative. Immunoisolation, with or without sucrose gradient purification, may be the best method for isolating exosomes from complex material such as biological fluids because the presence of common contaminating proteins or haematuria should not affect the quality of the sample [33, 64].

It may be possible to use limited clinical samples to perform differential proteomics analysis by tagging the peptides using iTRAQ (isobaric tag for relative and absolute quantitation) followed by LC-MS/MS. This would allow the relative expression of each protein identified to be quantified and any differences could be examined.

7.2.2 Validation of the GeneGo protein network

The auto expand tool in GeneGo provided information regarding the theoretical interactions of exosomal proteins identified in the current study. Further investigations are required to examine whether these interactions are real and if they are significant to exosome composition and/or function. Several MS-identified exosomal proteins were found to be prominent nodes in the network including EGFR, ubiquitin, Rac1, and RhoA.

Mutations in the EGFR gene are known to lead to upregulation of EGFR in BCa and has been suggested as a prognostic indicator [215]. Using a small interfering RNA (siRNA) to impede the expression of EGFR might allow any changes in the exosomal expression of proteins associated with EGFR in our network, such as guanine nucleotide-binding protein G(i), alpha-2 subunit (G-protein alpha-i2), glutathione S-transferase P (GSTP1), MUC1, β1 integrin (ITGB1), MHC Class I (HLA-Cw3), to be examined. This would demonstrate whether cellular EGFR has an influence on exosome protein composition and would be the first step I propose for validating this network.

Similar studies could be performed examining Rac1 and RhoA both of which are known to be involved in cancer [216]. The inhibition of RhoA in particular could theoretically have a negative effect on the expression of β1 integrin and sequentially effect the expression of 4F2 cell-surface antigen heavy chain and large neutral amino acids transporter small subunit 1. Furthermore the interactions of our proteins with other proteins exosomal or non-exosomal can also be explored using ExoCarta [140]. ExoCarta provides interaction network information for known exosomal proteins from BioGRID a protein interaction database [201]. GeneGo MetaDrug™ may also prove useful in identifying protein targets and which compounds can be used to disrupt their function. In addition MetaDrug™ can provide *in silico* predictions of toxicity. It can also present network information visualising signalling and metabolic pathways that may be useful in choosing a target and/or seeing its potential effect on a pathway [217].

Using the information provided by the network formed from our dataset may provide a highly novel approach to gain insight into the complex interactions and pathways of relevance to exosome biogenesis. It may also present other opportunities to intervene in a strategic manner to generate "designer exosomes" with enhanced or attenuated function.

7.2.3 Further investigations of the unique exosomal protein identifications

Out of the 353 proteins identified in HT1376 BCa exosomes 63 proteins have not been identified in exosomes from any other source. This suggests that there may be value in these 63 proteins as features unique to bladder epithelia or BCa. Further evaluation of this list is required to investigate their biomarker potential and/or their possible relevance in cancer biology and exosome function. As far as biomarker potential is concerned, literature searches looking for relationships between the protein and cancer may be a reasonable starting point to highlight candidates. Also analysis of the biological function of proteins in general would be informative in order to evaluate relationships with cellular events such as angiogenesis, cell adhesion, cell proliferation, cell migration and invasion, and immune modulation which are all affected in cancer.

One example could be the laminin complex. The dataset included three laminin subunit proteins (laminin subunit α-3, β-3 and γ-2) of the extracellular complex glycoprotein laminin. Laminins are involved in cell adhesion, cell migration, signal transduction and chemotaxis. The gene ontology information available categorises these proteins as cell membrane and extracellular. These three subunits in particular form laminin-5 which is expressed by epithelial cells [218]. In a review of the role of laminin-5 in epithelial tumour invasion, by Katayama and Sekiguchi (2003), the relationship between α6β4 and α3β1 integrins (all identified in the current exosomal protein dataset) and laminin-5 were described in detail. Bound laminin-5 and α6β4 integrins were particularly implicated in invasion and metastasis [218]. The information available suggests that laminin-5 along with α6β4 integrins may be of potential importance in the function of exosomes in tumour invasion and metastasis. It is not known whether laminin-5 is expressed by the exosome or that it is extracellular and bound to the exosomal integrins. Furthermore their reported increased expression in the tumour environment may be reflected in exosomes hence the laminin-5 subunits may be exosomal biomarkers for BCa [218].

Membrane proteins of potential interest in exosome biology include vesicle-associated membrane protein-associated protein A and syntaxin-4 as they are involved with vesicle transport and fusion. Hematopoietic cell protein CD70 was also identified and is normally involved in T-cell activation. However it has been found to be aberrantly expressed in epithelial cell carcinomas. This aberrant expression has sparked interest in CD70 as a target for therapy [219]. Therefore it may be useful to know if it is genuinely exosomally expressed and if this expression is BCa specific. Urokinase-type plasminogen activator (uPA) could also be of interest as an exosomal BCa marker as it has been previously found to be elevated (16 fold) in TCC tissue compared with matched normal tissue [220]. Furthermore, measuring uPA levels in urine in addition to NMP22 and urine cytology has been shown to improve their ability to predict TCC [221]. UPA may therefore be worth investigating further. There are many more examples of this sort buried within the 63 unique protein list that could be explored in the future in the context of clinical utility.

7.2.4 Do certain exosomal proteins influence cancer biology?

Several membrane proteins from the list of MS-identified proteins were verified within the study and were shown to be enriched in exosomes relative to whole cell lysates. This phenomenon is an aspect documented for several proteins that have exosome-related functions (such as MHC molecules). This suggests such proteins may therefore be particularly important in terms of the natural physiological functions of exosomes. The expression of functional membrane associated adhesion/signalling molecules or enzymes by exosomes offers a unique mechanisms for distributing these within the local microenvironment or even systemically.

Whilst there are many examples of molecular shedding from the plasma membrane an exosome route of dispersal holds the potential advantage of retaining the full functionality of the molecule. Soluble versions of membrane molecules are often relatively poorly efficient in their functions (e.g. CD59 [222]) or may even exhibit opposing functions compared to the membrane bound counterpart (e.g. betaglycan that sequesters TGFβ-functions [223, 224]). Thus the exosome pathway generates a vast surface area for

disseminating membranous proteins and hence aid in amplifying some of these functions. It would be interesting to examine the potential function of some of these exosomal proteins in and their potential influence on varied aspects of cancer biology. Some examples are briefly described below.

CD44

The cell surface glycoprotein CD44 is the principle surface receptor for hyaluronic acid (HA) (hyaluronan). CD44 is involved in many biological processes including regulation of growth, survival, differentiation and motility [214]. CD44 expression is upregulated in many cancers and can predict progression of several cancers [214, 225]. HA interaction with CD44 has been previously shown, by Misra *et al.,* (2008), to activate the ErbB2, phosphoinositide 3-kinase (PI3K)/AKT, β-catenin pathway. The activation of this pathway promotes cell survival [225]. Misra indicated a positive feedback loop between HA and PI3K/AKT, pathway induced by the interaction of CD44 with HA [226]. Exosomes derived from cancer cells may potentially aid cell survival through passing on their CD44 to another cell by a mechanism currently unknown, perhaps by fusion with a recipient cells plasma membrane. Although this process has not yet been convincingly demonstrated for exosomes such a mechanism has been shown for microvesicles bearing epidermal growth factor receptor (EGFR). The study shows a microvesicular mechanism for dissemination of EGFR with neighbouring cells resulting in hyper-responsiveness to EGF [227]. This type of delivery would increase the number of CD44 proteins available at the cell surface for binding to HA for the induction of the PI3K/AKT survival pathway. This may further enhance the positive feedback loop involving HA and the PI3K/AKT pathway. However, it is not known if exosomes sequester rather than enhance this HA-CD44 signalling. In addition CD44 is thought to interact with the glycoprotein basigin (CD147, emmprin) which was identified in the current study to be enriched in exosomes from various cancer cell lines.

Basigin

Basigin (EMMPRIN or CD147) also stimulates HA production and therefore downstream signalling cascades [207, 208] and is a member of the immunoglobulin superfamily capable of inducing matrix metalloproteinase (MMP) expression in fibroblasts. MMPs mediate the break down and remodelling of matrix for cell migration [228]. The function of exosomal basigin has been investigated with respect to cardiomyocyte progenitor cell-derived exosomes and their effect on the migratory capacity of endothelial cells. Exosomes were able to stimulate endothelial cell migration using an *in vitro* scratch wound assay [229]. Basigin has also been previously described in microvesicles (MV) and its release has been suggested to be involved in tumour-stromal interactions. This study showed the presence of basigin on the surface of MV but not exosomes. However, exosomes were a constituent of their MV sample and hence it might be the case that exosomal basigin is responsible for this effect [230]. The current study has shown unequivocal expression of basigin in HT1376-exosomes purified by continuous sucrose gradient using both immunoblotting and flow cytometry of exosome micro-bead complexes. This suggests that exosomes may influence tumour-stromal interactions aiding tumour invasion and metastasis.

Basigin has also been demonstrated to be associated with integrin isoforms $\alpha6\beta1$ and $\alpha3\beta1$ which are involved in several processes related to metastasis. It is thought that basigin may regulate the integrin/laminin-5 association mentioned in the previous section [231]. Finding such associations in the literature serves to underline the value of performing exosome proteomics as we have done. It also emphasises that such multi-molecular complexes, with previously defined roles, are likely present as functionally viable intact complexes in/on exosomes.

CD73

CD73 or 5'-nucleotidase is a glycosyl phosphatidylinositol (GPI) anchored enzyme and was the first protein to be identified in association with exosomes [232]. It is able to hydrolyse the phosphate group from adenosine monophosphate (AMP) to generate

extracellular adenosine. This adenosine can then be detected by adenosine binding receptors (A1, A2A, A2B, and A3) on the surface of assorted granulocytes and mononuclear cells and modulate inflammatory responses. For T cells, adenosine is a potent suppressor of effector functions [233].

Jin *et al.,* (2010) demonstrated that the CD73 on the tumour cell surface was able to impair T cell responses through the generation of extracellular adenosine [206], and that adenosine production may be an important immune suppressive mechanism for regulatory T cells [234]. We hypothesise that exosomally expressed CD73 may be functional in adenosine generation. Exosomally generated adenosine would contribute to adenosine in the tumour microenvironment and mediate immune suppressive/anti inflammatory effects.

7.3 *Concluding comment*

This thesis has demonstrated that it is possible to generate a proteomics dataset of high quality using BCa exosomes. This has not been previously done and therefore this data is of notable value. The data agrees with proteomics studies on exosomes from other sources in that the proteome is classically one of an exosome phenotype. Given the extensive quality control during exosome purification, detailed characterisation of exosomes and careful proteomics analysis performed this study is one of the very strongest proteomics analyses performed to date on exosomes.

Follow up studies based on some of these identifications will significantly aid our understanding of the biogenesis, interactions and functions that cancer derived exosomes may perform. In addition it is a valuable resource for further studies in the realm of biomarker discovery.

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Appendix 1:

Peer reviewed publications arising from the study

Journal of Translational Medicine BioMed Central

Research

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Can urinary exosomes act as treatment response markers in prostate cancer?

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Abstract

Background: Recently, nanometer sized vesicles (termed exosomes) have been described as a component of urine. Such vesicles may be a useful non-invasive source of markers in renal disease. Their utility as a source of markers in urological cancer remains unstudied. Our aim in this study was to investigate the feasibility and value of analysing urinary exosomes in prostate cancer patients undergoing standard therapy.

Methods: Ten patients (with locally advanced PCa) provided spot urine specimens at three time points during standard therapy. Patients received 3–6 months neoadjuvant androgen deprivation therapy prior to radical radiotherapy, comprising a single phase delivering 55 Gy in 20 fractions to the prostate and 44 Gy in 20 fractions to the pelvic nodes. Patients were continued on adjuvant ADT according to clinical need. Exosomes were purified, and the phenotype compared to exosomes isolated from the prostate cancer cell line LNcaP. A control group of 10 healthy donors was included. Serum PSA was used as a surrogate treatment response marker. Exosomes present in urine were quantified, and expression of prostate markers (PSA and PSMA) and tumour-associated marker 5T4 was examined.

Results: The quantity and quality of exosomes present in urine was highly variable, even though we handled all materials freshly and used methods optimized for obtaining highly pure exosomes. There was approx 2-fold decrease in urinary exosome content following 12 weeks ADT, but this was not sustained during radiotherapy. Nevertheless, PSA and PSMA were present in 20 of 24 PCa specimens, and not detected in healthy donor specimens. There was a clear treatment-related decrease in exosomal prostate markers in 1 (of 8) patient.

Conclusion: Evaluating urinary-exosomes remains difficult, given the variability of exosomes in urine specimens. Nevertheless, this approach holds promise as a non-invasive source of multiple markers of malignancy that could provide clinically useful information.

Background

Prostate cancer (PCa) remains the most prevalent male cancer in the west, with projected 186,000 new cases, and 28,000 deaths in the USA expected in 2008 (American Cancer Society, Atlanta, Georgia 2008). Whilst advances are being made in understanding the biology underlying this disease, and in many respects in its treatment, there remains a need for better tools for PCa diagnosis and monitoring.

Disease-related biomarker(s) should ideally be non-invasively available; urine-analysis fits this requirement well. Several urine-borne molecules are currently being evaluated as PCa-indicators [1-10], but recently, approaches measuring several candidate urine-markers at once may give a more complete clinical picture [11-13].

Nano-meter sized vesicles (termed exosomes) are an additional component of urine [14], which have been proposed as a possible source of multiple biomarkers of renal disease [14,15] in particular, but perhaps also of interest in urological cancer. Exosomes are a notable feature of malignancy, with elevated exosome secretion [16] and tumour-antigen enrichment of exosomes associated with cancer cells [17,18]. The physiological importance of cancer exosomes remains unclear. There are several studies suggesting they may act as an advantageous source of multiple tumour rejection antigens for activating anti-cancer immune responses [17-19]. Cancer exosomes have been proposed by some as possible therapeutic vaccines [20]. Paradoxically, however, there is also a growing number of reports demonstrating active immune-suppressive functions for cancer exosomes, assisting cancers evade immune attack [21-24]. Cancer exosomes may also contribute to angiogenic processes [25], may disseminate metastatic potential in certain settings [26] and could play roles in drug resistance [27].

From a biomarker perspective, the expression of tumour-associated antigens by exosomes naturally raises questions about the possible value of these nano-vesicles as markers of malignancy. Furthermore, exosomes may be a source of important cancer-associated antigens not available as soluble molecules within biological fluids, such as the oncofetal glycoprotein-5T4; which is over expressed by epithelial cancers but not shed from the cell surface [28]. Biological changes related to malignancy of the genitourinary tract, or to therapy, may perhaps be mirrored by changes in urinary exosomes.

In this report, we present a pilot study with the key aim of evaluating the feasibility of studying urine exosomes of PCa patients, as tools for monitoring response to treatment. Whilst we have discovered some difficulties such as variability and low quantity of urine-borne exosomes, the study provides the first encouraging evidence suggesting that further molecular analyses of urine exosomes in PCa are warranted.

Methods

Prostate Cancer patients and healthy donors

Ten PCa patients, participating in a local Phase II Clinical Trial, were recruited, together with 10 healthy male volunteers. The patients were confirmed positive for PCa by biopsy, and the tumour stage, Gleason score, serum-PSA and age is summarised in Table 1. Patients received 3–6 months neoadjuvant androgen deprivation therapy (ADT) prior to radical radiotherapy (RT), which consisted of a single phase delivering 55 Gy in 20 fractions to the prostate and 44 Gy in 20 fractions to the pelvic nodes. Patients were continued on adjuvant ADT according to clinical need. The trial was approved by the South East Wales Ethics Committee.

Urine sample collection

Urine, up to 200 ml volume, collected into sterile containers (Millipore), was brought to the laboratory for processing within 30 minutes. Samples were collected mid to late morning, and these were not first-morning urine. Urine was tested for blood, proteins, glucose and Ketones and the pH was measured; (by Combur^5 Test®D, dipstick (Roche)) (summarised in Table 2). PCa-patient urine was collected at three time points: "ADT_4" (0–4 weeks after initiation of ADT), "ADT_{12}" (following three months of ADT) and "RT_{20}" (after 20 fractions of Radiotherapy). At intervals during treatment (ADT_4, ADT_{12} and at 4 weeks post Radiotherapy), serum PSA levels were measured.

Exosome purification

Urine was subjected to serial centrifugation, removing cells (300 g, 10 min), removing non-cellular debris (2000 g, 15 min). The supernatant was then underlayed with a 30% sucrose/D2O cushion, and subjected to ultracentrifugation at 100,000 g for 2 h as described [17,23,29]. The cushion was collected, and exosomes washed in PBS. Exosome pellets were resuspended in 100–150 ul of PBS and frozen at -80°C. The quantity of exosomes was determined by the micro BCA protein assay (Pierce/Thermo Scientific).

Cell culture

LNCaP and DU145 prostate cancer cell lines (from ATCC), were seeded into bioreactor flasks (from Integra), and maintained at high density culture for exosome production as described [30].

Electrophoresis and Immuno-blotting

Cell lysates were compared to exosomes by immuno-blotting as described [31]. Primary monoclonal antibodies included mouse anti-human PSA (a gift from Dr Atilla

Table 1: Details of patients participating in this study

Patient	Clinical Stage (all N0)	Gleason Score	Age (years)	Serum PSA ADT_4 (ng/ml)	Serum PSA ADT_{12} (ng/ml)	Serum PSA at 6 months (ng/ml)
1	T2b	7 (3+4)	66	10.5	2.10	1.2
2	T2b	7 (3+4)	62	134.0	0.20	<0.01
3	T2	8 (3+5)	70	8.3	1.40	<0.1
4†	n/d	7 (3+4)	65	83.2	83.40	†
5	T2c	7 (3+4)	69	95.2	4.10	<0.1
6	T2	8 (4+4)	70	10.8	0.10	<0.1
7	T3a	7 (3+4)	53	36.5	7.20	0.3
8	T3b	6 (3+3)	61	14.1	0.80	0
9	T2	7 (4+3)	66	21.1	0.20	<0.1
10	T2	8 (4+4)	71	28.1	1.3	n/d

† Patient died from an unrelated brain tumour prior to Radiation Treatment.
n/d not determined.

Turkes, Cardiff and Vale NHS Trust, Cardiff), anti-TSG101, anti-LAMP-1, anti-HSP90, anti-Calnexin, anti-CD81 and anti-PSMA (from Santa Cruz Biotechnology), anti GAPDH (from BioChain Institute, Inc), anti CD9 (from R&D systems). Anti-5T4 was a gift from Dr R Harrop (Oxford BioMedica UK Ltd). Goat polyclonal anti-Tamm Horsfall Protein (THP) was from Santa Cruz, and bands were detected using anti-goat-HRP (Dako). Membranes were stripped using the Restore Plus™ western blotting stripping buffer (Pierce/Thermo Scientific), blocked overnight, and re-probed.

Examining exosome membrane integrity

To investigate if urine damages exosome-membranes, exosomes isolated from B-cell lines, were immobilised onto anti-MHC Class-II coated dynal-beads (Dynal/Invitrogen) [32]. The exosome-bead complexes incubated overnight at 37°C in 25 mM Calcein-AM as described [31]. Calcein-loaded exosome-bead complexes were exposed to various salt-solutions or to fresh urine, at room temperature for 1 h. Fluorescence was analysed by flow cytometry (FACScan, BD), running Cell Quest software (BD). Calcein-fluorescence was compared to fluorescence of anti-Class-I (RPE) stained exosome-beads, in parallel tubes; a measure of whether exosomes remain attached to the bead surface. Results are expressed as the ratio of Calcein: Class-I fluorescence.

Examining proteolytic damage of exosomes by urine

Exosomes purified from LNCaP cells, were treated with fresh urine in the presence or absence of protease inhibitors (including EDTA, Pepstatin-A, Leupeptin and PMSF). After 2 h or 18 h, samples were examined by western blot for expression of CD9, PSA and TSG101. As a positive control for proteolysis, exosomes were treated with trypsin (Cambrex).

Results

Purification of urinary exosomes

We used a standardised method, designed for exosome-purification from cell culture supernatant, and have applied this to fresh-urine as an exosome source. With this method, exosomes are isolated based on their buoyancy characteristics [33]. Analysis of protein content of urine at multiple steps throughout purification, revealed the method was effective in eliminating principal contaminants (Fig 1a), (such as the band at 80 Kd) while significantly concentrating vesicles bearing a distinct protein repertoire, across the entire molecular weight spectrum (Fig 1a). Performing immuno-blot analyses on parallel gels revealed typical exosomal proteins were only detected in the final exosome-product (Fig 1b).

Comparing this method with the method of Pisitkun *et al* [14], using cell culture supernatants (Fig 1c) or healthy donor urine (Fig 1d) as source material, showed the

Table 2: Details of urine specimens collected from PCa patients

Patient	Time Point	Dip-Stick Blood, Protein, Glucose, Ketones, pH					Specimen Volume (ml)	Total Exosomes Recovered (μg)	Exosome Concentration (ng/ml)
1	ADT_4	1	1	0	0	7	90	72.9	810.0
	ADT_{12}	0	1	0	0	5	180	141.9	788.3
	RT_{20}	0	0	0	0	7	180	19.6	109.3
2	ADT_4	1	1	0	0	-	170	125.5	738.2
	ADT_{12}	1	2	0	0	7	180	2.61	14.5
	RT_{20}	0	0	0	0	5	90	39.2	435.7
3	ADT_4	4	2	4	0	5	180	72.9	405.3
	ADT_{12}	2	1	1	0	5	180	70.9	393.9
	RT_{20}	0	1	1	0	5	60	8	133.3
4†	ADT_4	0	1	0	0	5	95	25.4	268.0
	ADT_{12}	1	3	3	0	5	55	6.54	118.9
	RT_{20}	-	-	-	-	-	-	-	-
5	ADT_4	4	0	0	0	5	180	38.4	213.6
	ADT_{12}	1	2	0	0	7	90	27.1	301.2
	RT_{20}	1	1	0	0	6	150	5.1	34.5
6	ADT_4	0	0	0	0	6	180	19.4	108.1
	ADT_{12}	1	0	0	0	5	180	6.2	34.7
	RT_{20}	1	1	0	0	5	120	9.1	76.1
7	ADT_4	3	1	1	0	6	97	39	402.1
	ADT_{12}	0	1	0	0	5	120	12.1	101.0
	RT_{20}	1	1	0	0	5	45	17.7	395.1
8	ADT_4	0	1	1	0	6	150	125.1	834.4
	ADT_{12}	0	1	0	0	5	110	26	236.4
	RT_{20}	1	3	0	0	7	60	34.4	574.0
9	ADT_4	0	1	0	0	5	120	8.2	68.3
	ADT_{12}	0	1	0	0	6	180	17	94.4
	RT_{20}	2	3	4	0	6	60	133.1	2218.7
10	ADT_4	0	1	0	0	5	120	19.4	162.3
	ADT_{12}	0	0	0	0	7	180	11.4	63.4
	RT_{20}	0	0	0	0	6	170	88.3	519.4

† Patient 4 died before RT
- Not recorded, or sample unavailable

sucrose method results in a pellet which is more enriched in exosomes, evident by strong band intensity for exosome markers such as CD9, TSG101 and LAMP-1. Importantly, the sucrose method resulted in good enrichment of tumour associated antigens; in this case 5T4 (Figure 1c), indicating an important advantage in analysis of exosomes over pelleted sediment [14]. Although many markers were detected in the comparator preparation, these were at a lower level. The more intense band for calnexin (a non-exosomally expressed marker), is evidence for more contaminants when using the comparator method (Fig 1c). Similarly, with urine as the source material, the sucrose-cushion method again proved advantageous (Fig 1d), showing higher levels of exosome expressed proteins, and reduced contamination with Tamm Horfsall protein (THP). The data support this approach for enriching exosomes from fresh urine specimens; and confers some advantages over previously published urine-exosome protocols.

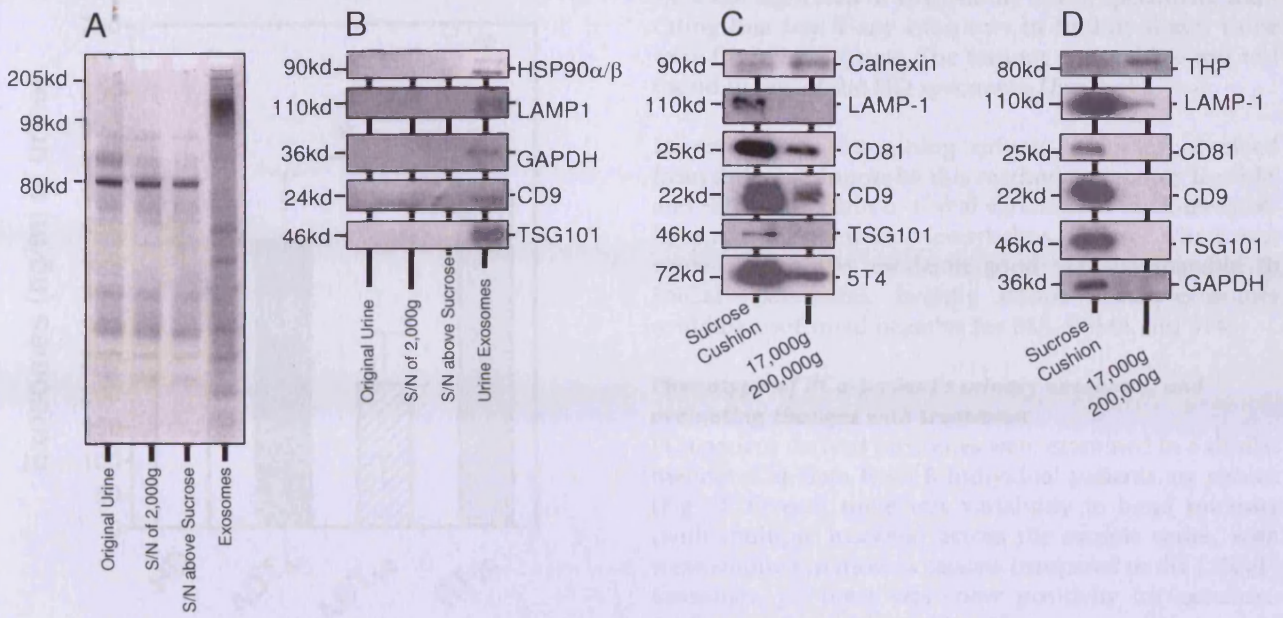

Figure 1

Purification of urine-derived exosomes. Healthy donor urine was subjected to exosome purification, and at each step, 10 μl of sample was kept for electrophoretic analysis (4–20% gradient polyacrylamide gel, silver stained) (A), demonstrating effective removal of the principal non-exosomal protein bands such as that at ~80 Kd, and significant enrichment of diverse protein species in the final exosome product (A). Parallel gels were run for immuno-blot analyses, using antibodies against typical exosome proteins as indicated (B). Comparing the sucrose cushion method, with a simpler method of Pisitkun et al, where cell culture media (C) or fresh urine (D) were subject to centrifugation at 17,000 g followed by pelletting at 200,000 g. Exosomes (from sucrose method) and the 200,000 g pellet were normalised for protein differences, and 2.5 μg/well analysed by western blot for markers as indicated.

Changes in urine-exosome quantity during PCa therapy

The quantity of exosomes present in each preparation was measured, corrected for starting urine volume, and values compared across the patient (Table 2) and healthy donor (Table 3) groups are summarised in figure 2. Prostate cancer patients on average had 1.2-fold higher levels of urinary exosomes (at ADT_4) compared to healthy men. There was broad variation in the exosome-content across both the healthy donors (366.8 ± 92.56, n = 10 mean ± SE) and patients (443.2 ± 109.7, n = 10, ADT_4). After three months of androgen deprivation therapy (ADT_{12}) there was a ~2-fold decrease in exosome levels (224.9 ± 82.7, n = 10), with 8 out of 10 patients showing a decrease in exosome quantity. In terms of radiation treatment (RT_{20}, 499.6 ± 225.6, n = 9), there was no significant difference compared to ADT_4 or to ADT_{12}, as 3 out of 9 patients demonstrated a further decrease in exosome levels, whilst 6 out of 9 had increasing urinary exosome levels. There was a decrease in serum PSA levels in 9/10 patients, demonstrating that standard therapy was successful in tumour bulk reduction.

In conclusion it is not possible to demonstrate a correlation between locally advanced PCa with the quantity of exosomes present in urine, and there is no correlation between serum PSA and urinary-exosome levels. From the current data set, there is some suggestion however, that at ADT_{12} there is a decrease in the amount of exosomes present.

Prostate Cancer cell lines produce typical exosomes, positive for prostate and cancer-associated antigens

Two prostate cancer cell lines were maintained in culture, as a source of PCa-exosomes, and the expression of typical exosome-markers (e.g. the tetraspanin CD9) and some known markers of prostate (PSA and PSMA) were examined. The LNCaP cells (whole cell lysates) were directly compared to LNCaP-exosomes by immuno-blot, revealing positive exosomal expression of PSA and PSMA. There was also clear positive exosomal expression of 5T4 by LNCaP-exosomes. Both PSA and 5T4 were particularly enriched in exosomes, compared to the parent cell (Fig 3A). The DU145 cell line, which does not express PSA or PSMA served as a control demonstrating specific staining.

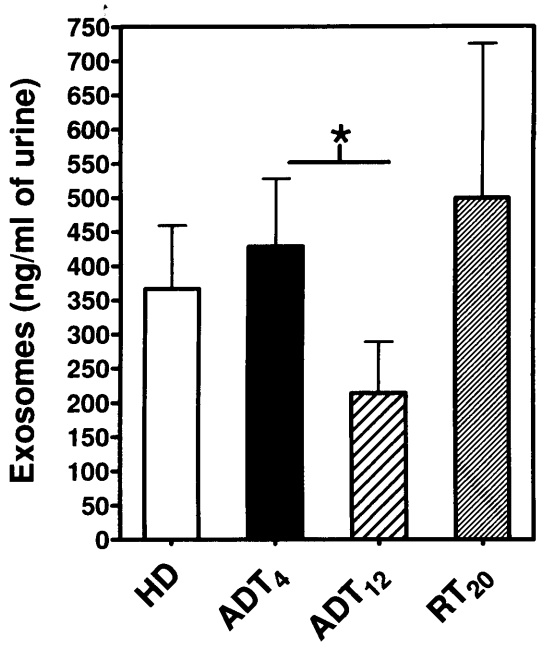

Figure 2
Quantification of urine-derived exosomes, in healthy donors, and Prostate Cancer patients. The quantity of exosomes present in each preparation was measured using the BCA protein assay. Values were corrected for urine-specimen volume, and are represented as ng Exosomes per ml of urine. Preparations from 10 healthy donors and 10 PCa patients undergoing standard therapy, at ADT_4 (after 4 weeks ADT), ADT_{12} (after 3 months of ADT), and at RT_{20} (and after 20-fractions of radiotherapy) are compared. Bars represent mean+SE. *$p < 0.5$ using the Wilcoxon matched pairs test are shown.

Staining for GAPDH showed equal loading of wells. We concluded that exosomes isolated from PCa cells express molecules typical of exosomes from other cellular sources together with prostate markers and tumour-associated antigen(s). This immuno-blot panel was considered suitable for analysis of urinary exosomes in following studies.

The phenotype of healthy donor urinary exosomes

We performed analyses of urinary-exosomes from healthy donors (HD), and compared expression levels for these molecules to those of LNCaP-derived exosomes. Markers such as TSG101 and CD9 were detected in most HD-specimens by western blot, albeit at low levels compared to the LNCaP standard, suggesting that at least some exosomes were present in these specimens. There was considerable variability in band intensity obtained across these donors, even though analyses were all normalised for differences in protein. Prostate markers (PSA and PSMA) were not expressed in any healthy donor specimens, indicating that few if any exosomes in healthy donor urine arise from the prostate. The tumour antigen 5T4 was not found in any of the HD specimens (Figure 4).

In conclusion, examining urinary-exosomes obtained from different donors by this method is certainly feasible, and this is sufficient to reveal variation in exosome-quality across the samples. Nevertheless, in cases where exosome-quality was moderate/good (i.e. comparable to LNCaP exosomes), healthy donor urinary-exosomes could be confirmed negative for PSA, PSMA and 5T4.

Phenotype of PCa-patient's urinary exosomes, and evaluating changes with treatment

PCa patient derived exosomes were examined in a similar manner. The data from 8 individual patients are shown (Fig 5). Overall there was variability in band intensity (with multiple markers) across the sample series, with weak staining in most occasions compared to the LNCaP-exosomes, yet there was some positivity for exosome-markers in 20 of 24 samples. There was variation across the patient cohort, and variation from within an individual's sample series (ADT_4, ADT_{12} and RT_{20}). As great attention was paid towards loading 5 μg of sample per well, we believe the results more likely reflect the variable exosomal content of the sample, rather than technical issues of sample loading. Bands for prostate-derived proteins PSA or PSMA were evident in 5 patients (p1, p7, p8, p9, p10), indicating that at least some of the exosomes present in the urine were of prostate origin. Given the variation in band intensity across the three time points in most of these samples it is not possible to demonstrate phenotypic changes in response to treatment. The exception to this is shown by patient 8, in which band intensities for exosome-markers were stable at all three time points. This patient demonstrated a strong band for PSA at ADT_4, which diminished with treatment, becoming undetectable at RT_{20}. The band for PSMA also followed this pattern to an extent, whilst the tumour-antigen 5T4 remained detectable at RT_{20}, suggesting that there may be some element of residual disease present, and that exosomal 5T4 may reflect this. The data are summarised in Table 4.

Urine does not osmotically damage exosome membrane integrity

Our study highlighted variable quantity of exosomes in urine specimens. This was ~10-times lower than expected, according to others [34]. We hypothesised that variable hydration state of individuals providing urine specimens may lead to some differences in water/salt content of urine; and that this may damage exosomes present in urine. This would impact on exosome-flotation character-

Table 3: Details of urine specimens collected from healthy donors

Healthy Donor	Age of donor	Dip-Stick Blood, Protein, Glucose, Ketones, pH					Specimen Volume (ml)	Exosomes Recovered (μg)	Exosome Concentration (ng/ml)
1	29	0	0	0	0	7	180	9.8	54.4
2	37	0	1	0	0	7	180	115.2	640.0
3	37	0	1	0	0	7	180	32.3	179.4
4	63	2	0	0	0	5	180	55.4	307.8
5	61	0	1	0	0	7	180	154.7	859.4
6	50	0	1	0	0	7	180	8.7	48.3
7	49	0	0	0	0	6	150	61.2	408.0
8	55	0	1	0	0	6	180	37.2	206.7
9	56	0	0	4	0	7	145	28.5	196.6
10	57	0	1	0	0	8	170	130.3	766.5

istics, and may explain the variability and low quantity we observed using the sucrose-cushion method.

Experiments were performed, using exosomes loaded with a fluorescent dye, to assess how various osmotic conditions might damage exosome membranes; revealing that exosomes are surprisingly resistant to high and low salt solutions (Figure 6a). Incubating exosomes in urine specimens had no impact on the integrity of the membrane (Figure 6b). We conclude that urine does not osmotically damage the exosome membrane, and this is unlikely to impact on the buoyancy characteristics of exosomes.

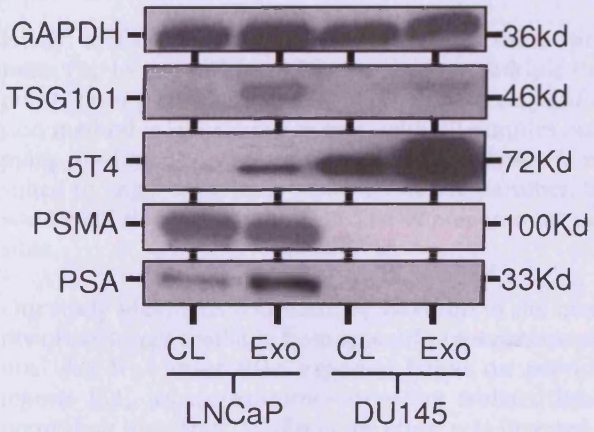

Figure 3
Characterising exosomes produced by LNCaP-prostate cancer cell line. Prostate cancer cell lines (LNCaP and DU145), as indicated, were maintained in culture as a source of positive-control prostate cancer exosomes (for subsequent analyses). Whole cell lysates (CL) or exosomes (Exo) were analysed by SDS-PAGE (5 μg/well), with a panel of antibodies as indicated.

Exosomes are not prone to proteolysis by urine

Proteolytic damage of exosomal constituents, by urine-proteases, may also explain low exosome levels we observed. Unlike Pisitkun *et al*, we used fresh urine specimens without protease inhibitors. To test this, we purified exosomes from LNCaP cultures, and incubated these with urine specimens in the presence/absence of protease inhibitors. Analysis of exosome markers by western blot revealed fresh urine specimens did not cause degradation of exosome-markers tested. We conclude that exosomes can largely resist endogenous proteolytic activity of urine (for at least 18 hours at 37°C) (Figure 6c).

Discussion

We present the findings of a pilot study, investigating urinary exosomes in prostate cancer patients. We had two main aims in the study; firstly to assess the feasibility of using urine as an exosome source in the context of a clinical trial, and secondly to demonstrate changes occurring in response to standard PCa-therapy. We anticipated being able to show differences in urinary exosome quantity, between healthy individuals, and individuals with

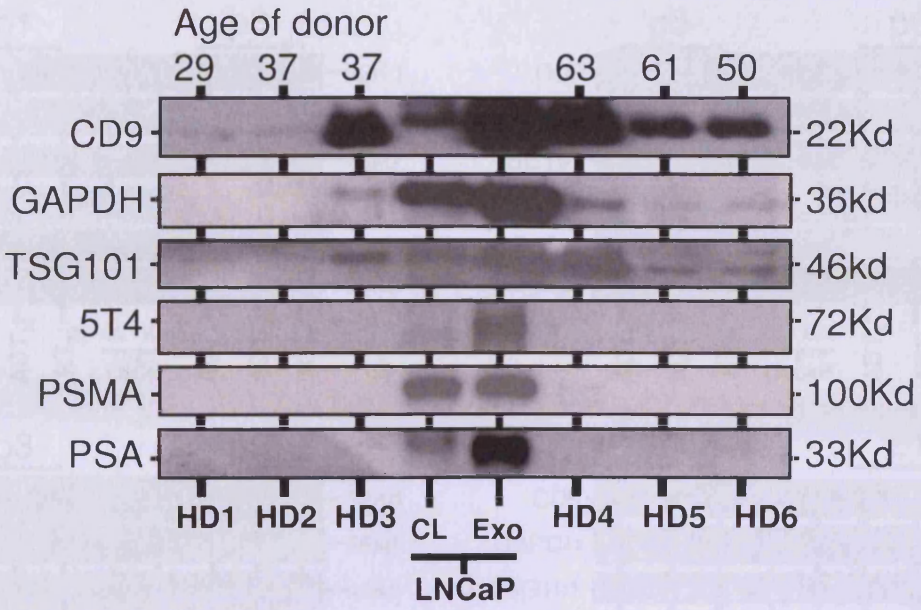

Figure 4
Characterising exosomes from healthy donor urine. Six healthy donors (detailed in Table 3), provided urine specimens and exosomes were purified. Western blots were performed with 5 μg urine-derived exosomes/well, or with 5 μg LNCaP-derived exosomes (Exo) or 5 μg LNCaP whole cell lysates (CL). Blots were probed with antibodies against PSA, TSG101, 5T4, CD9 and GAPDH, as indicated.

locally advanced prostate cancer, together with diminishing exosomally expressed PCa-markers in response to therapy.

Firstly, it is certainly feasible to collect spot urine specimens (up to 200 ml) from PCa patients, at multiple time points during standard treatment. The exosome purification method is laborious however, with 30 samples occupying 30-days of preparation time. This approach is not suited to larger scale trials or screening programmes, but was aimed at achieving the best quality preparations possible.

Our study highlights considerable variation in the quantity of exosomes available from spot urine specimens, and this was 10× lower than expected based on previous reports [34], where exosomes were not isolated based upon their buoyancy. Whilst some effort was invested in accounting for this discrepancy, such as evaluating the impact of urine protease activity on exosomes, or the effect of osmotic conditions on exosome membrane integrity, this discrepancy may simply be due to the presence of more non-exosomal contaminants present when using a simple pelletting approach; and that exosomes are therefore less abundant in urine than originally thought.

Comparing urinary-exosome quantity as we have done here is unlikely to provide meaningful information to the clinic, as there was no real difference between healthy men and those with locally advanced disease. We did observe a 2-fold decrease in urinary exosomes following 3-months ADT, where 8 of 10 patients showed a reduction in their urinary exosome content, and of these, 6 had reductions of >50%. This lower exosome level was not well maintained, with 5 of 9 patients showing elevated exosome levels with radiotherapy. In contrast, serum PSA levels demonstrated that all but one patient had responded well to treatment, with levels below 1.5 ng/ml at 6 months post treatment. There was no correlation between this surrogate cancer-marker, and the quantity of urinary exosomes. One may speculate that the reduction in prostate volume caused by ADT may explain the decrease in urinary-exosomes, and that radiation, a documented stimulus for exosome secretion [16], and a potent inducer of a robust local inflammatory response, may elevate exosomal urine content following radiotherapy. These aspects require further investigation.

Measuring protein quantity (present in purified exosome preparations), is clearly not sufficient to discriminate cancer cell derived exosomes, from a "high background" of non-cancer cell exosomes present in this complex mixed

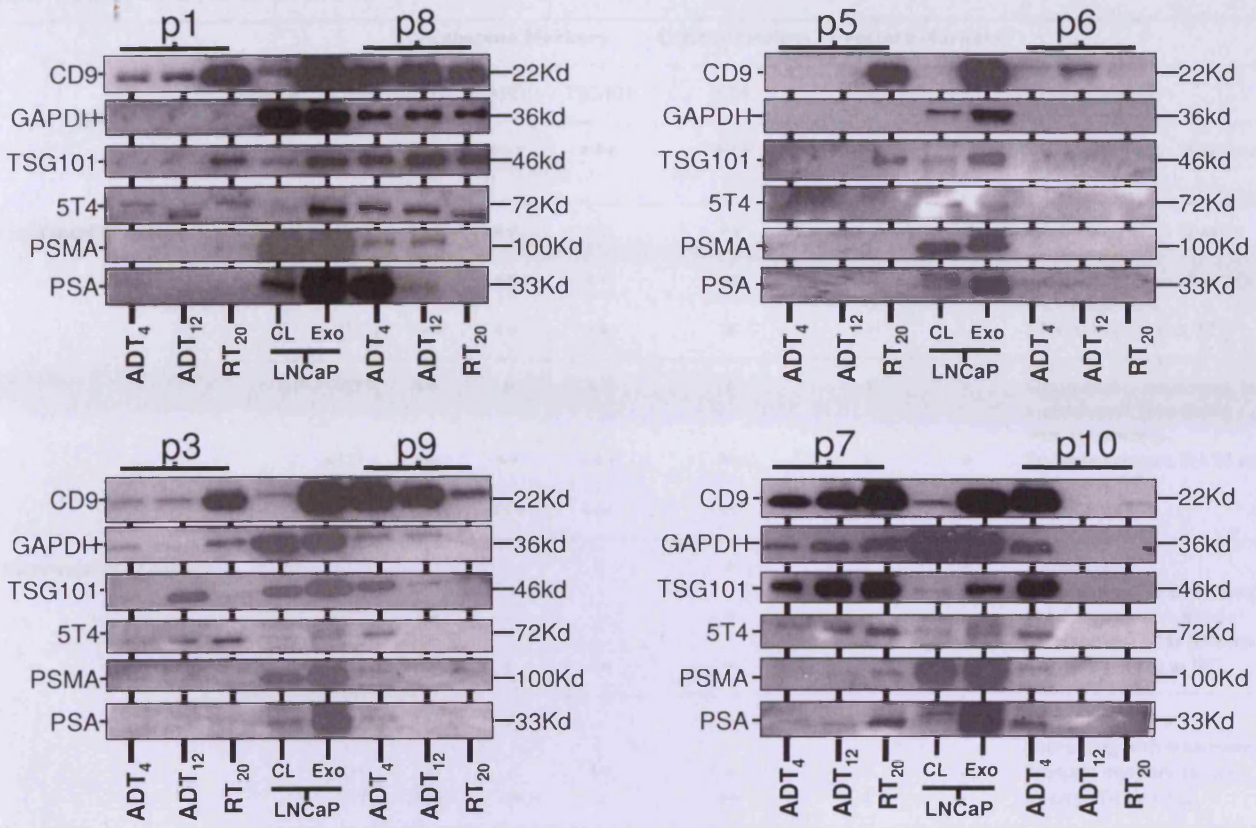

Figure 5
Characterising exosomes from PCa patients. Urinary exosomes (5 μg/well), isolated from 8 PCa patients (at ADT_4, ADT_{12} or RT_{20}), were subject to western blot analyses with a panel of antibodies as indicated. Whole cell lysates (CL) or exosomes (Exo) of LNCaP (5 μg/well) was included on each gel as positive controls.

exosome population in urine. A future approach could involve an immuno-affinity based method, for identifying (and quantifying) the proportion of tumour marker positive exosomes present in urine. One group has previously reported an approach, based upon EpCAM expression by ovarian cancer derived exosomes, for analysing exosomes present in the circulation [35]. We and likely others are working to develop an ELISA-like approach, better suited as a screening tool for cancer-derived exosomes in urine and other body fluids. Knowledge from this study will assist us in developing this tool.

In terms of exosome-phenotype, this study has highlighted some interesting observations from some of the PCa patients' specimens. Firstly, it was not previously known that the prostate can contribute any exosomes to the total urine exosome-pool. In healthy donors there was no positive staining for the prostate markers PSA or PSMA, and the tumour marker 5T4 was also negative. In the patient cohort, PSA was evident in 8/20, and PSMA present in 9/20 specimens (where 20/24 specimens were positive for one or more exosome-markers; i.e. evaluable as exosome-positive). Staining for 5T4 showed positivity in 14/20 samples. Together, this demonstrates for the first time, expression of prostate and cancer-associated markers by urinary exosomes.

One particular patient (p8) demonstrated comparable exosomes at each of the three time points, and a clear loss of exosomal-PSA in response to therapy. Unexpectedly, 5T4 remained strongly expressed, even following 20-fractions of radiotherapy, suggesting this may be a candidate marker for assessing the presence of residual malignant cells, refractory to the effects of androgen-ablation or radiotherapy. This aspect certainly warrants follow up studies,

Table 4: Summary of patient's western blot data

			Exosome Markers			Cancer Marker	Prostate Markers		
	Patient	**Time**	CD9	GAPDH	TSG101	5T4	PSMA	PSA	**Summary**
	LNCap	N/A	++++	+++	+++	+++	++++	++++	The Comparator "Standard" Sample
Good Quality	**p8**	ADT_4	+++	++	++	++	++	+++	Consistent, High Quality Exosomes.
		ADT_{12}	+++	++	++	++	++	+	Prostate markers diminish with treatment.
		RT_{20}	+++	++	++	+	-	-	5T4 still evident at RT_{20}
	p7	ADT_4	++	+	++	+	+	+	Good quality exosomes, but inconsistent, (increasing with treatment).
		ADT_{12}	++	++	+++	++	+	+	Prostate markers & 5T4 still evident at RT_{20}
		RT_{20}	+++	+++	+++	++	++	++	
Intermediate Quality	**p1**	ADT_4	+	-	-	+	-	+	Inconsistent, (increasing with treatment)
		ADT_{12}	++	-	-	+	-	-	Prostate markers barely detected, no clear pattern.
		RT_{20}	+++	+	++	+	+	-	5T4 still evident at RT_{20}
	p3	ADT_4	+	+	-	+	-	-	Inconsistent, (increasing with treatment)
		ADT_{12}	+	-	++	+	-	-	Prostate markers absent.
		RT_{20}	+++	++	-	++	-	-	Strong 5T4 at RT_{20}
Poor	**p9**	ADT_4	+++	+	+	+	+	+	Inconsistent, (decreasing with treatment)
		ADT_{12}	++	+	-	-	-	-	Prostate markers barely detected, no clear pattern.
		RT_{20}	+	-	-	-	+	-	No 5T4 at RT_{20}
	p5	ADT_4	-	-	-	-	-	-	Poor quality at 2/3 time-points
		ADT_{12}	-	-	-	-	-	-	Not Evaluable
		RT_{20}	+++	-	++	-	-	-	
Very Poor Quality	**p10**	ADT_4	+++	++	+++	+	+	+	Poor quality at 2/3 time-points
		ADT_{12}	-	-	-	-	-	-	Not Evaluable
		RT_{20}	+	-	-	-	-	-	
	p6	ADT_4	+	-	-	-	-	-	Poor quality at 3/3 time-points
		ADT_{12}	++	-	-	-	-	-	Not Evaluable
		RT_{20}	-	-	-	-	-	-	

as there is a need for markers suited to identifying the presence of treatment-resistant cells.

The future of urine-exosome analysis in prostate cancer remains uncertain. This study has demonstrated that extensive steps taken to freshly process and highly purify exosomes from urine are labour intensive, yet results in a variable product with only 17% of attempts containing exosomes of comparable quality to those obtained from cell culture. When the exosome content of source material is consistent, variation due to the preparation method used is <1% [30]. It may be possible to overcome this degree of heterogeneity in the exosome content of the source material, for example by 24 hr urine collection or

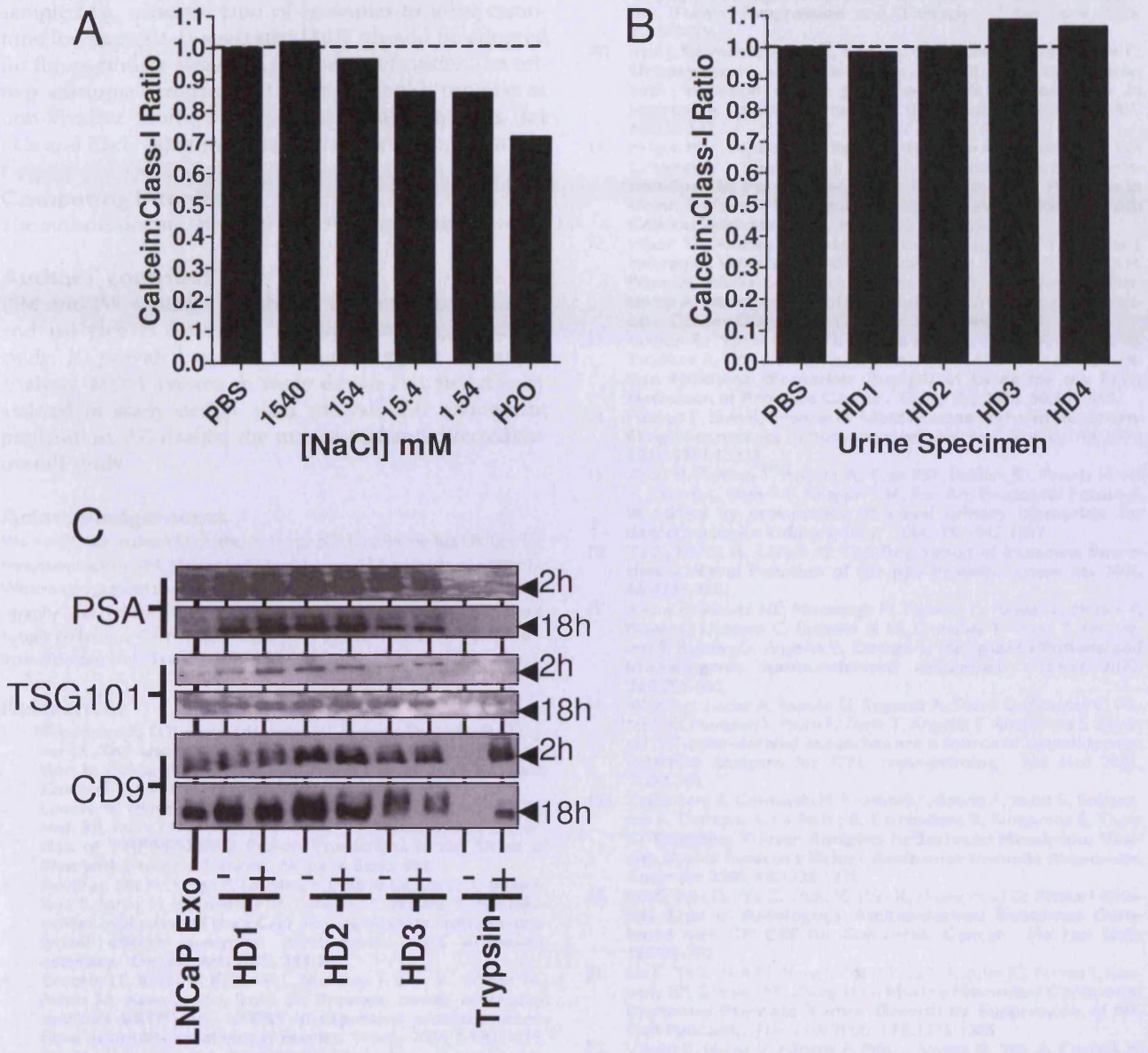

Figure 6

Evaluating urine-mediated damage of exosomes. Exosomes coupled to microbeads were labelled with a luminal fluorescent dye (Calcein-AM), prior to incubation with various concentrations of NaCl (A) or with fresh urine specimens from four healthy donors (HD1-4) (B). In parallel, identical beads were set up, in the absence of Calcein-AM dye, stained instead with anti-MHC Class-I (RPE) conjugated antibody. After 1 h at room temperature, the fluorescence signal present in the FL-1 channel (Calcein) was compared to FL-2 fluorescence (Class-I-RPE). Graphs show ratio of Calcein to Class I fluorescence. To examine proteolytic damage of exosomes (C), western blot was performed for CD9, TSG101 and PSA on LNCaP-derived exosomes; which were incubated for 2 h or 18 h with fresh urine specimens (from three healthy donors), in the presence or absence of protease inhibitors. Trypsin was used as a positive control for proteolysis.

by collection after prostate massage. Such modifications together with improved methods for normalisation of the sample (e.g. compare ratio of exosomes to urine creatinine for example as suggested [34]), should be adopted for future studies. Regardless of these difficulties, the urinary exosome compartment genuinely holds promise as non-invasive source of tumour-associated antigens, for PCa and likely other malignancies of the urological tract.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PJM and JW equally contributed to sample preparation and analyses. JS conceived, designed and organised the study. JC provided general technical support in sample analysis. MDM assisted in study design and analysis. ZT assisted in study design, data analysis and manuscript preparation. AC drafted the manuscript and directed the overall study.

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Proteomics Analysis of Bladder Cancer Exosomes*[S]

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Exosomes are nanometer-sized vesicles, secreted by various cell types, present in biological fluids that are particularly rich in membrane proteins. *Ex vivo* analysis of exosomes may provide biomarker discovery platforms and form non-invasive tools for disease diagnosis and monitoring. These vesicles have never before been studied in the context of bladder cancer, a major malignancy of the urological tract. We present the first proteomics analysis of bladder cancer cell exosomes. Using ultracentrifugation on a sucrose cushion, exosomes were highly purified from cultured HT1376 bladder cancer cells and verified as low in contaminants by Western blotting and flow cytometry of exosome-coated beads. Solubilization in a buffer containing SDS and DTT was essential for achieving proteomics analysis using an LC-MALDI-TOF/TOF MS approach. We report 353 high quality identifications with 72 proteins not previously identified by other human exosome proteomics studies. Overrepresentation analysis to compare this data set with previous exosome proteomics studies (using the ExoCarta database) revealed that the proteome was consistent with that of various exosomes with particular overlap with exosomes of carcinoma origin. Interrogating the Gene Ontology database highlighted a strong association of this proteome with carcinoma of bladder and other sites. The data also highlighted how homology among human leukocyte antigen haplotypes may confound MASCOT designation of major histocompatability complex Class I nomenclature, requiring data from PCR-based human leukocyte antigen haplotyping to clarify anomalous identifications. Validation of 18 MS protein identifications (including basigin, galectin-3, trophoblast glycoprotein (5T4), and others) was performed by a combination of Western blotting, flotation on linear sucrose gradients, and flow cytometry, confirming their exosomal expression. Some were confirmed positive on urinary exosomes from a bladder cancer patient. In summary, the exosome proteomics data set presented is of unrivaled quality. The data will aid in the development of urine exosome-based clinical tools for monitoring disease and will inform follow-up studies into varied aspects of exosome manufacture and function. *Molecular & Cellular Proteomics 9:1324–1338, 2010.*

Bladder cancer is one of the eight most frequent cancers in the Western world, and the frequency of transitional cell carcinoma (TCC),[1] which accounts for 90% of bladder cancers, is second only to prostate cancer as a malignancy of the genitourinary tract. Urine cytology and cystoscopy remain the predominant clinical tools for diagnosing and monitoring the disease, but cytology is poorly sensitive, particularly for low grade tumors, and does not serve as a prognostic tool. Cystoscopy is an invasive procedure, and there is pressing need to identify informative molecular markers that can be used to replace it.

Recently, small cell-derived vesicles termed exosomes that are present in body fluids (1–5) have been proposed as a potential source of diagnostic markers (2, 6–8). These nanometer-sized vesicles, which are secreted by most cell types, originate from multivesicular bodies of the endocytic tract and reflect a subproteome of the cell. Exosomes are enriched in membrane and cytosolic proteins, and this molecular repertoire appears to be of particular functional importance to the immune system (9). Exosomes also comprise an array of lipids, mRNA, and microRNA, which are likely involved in conveying intercellular communication processes (10). Importantly, many exosomal components are simply not present as free soluble molecules in body fluids, such as certain microRNA species, which are encapsulated within the exosome lumen (6, 10). Therefore, the ability to isolate exosomes from urine (2), plasma (1), saliva (11), or other physiological sources (3) holds significant potential for obtaining novel and complex sets of biomarkers in a non-invasive manner. Exosome analysis may therefore be of value in disease diagnosis and monitoring in a variety of settings (6, 7, 12–14).

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[1] The abbreviations used are: TCC, transitional cell carcinoma; 5T4, trophoblast glycoprotein; BCA, bicinchoninic acid; FDR, false discovery rate; HLA, human leukocyte antigen; hsp90, heat shock protein 90; LAMP, lysosome-associated membrane protein; MHC, major histocompatability complex; TEAB, triethylammonium bicarbonate; TSG101, tumor susceptibility gene 101; NHS, National Health Service; FBS, fetal bovine serum; RT, room temperature; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; hnRNP, heterogeneous nuclear ribonucleoprotein; 2DE, two-dimensional electrophoresis; ID, identifier; ORA, overrepresentation analysis.

Exosomes as indicators of pathology were first documented in the context of renal injury where a differential proteomics approach revealed changes in urinary exosome phenotype following renal injury (7). The researchers identified exosomally expressed Fetuin-A as a marker that became elevated 50-fold within hours following nephrotoxin exposure in rodents. Exosomal Fetuin-A elevation was also apparent in patients with acute renal injury before changes in urinary creatinine were observed (7). Clinical exosome analysis may also prove useful for solid cancers, such as ovarian or lung cancer, where the quantity of epithelial cell adhesion molecule-positive serum exosomes may correlate with tumor stage/grade. Such disease-associated exosomes express microRNA species not detected in healthy subjects (6, 12), although in this respect, there is little correlation between microRNA and disease bulk (6, 12). Other recent examples include studies of urinary exosomes in prostate cancer with exosomes expressing protein markers 5T4 (15), prostate cancer gene 3 (PCA-3) (8), or mRNA (TMPRSS2-ERG) (8, 16) associated with prostate cancer. To our knowledge, exosomes have not yet been studied in the context of other urological malignancies such as renal cancer, and to date, only one report describes the urine-derived microparticles from bladder cancer patients (17). In that report, they examined the proteome of a highly complex mixture of microvesicles, exosomes, and other urinary constituents that can be pelleted by high speed ultracentrifugation, identifying eight proteins that may be elevated in cancer. However, given the nature of the sample analyzed, it is unknown whether these proteins are exosomally expressed.

Identification of the principal and most relevant molecular markers in these and other clinical scenarios remains a major challenge. In part, this is because exosomes present within complex body fluids originate from heterogeneous cell types. For example, plasma exosomes may be derived from platelets, lymphocytes, or endothelial cells (1), and a proportion may arise from well perfused organs such as the liver (18) and likely other organs as well (16). Similarly, exosomes present in urine arise from urothelial cells of the kidney and downstream of the renal tract (2, 8, 15).

Importantly, all proteomics studies of exosomes isolated from body fluids are unavoidably complicated by the presence of high abundance non-exosomal proteins contaminating the preparations. Examples include albumin, immunoglobulin, and complement components present in exosomes prepared from malignant effusions (5) and Tamm-Horsfall protein present in exosomes purified from urine (2). As such, great care must be taken in the interpretation of the large data sets produced by proteomics studies, requiring careful validation of the proteins of interest. The protein composition of exosomes using a single homogenous cell type is one approach that may be used to uncover the protein components of exosomes produced by various cell types.

There remain two major issues in the realm of exosome proteomics that complicate our interpretation of lists of identified proteins. Foremost are the diverse methods chosen for exosome purification that in some studies have involved attempts to remove contaminants through a key biophysical property of the vesicles, *i.e.* their capacity to float on sucrose (19, 20) or other dense media (21). Not all published studies, however, have taken such steps, preferring a far simpler pellet (or pellet and wash) approach. These latter preparations may be significantly contaminated by components of the cellular secretome, cell fragments, and other components. All of these factors could lead to false positive identifications of exosome proteins. The second key issue centers on the MS approaches utilized in various exosome proteomics studies. Many early examples relied only on a peptide mass fingerprinting approach, lacking robust peptide sequence data (22, 23), and more recently, search criteria that are generally recommended for MS-derived sequence data have not been specified in all studies. In this study, we have listed only those proteins identified by good quality MS/MS data for two or more peptides. Variability in the robustness and bias in bioinformatics analysis of data sets and in the steps taken to validate identified proteins is an additional factor that impacts the confidence in the identification lists produced.

In this study, we aimed to perform the first proteomics analysis of human bladder cancer exosomes. We took extensive steps to produce high purity and quality-assured exosome preparations prior to beginning proteomics workflows. Solubilizing the sample with SDS and a reducing agent (DTT) was a critical step that allowed for global protein identification using nanoscale liquid chromatography followed by MALDI-TOF/TOF mass spectrometry. In this study, we present the identification of a significant number of exosomally expressed proteins (353 in total) of unrivaled quality. Critical manual examination of these identifications revealed issues with multiple (physiologically impossible) MHC Class I identifications that were attributed to a misdesignation of nomenclature by MASCOT due to peptide (and target protein) homology. The data were subjected to unbiased overrepresentation analysis (examining ExoCarta and Gene Ontology databases) to reveal a proteome consistent with exosomes, particularly of carcinoma origin. Validation of several identified proteins, by combining ultracentrifugation on a linear sucrose gradient with Western blotting and/or analysis of exosome-coated latex beads, demonstrated correct surface orientation of several MS-identified membrane proteins at densities consistent with exosomes.

The robust approaches taken emphasize our confidence in the validity of the identifications generated and highlight that 72 (of 353) proteins have not been previously shown to be exosomally expressed by other human proteomics studies. The data will be useful for future studies in this underinvestigated disease and will form a platform not only for future clinical validation of some of these putative markers but also

to aid further investigations into novel aspects of exosome function and manufacture.

EXPERIMENTAL PROCEDURES

Cell Culture—HT1376 is a cell line originating from a primary TCC of the bladder (Stage T2, Grade G4) (24). *In vitro* cultured HT1376 cells were used as the exosome source for this study because they have been extensively characterized previously and are representative of the behavior and phenotype of TCC (24, 25). The cells were maintained in Dulbecco's modified Eagle's medium (Lonza) supplemented with penicillin/streptomycin and 5% FBS (which had been depleted of exosomes by overnight ultracentrifugation at 100,000 × *g* followed by filtration through 0.2-μm and then 0.1-μm vacuum filters (Millipore)). The cells were seeded into bioreactor flasks (from Integra) and maintained at high density culture for exosome production as described (26). Cells were confirmed negative for mycoplasma contamination by monthly screening (Mycoalert, Lonza). Additional well characterized bladder cancer cell lines (HT1197, RT4, RT112, and T24) (25, 27) were obtained from ATCC or from Cancer Research UK cell bank and cultured similarly.

Exosome Purification—The culture medium of HT1376 cells (typically 15–30 ml) was subjected to serial centrifugation to remove cells (400 × *g* for 10 min) and cellular debris (2000 × *g* for 15 min). The supernatant was then centrifuged at 10,000 × *g* for 30 min, and the supernatant was further purified by underlaying with a 30% sucrose, D_2O cushion and subjected to ultracentrifugation at 100,000 × *g* for 2 h. The cushion was collected, and exosomes were washed in PBS as described previously (20, 28, 29). Exosome pellets were resuspended in 100–150 μl of PBS and frozen at −80 °C. The quantity of exosomes was determined by the micro-BCA protein assay (Pierce/Thermo Scientific), and this gave an average of 12 μg (±2.2 S.E., n = 5) exosomes/ml of culture medium for the HT1376 cell line. Transmission electron microscopy of exosomal preparations was performed as described (29).

Determinination of Exosome Density—To quantify the density of exosomes produced by HT1376, we used a protocol similar to that described previously based on ultracentrifugation on a linear sucrose gradient (19, 30). Briefly, cell culture supernatant was subjected to differential centrifugation, and the pellet at 70,000 × *g* was overlaid on a linear sucrose gradient (0.2 M up to 2.5 M sucrose). Specimens were centrifuged at 4 °C overnight at 210,000 × *g* using an MLS-50 rotor in an Optima-Max ultracentrifuge (Beckman Coulter). The refractive index of collected fractions was measured at 20 °C using an automatic refractometer (J57WR-SV, Rudolph Scientific), and from this, the density was calculated as described previously (19). Fractions were washed in buffer (PBS or MES buffer; discussed below) by ultracentrifugation at 150,000 × *g* (in a TLA-110 rotor in an Optima-Max ultracentrifuge), and pellets were resuspended in MES buffer for coupling to microbeads or in SDS sample buffer for analysis by Western blot.

Flow Cytometric Analyses of Exosome-coated Beads—One microgram of purified exosomes was incubated with 1 μl of latex beads (surfactant-free, aldehyde sulfate 3.9-μm beads, Interfacial Dynamics) that had been washed twice in MES buffer (0.025 M MES, 0.154 M NaCl, pH 6). For analysis of sucrose gradient fractions, 30% of each fraction was coupled to 0.5 μl of stock beads. Exosome beads were incubated in a final volume of 100 μl of MES buffer at room temperature (RT) for 1 h on a shaking platform followed by rolling overnight at 4 °C. Beads were blocked by incubating with 1% BSA, MES buffer for 2 h at RT. Blocking buffer was washed away, and beads were resuspended in 0.1% BSA, MES buffer. Primary monoclonal antibodies were used (at 2–10 μg/ml) for 1 h at 4 °C. After one wash, goat anti-mouse Alexa Fluor 488-conjugated antibody (Invitrogen) diluted 1:200 in 0.1% BSA, MES buffer was added for 1 h. After washing, beads were analyzed by flow cytometry using a FACSCanto instrument configured with a high throughput sampling module running FACSDiva Version 6.1.2 software (BD Biosciences). The conditions used for exosome coupling to beads and subsequent antibody staining were determined experimentally as described previously (30).

One-dimensional Electrophoresis and Immunoblotting—Cell lysates were compared with exosome lysates by immunoblotting as described (31) where protein (up to 20 μg/well) was solubilized by the addition of a 30% volume of 6 M urea, 50 mM Tris-HCl, 2% SDS, 20 mM DTT, and 0.002% (w/v) bromphenol blue. Samples were electrophoresed through 4–12% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes that were blocked and probed with antibodies using the Qdot® system (Invitrogen). Bands were visualized using the MiniBIS Pro imaging system (DNR Bio-Imaging Systems). The following primary monoclonal antibodies were used: TSG101, lysosome-associated membrane protein 1 (LAMP-1), hsp90, calnexin, HLA-G, galectin-3, basigin, hnRNPK, gp96, cytokeratins 18 and 17, and CD44 (Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (BioChain Institute, Inc.), CD9 (R&D Systems), and CD63 and CD81 (Serotec). Anti-5T4 was a gift from Dr. R. Harrop (Oxford BioMedica UK Ltd.).

Two-dimensional Electrophoresis and MS—A gel-based approach was used to examine the exosome protein profile using a standard 2DE protocol. Briefly, exosomes (750 μg) were solubilized for 1 h at RT in 150 μl of lysis buffer (7 M urea, 2 M thiourea, 20 mM DTT, 4% (w/v) CHAPS, 0.005% (w/v) bromphenol blue, and 0.5% (v/v) IPG buffer pH 3–10 non-linear (GE Healthcare)). Extracted proteins were then solvent-precipitated using the 2D Clean-Up kit (GE Healthcare) before the pellet was resuspended in lysis buffer. From this, 500 μg of protein was recovered, and this was subjected to isoelectric focusing using 18-cm pH 3–10 non-linear IPG rehydrated strips, an Ettan IPGphor III IEF system (GE Healthcare), and recommended voltages. Subsequently, the IPG strip was equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 0.002% (w/v) bromphenol blue) containing 1% (w/v) DTT followed by 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. Equilibrated IPG strips were subjected to second dimension separation using the Ettan™ DALTsix system (GE Healthcare). Silver staining was performed, and randomly selected gel spots were excised, subjected to trypsin digestion, and MALDI-TOF/TOF mass spectrometry analysis as described previously (32). The database search settings used were the same as described later for LC-MALDI protein identification except that a precursor mass tolerance of 50 ppm was used.

Preparation of Exosome-derived Peptides for Nano-LC—HT1376-derived exosome preparations were repelleted at 118,000 × *g* for 45 min at 4 °C in a TLA-110 rotor in an Optima-Max ultracentrifuge (Beckman Coulter). The pellets were solubilized in 100 μl of triethylammonium bicarbonate (TEAB) lysis buffer (20 mM TEAB) containing 20 mM DTT and 1% (w/v) SDS at RT for 10 min, then heated to 95 °C for 10 min, and then left for a further 10 min at RT. The samples were subjected to an additional ultracentrifugation step (118,000 × *g* for 45 min at RT), and supernatants (now free of insoluble material) were subjected to solvent precipitation to remove salts, lipids, and detergent (using the 2D Clean-Up kit, GE Healthcare). The pellets were resuspended in 20 mM TEAB and left overnight at 4 °C. The protein content was then determined using a BCA protein assay kit (Sigma). Samples were then reduced, denatured, and alkylated using an Applied Biosystems iTRAQ (isobaric tags for relative and absolute quantitation) labeling kit and standard protocol. The proteins were subjected to digestion with trypsin (0.8 μg/sample) and incubated at 37 °C for 12–16 h. The samples were then dried and resuspended in water with 0.1% (v/v) TFA.

LC-MALDI and Protein Identification—Digested peptides were separated on a nano-LC system (UltiMate 3000, Dionex, Sunnyvale, CA) using a two-dimensional salt plug method as described previously (32). Mass spectrometry was performed using an Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer as described (32). The MS/MS data were used to search the Swiss-Prot database (Version 57.7; release date, September 1, 2009; 497,293 sequences; human taxonomy) using MASCOT database search engine Version 2.1.04 (Matrix Science Ltd., London, UK) embedded into GPS Explorer software Version 3.6 Build 327 (Applied Biosystems) (default GPS Explorer parameters; one missed cleavage allowed; fixed modification of methyl methanethiosulfonate (Cys); variable modifications of oxidation (Met), pyro-Glu (N-terminal Glu), and pyro-Glu (N-terminal Gln); 150-ppm mass tolerance in MS and 0.3-Da mass tolerance for MS/MS, which are recommended published tolerances for LC-MALDI (32)). For a protein to be identified, there needed to be a minimum of two peptides with MASCOT E-values less than 0.05. There was a false discovery rate (FDR) of 0%, which was determined using the same Swiss-Prot database with the entire sequence randomized. Where more than one protein was identified, the protein with the highest MOWSE (molecular weight search) score in MASCOT is reported. The analysis was performed with two biological replicates, each including a technical replicate.

MS Data Analysis—The resultant protein list was analyzed for any biological enrichment against previously defined lists using MetaCore GeneGO (Version 5.4) and selected ExoCarta submissions (33) (MS-based data containing 10 or more matching gene identifiers). For analysis using 44 studies from ExoCarta gene sets, our protein list was converted from Swiss-Prot accession numbers to EntrezGene IDs using BioMart before overrepresentation analysis (ORA) using the hypergeometric distribution in R against a background of all human genes with EntrezGene IDs. For ORA in MetaCore, data were first converted into Swiss-Prot IDs (using BioMart) before analysis, again using hypergeometric tests.

Urinary Exosomes—Freshly collected urine specimens (up to 250 ml) were subjected to the same exosome purification protocol as described earlier. Fresh urine was collected from three patients with confirmed diagnoses of transitional carcinoma of the bladder. The specimens were obtained following transurethral resection of bladder tumor prior to the start of any other treatment. Purification of exosomes commenced within 30 min of sample collection. As controls, urine specimens were also collected freshly from four healthy volunteers. Ethical approval was obtained from South East Wales Ethics Committee, and institutional approval for the study was obtained from the Velindre NHS Trust Research Committee and Cardiff and Vale NHS Trust.

RESULTS

Characterization of HT1376 Exosomes—Exosomes were purified from HT1376 cells, and preparations were subjected to several forms of analysis to evaluate sample quality/purity prior to analysis using proteomics workflows.

First, Western blots were performed to compare whole cell lysates with exosomes to examine the expression of expected published exosomal markers (30) and to evaluate the relative expression of these markers compared with the parent cell as a whole. As we expected, the multivesicular body marker TSG101 was strongly enriched in exosome preparations compared with cell lysates (Fig. 1*A*). Additionally, a number of other molecules, including MHC Class I, the tetraspanins CD9 and CD81, the lysosomal protein LAMP-1, and to some extent glyceraldehyde-3-phosphate dehydrogenase, were similarly enriched. Such features are typical of exosomes produced by varied cell types (30). The heat shock protein hsp90 was not exosomally enriched, and this is typical of cells that are not under stress conditions (26, 34, 35). Staining for cytokeratin 18 revealed a strong band in cell lysates but little or no detectable band in exosomes. Similarly, the endoplasmic reticulum-resident gp96 was readily detected in cell lysates but just detectable in exosomes, which indicated that little if any contaminating cellular debris was present in the exosome preparations.

The markers present on the exosome surface were also examined, following coupling of exosomes to latex beads, by flow cytometry (Fig. 1*B*). This was performed to demonstrate the expression of correctly oriented proteins on the exosome surface. Tetraspanins were the choice markers for this because their expression is a well documented feature of exosomes from multiple cell types. The analyses showed very strong expression of the tetraspanin CD9 and readily detectable expression of CD81 and CD63 (Fig. 1*B*) for this and other bladder cancer cell lines (see Fig. 5*A*). Moreover, this assay can also highlight the presence of significant contaminating proteins in the preparations. When contaminants, rather than exosomes, bind to the bead surface during the coupling reaction, the assay subsequently yields low fluorescence signal for exosomal markers like CD9 (Fig. 1*B*, *line graph*). Intentional contamination of purified exosomes with FBS (the likeliest source of contaminants in a cell culture model) revealed that adding 0.01% FBS is sufficient to decrease CD9-specific staining by around 30%. Thus, we set an arbitrary threshold for the purity of exosome preparations: those with a CD9 staining below 5000 median fluorescence units were deemed low quality and not utilized further.

As well as expression of a typical exosomal molecular profile, we also investigated another key feature of exosomes, that is their density characteristics. HT1376 exosomes, pelleted at 70,000 × *g*, were overlaid on a linear sucrose gradient and subjected to ultracentrifugation for 18 h. Fifteen fractions were collected, and analysis by Western blot revealed the presence of TSG101 floating at a density range around 1.1–1.19 g/ml (Fig. 1*C*). Such analysis confirms that HT1376 cells produce exosomes of typical density similar to that described for exosomes from other cell types (19). This method, in combination with the latex microbead assay (above), was also used as a tool for validating MS protein identifications (see Results, Validation of Exosomal proteins identified in Fig. 4*B*). Electron microscopy of exosome preparations was also performed (Fig. 1*D*), revealing nanovesicular structures within a size range consistent with their definition as exosomes (30–100 nm). Taken together, the data indicate that HT1376 bladder cancer cells produce exosomes that have molecular and biophysical characteristics similar to exosomes of other cell types and that our exosome preparations are of high quality and virtually free of contaminating cellular debris.

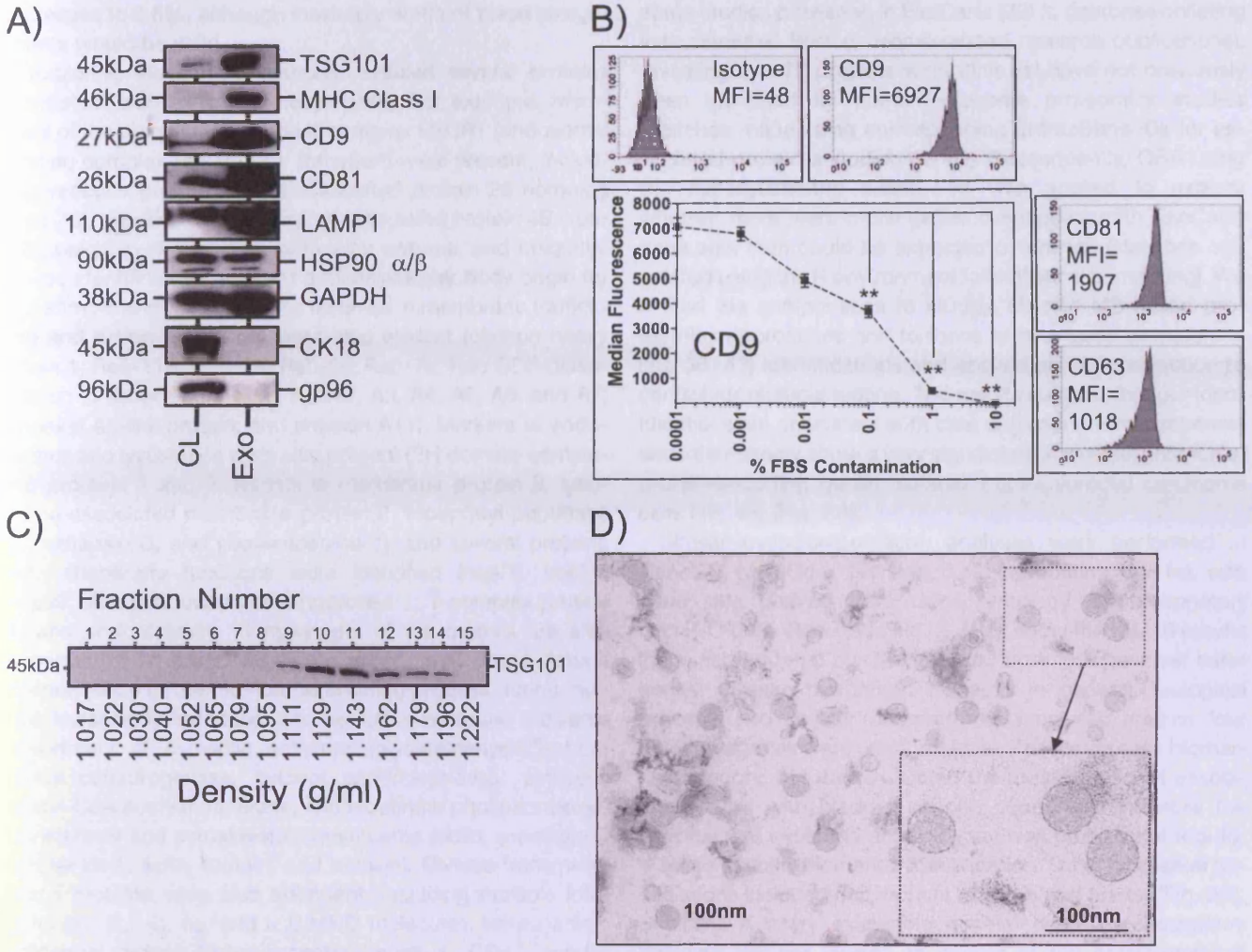


FIG. 1. **Characterization of HT1376-derived exosomes using Western blotting, flow cytometry, and electron microscopy.** Cell (*CL*) and exosome (*Exo*) lysates (5 μg/well) were compared by Western blotting using a range of antibodies as indicated. This demonstrated relative enrichment of several proteins in exosomes. Some markers, such as gp96, were absent from exosomes, indicating negligible contamination of the preparations by cellular debris (this is representative of three experiments) (*A*). Exosomes coupled to latex beads were analyzed by flow cytometry, and this revealed positive expression of tetraspanin molecules on the exosome surface. Median fluorescence intensity values (*MFI*) are shown (representative of >5 experiments) (*B*). Intentional contamination of purified exosomes with increasing amounts of FBS prior to coupling to latex beads reveals a decrease in signal intensity for CD9 (mean ± S.E.; $n = 6$; **, $p < 0.001$, one-way analysis of variance with Tukey's post test) (*B*, *line graph*). Material pelleted at 70,000 × *g* from cell-conditioned medium was overlaid on a linear sucrose gradient (0.2–2.02 M) and ultracentrifuged for 18 h at 210,000 × *g*. Collected fractions were analyzed by refractometry to ascertain fraction density and thereafter by Western blot using antibodies to TSG101, which is an exosome marker. TSG101 floats at typical exosome densities of between 1.1 and 1.2 g/ml (representative of four experiments) (*C*). Transmission electron micrograph of a typical exosome preparation reveals heterogeneous vesicles between 30 and 100 nm in diameter (*D*). *CK*, cytokeratin.

Identification of Exosomal Proteins by LC-MALDI MS—To obtain exosome-derived trypsin digest peptides for nano-LC, we used a protocol encompassing a 1% (w/v) SDS extraction that would normally be sufficient to solubilize membrane proteins (36). However, our initial attempts with this standard protocol revealed some major issues with the efficacy of exosome solubilization, resulting in very low numbers of proteins identified (three in total with multiple peptide assignments) compared with other cell types commonly processed in the laboratory (we usually identify 300–500 proteins for cultured cell lysates). We therefore modified the sample preparation protocol to achieve more efficient solubilization of exosomes by simply including DTT in the solubilization buffer.

This process resulted in the identification of 353 proteins (supplemental Table 1). Importantly, we include only proteins identified with two or more peptides and an expect value of less than 0.05, criteria that produce an FDR of 0%. By including identifications based upon a single peptide with an expect value of less than 0.0025 (an additional 261 proteins), the FDR

increases to 2.6%, although inevitably some of these assignments would be valid.

Exploring these identifications revealed several proteins consistent with exosome biosynthesis. For example, members of the ubiquitin-dependent complex ESCRT (endosomal sorting complex required for transport) were present, including vacuolar protein sorting-associated protein 28 homolog (vps-28), vacuolar protein sorting-associated protein 4B (vps-4B), ubiquitin-like modifier-activating enzyme, and ubiquitin. These identifications suggest a multivesicular body origin for the sample analyzed. Proteins involved in membrane trafficking and fusion processes were also evident (clathrin heavy chain 1; Rab-11B; Rab-5A; Rab-6a; Rab-7a; Rab GDP dissociation inhibitor β; annexins A1, A2, A3, A4, A5, A6, and A7; annexin A8-like protein; and annexin A11). Markers of endosomes and lysosomes were also present (EH domain-containing proteins 1 and 2, lysosome membrane protein 2, lysosome-associated membrane protein 2, tripeptidyl-peptidase 1, cathepsin-D, and sequestosome-1), and several proteins with chaperone functions were identified (hsp70, hsc70, hsp90, stress-induced phosphoprotein 1, T-complex protein 1, and endoplasmin). Components of the cytosol are also expected to be found within the exosome lumen, a natural consequence of the membrane budding process during multivesicular body formation, and here also we found a diverse assortment of cytosolic enzymes (glyceraldehyde-3-phosphate dehydrogenase, cytosol aminopeptidase, cytosolic acetyl-CoA acetyltransferase, and nicotinate phosphoribosyltransferase) and cytoskeletal constituents (actin, α-actinin-4, cytokeratins, ezrin, tubulin, and myosin). Diverse transmembrane proteins were also abundant, including multiple integrins (β_1, β_4, α_3, α_6, and α_v), MHC molecules, tetraspanins, epidermal growth factor receptor, mucin-1, CD44, syndecan-1, and various membrane transporters such as solute carrier families 2 and 3, 4F2 cell surface antigen heavy chain, choline transporter-like protein, and sodium/potassium-transporting ATPase subunit β-3. The proteome identified here is therefore broadly consistent with that expected for exosomes; it is comparable with proteomics identifications highlighted by other researchers investigating exosomes from other cellular or physiological sources (37). Of interest, when comparing our data set with MS identifications obtained from microparticles isolated from the urine of bladder cancer patients (17), proteins common to each study were only 7.5% (detailed in supplemental Fig. 1). This is perhaps not surprising given the differences in source material and sample preparation approaches, but it does indicate that some exosomal proteins are present within such microparticle preparations.

Exocarta and Gene Ontology Analysis—Having manually reviewed the MS/MS identifications for interesting hits related to exosome biology, we next subjected our results to a less biased assessment focused on characterizing the key biological themes within the protein list. Our 353 protein identifications were first compared with the multiple proteomics exosome studies published in ExoCarta (33) (a database collating lists extracted from exosome-related research publications), revealing that 72 proteins within this list have not previously been identified by human exosome proteomics studies (matches made using corresponding EntrezGene IDs for associated protein-encoding genes). Subsequently, ORA using the hypergeometric distribution was applied to explore whether there were more genes overlapping with ExoCarta gene sets than could be expected by chance (statistics calculated using the R environment for statistical computing). We limited the comparisons to studies utilizing MS-based proteomics approaches and to those with at least 10 matching (23, 38–43) identifications and applied an FDR correction to control for multiple testing. The results suggest that our identifications are consistent with data originating from exosomes and interestingly show a very significant overrepresentation of protein-encoding genes isolated from colorectal carcinoma cells (42, 43) (Fig. 2*A*).

Similar overrepresentation analyses were performed in GeneGO MetaCore (Version 5.4), contrasting our list with gene sets derived from Gene Ontology and proprietary GeneGO data. Results in Fig. 2, *B–E*, show the top 10 results (gene sets ordered by ORA p value) from four gene set categories: disease biomarker, diseases in general, biological process, and cellular compartment (analyses against four other categories were uninformative). For the disease biomarker category, our data indicated the most significant association to be with bladder cancer, supporting therefore the premise that exosome analysis may well be a useful tool for disease-specific biomarker identification. Other biomarker associations included carcinomas of colon and breast (Fig. 2*B*). Similarly, a query examining general disease associations revealed features related to cancer of the gastrointestinal tract, metastatic cancer, respiratory tract diseases (including lung cancer), and carcinoma (Fig. 2*C*). Significant overrepresentation of encoding genes within our data related to genitourinary tract gene sets (including bladder neoplasm) was identified, but within the top 40. Although the accuracy of ORA can be limited by the quality and size of the gene sets queried, our analysis suggests that HT1376 exosomes express proteins strongly related to neoplastic diseases in general and to carcinomas in particular (Fig. 2, *B* and *C*).

Our proteome contained proteins whose encoding genes are located within membranous vesicles, the cytoplasm, and the cytoskeleton (Fig. 2*E*). Examination of the biological processes associated with this proteome revealed significant associations with the control of the cytoskeleton, intercellular adhesion, matrix adhesion processes, and protein folding-related processes (Fig. 2*D*). In summary, the statistically based, unbiased analyses undertaken reveal aspects of a bladder cancer exosome proteome that shows similarity to those determined from other exosome sources and emphasize a proteome particularly implicated in carcinoma.

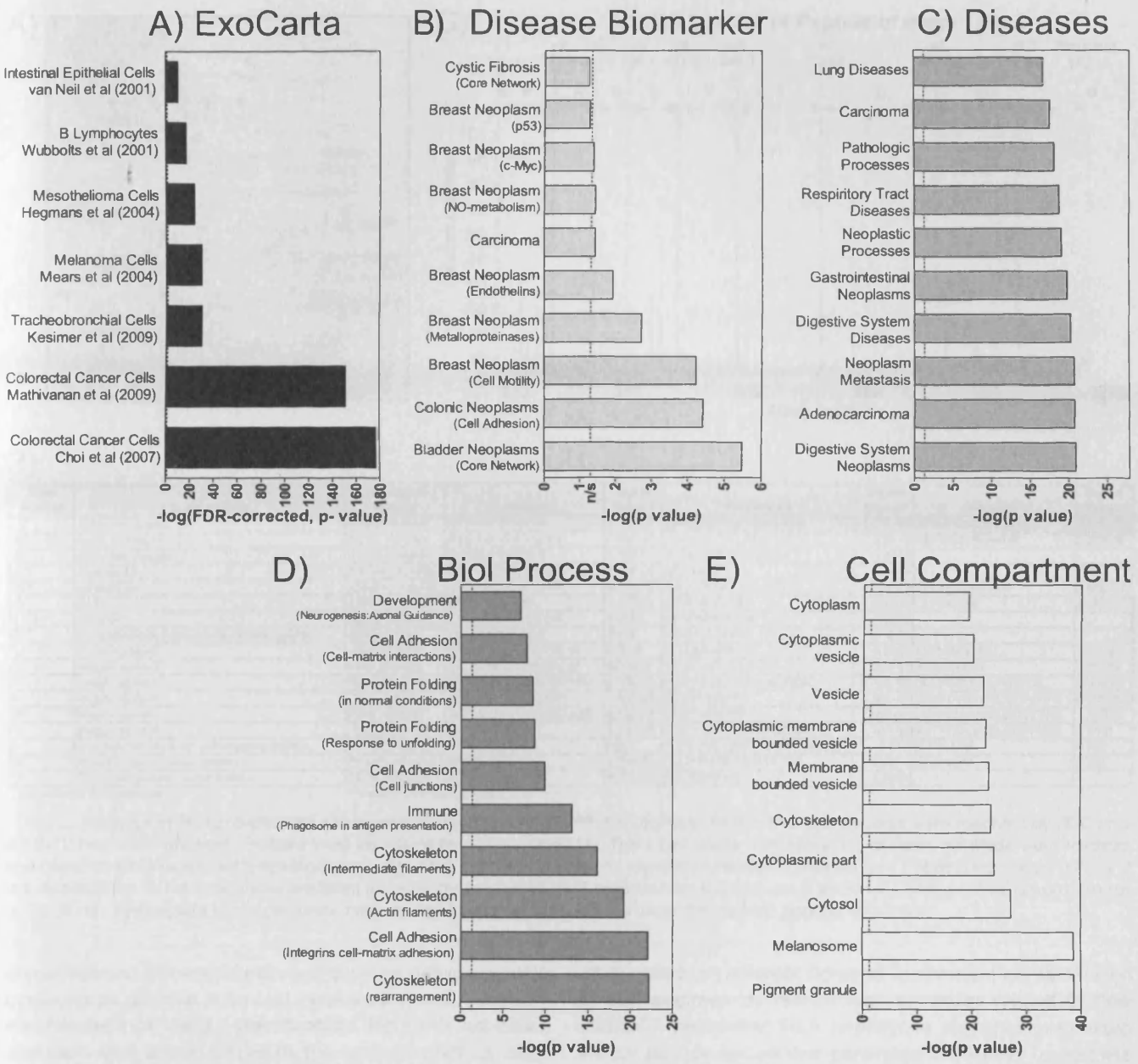
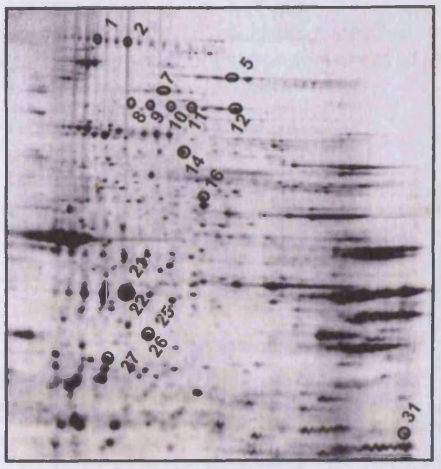


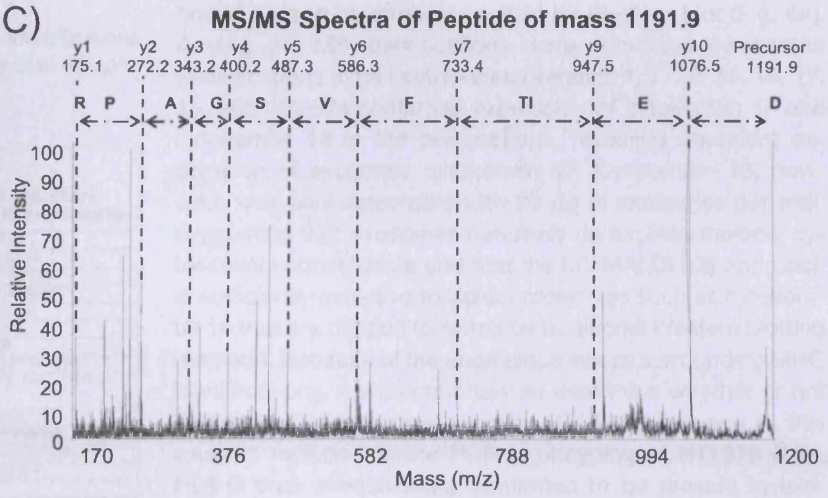
FIG. 2. **Summary of overrepresentation analysis of nano-LC/MS-derived protein identifications against gene sets from ExoCarta and GeneGO.** To facilitate comparison with ExoCarta gene sets, our protein list was first converted to an EntrezGene-identified gene list before undertaking ORA using the hypergeometric distribution. Results were filtered to include comparisons with MS-based studies only and with those reporting 10 or more matching genes, yielding seven studies (23, 38–43). This demonstrates how well our MS data compare with exosome protein profiles from specified cell types, displayed as the $-\log(p \text{ value})$ corrected for false detection rate (*A*). ORA analysis using MetaCore utilized the Swiss-Prot IDs for the identified protein list. For clarity, we report the top 10 overrepresented genes contained within each of the following group headings: disease biomarker (*B*), diseases (*C*), biological (*Biol*) process (*D*), and cellular compartment (*E*). The *dotted line* indicates $p = 0.05$; hence, *columns to the left of this are not statistically significant* (*ns*).

Validation of Nano-LC Approach Using 2DE—We performed 2DE with the aim of selecting random spots for MS identification and to confirm the absence/presence of these proteins in the main identification list. Running preparative gels, with 100 μg of purified exosomes per gel, was problematic because the spots picked contained too little material to yield confident protein identifications by MS. Increasing the amount of protein to 500 μg of exosomes per gel, however, resulted in an identification hit rate of >53%. Seventeen spots of intermediate staining intensity (silver-stained) were successfully identified by MS analysis (Fig. 3). These included integrins α_3 and α_6, gelsolin, cytosolic enzymes lactate dehy-

A)



C)



B)

Spot Number	Protein Name	Accession Number	Sequence 1	Expect Value 1	Sequence 2	Expect Value 2	Sequence 3	Expect Value 3
21*	Actin, cytoplasmic 1	ACTB_HUMAN	QEYDESGPSIVHR	2.00E-07	VAPEEHPVLLTEAPLNPK	1.00E-06	SYELPDGQVITIGNER	4.90E-06
22*	Actin, cytoplasmic 1	ACTB_HUMAN	SYELPDGQVITIGNER	7.00E-10	VAPEEHPVLLTEAPLNPK	7.90E-10	QEYDESGPSIVHR	3.80E-08
27	Actin, cytoplasmic	ACTG_HUMAN	QEYDESGPSIVHR	5.10E-05	SYELPDGQVITIGNER	0.0042		
26**	Annexin A4	ANXA4_HUMAN	GAGTDEGCLIEILASR	1.60E-05	AEIDMLDIR	0.0048		
25**	Annexin A4	ANXA4_HUMAN	GLGTDEDAIISVLAYR	4.10E-12	GAGTDEGCLIEILASR	8.70E-12	NHLLHVFDEYKR	8.00E-11
12	Ezrin	EZRI_HUMAN	KAPDFVFYAPR	1.00E-08	APDFVFYAPR	1.90E-06	QLFDQVVK	0.00012
7	Gelsolin	GELS_HUMAN	EVQGFESATFLGYFK	0.0022	HVVPNEVVVQR	0.0035		
31	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	LVINGNPITIFQER	0.018	AGAHLQGGAK	0.03		
1	Integrin alpha-3	ITA3_HUMAN	EAGNPGSLFGYSVALHR	0.0004	YLLLAGAPR	0.0021	ARPVINIVHK	0.0022
2	Integrin alpha-3	ITA3_HUMAN	EAGNPGSLFGYSVALHR	3.00E-07	YTQVLWSGSEDQR	7.00E-05	YLLLAGAPR	0.00019
8	Integrin alpha-6	ITA6_HUMAN	NSYPDVAVGSLSDSVTIFR	7.10E-09	DGEVGGAVYVYMNQQGR	5.90E-07	DGWQDIVIGAPQYFDR	8.60E-07
9	Integrin alpha-6	ITA6_HUMAN	GIVSKDEITFVSGAPR	1.90E-09	DEITFVSGAPR	3.30E-09	NSYPDVAVGSLSDSVTIFR	6.60E-09
10	Integrin alpha-6	ITA6_HUMAN	DGEVGGAVYVYMNQQGR	9.70E-10	DEITFVSGAPR	2.60E-08	NSYPDVAVGSLSDSVTIFR	1.20E-07
11	Integrin alpha-6	ITA6_HUMAN	DEITFVSGAPR	5.10E-06	DGEVGGAVYVYMNQQGR	1.30E-05	NSYPDVAVGSLSDSVTIFR	0.0016
5	Programmed cell death 6-interacting protein	PDC6I_HUMAN	FYNELTEILVR	0.002	FLTALAQDGVINEEALSVTELDR	0.019	ELPELLQR	0.038
16	Rho GTPase-activating protein 1	RHG01_HUMAN	NPEQEPIPIVLR	7.30E-05	FLLDHQGELFPSPDPSGL	0.0074	LEQLGIPR	0.048
14	T-complex protein 1 subunit alpha	TCPA_HUMAN	AFHNEAQVNPER	4.40E-06	EQLAIAEFAR	0.0046		

* excised from the same spot

** excised from the same spot

FIG. 3. **Analysis of HT1376-derived exosomes using 2DE and MS.** Protein extracts from HT1376 exosomes were resolved by 2DE on a pH 3–10 non-linear gradient. Proteins were visualized by silver staining (*A*). Thirty-two spots were randomly chosen, gel plugs were excised, and peptides were recovered following trypsin digestion. Of these, successful identifications were obtained for 17 spots (annotated in *A*), and the details of the MS identifications are listed (*B*). A representative MS/MS analysis from the data set is shown in *C*; the peptide is from integrin α_6 (*spot 10*). The peptide has a precursor mass of 1191.9 and is annotated to show the derived peptide sequence.

drogenase and glyceraldehyde-3-phosphate dehydrogenase, cytoskeleton proteins actin and cytokeratins, ezrin, and others. Nineteen of the 21 identifications from this gel-based approach were also identified by the nano-LC method, demonstrating excellent agreement (90%) between these different methods for resolving exosomal proteins or peptides.

Validation of Proteins Identified: Anomalous MHC Class I Identifications—As with any such proteomics data set, it is important to evaluate the list manually for any unexpected or unexplainable MS identifications and to question the validity of any anomalies discovered in the data. In the current analysis, the LC-MALDI MS data contained multiple identifications for HLA molecules that passed our quality criteria (Expect values <0.05 and IDs based on more than one peptide). These identifications, however, were not physiologically possible as they included five HLA-B alleles and five HLA-C alleles (Table I) from a homogenous cell line. Explanations for this could include contamination of the source cell line with other cells from different donor(s), inadvertent contamination of the specimen by researchers, or issues related to how MASCOT designated HLA haplotypes nomenclature based on the peptide sequences generated from MS. To address these possibilities, a clinical diagnostic service (Welsh Blood Service, Llantrisant, Wales, UK) carried out haplotype analysis of the researcher and the HT1376 cell line. The researcher had no HLA alleles that corresponded to those in the MS list, whereas the HT1376 cells were haplotyped as HLA-A*24; -B*15(62); -Cw*03(9), confirming a homogenous cell line. This led us to examine in more detail the peptide sequences obtained and to evaluate how these were assigned by MASCOT to a given HLA nomenclature (Table I). It was apparent that several peptide sequences had been assigned to multiple HLA types. For example, sequence FDSDAASPR was designated to HLA-B15, -B52, -B54, and -B59 and to HLA-C01, -C12, -C17, and -C03. In contrast, however, there were some peptides that appeared in only a single designation. These

TABLE I

Examination of MASCOT-designated MHC class I identifications, highlighting assignment of peptide sequences to more than one protein identification

MASCOT Designated HLA-identifications														
A24[a]	A80	B15[a]	B52	B54[a]	B59	B08	C01	C12	C14	C17	C03[a]	HLA-G[c]	Peptide Sequences assigned a HLA-designation
■		■	■	■	■			■	■	■			FIAVGYVDDTQFVR
							■						APWVEQEGPEYWDR
												■	**APWVEQEGPEYWEEETR[b]**
■								■	■				**APWIEQEGPEYWDEETGK[b]**
		■	■		■	■			■			■	APWVEQEGPEYWDR
■													**AYLEGTCVDGLR[b]**
		■	■									■	AYLEGLCVEWLR
		■	■		■	■							DGEDQTQDTELVETRPAGDR
■	■							■		■			DGEDQTQDTELVETRPAGDGTFQK
		■											FDSDAASPR
												■	**FIAMGYVDDTQFVR[b]**
												■	**GEPHFIAVGYVDDTQFVR[b]**
		■	■	■	■								GGSYSQAASSDSAQGSDVSLTA
■													GYHQYAYDGKDYIALK
■													GYHQYAYDGK
■	■												KGGSYSQAASSDSAQGSDVSLTACK
■													KWEAAHVAEQQR
		■										■	MYGCDVGPDGR
■	■												SWTAADMAAQITK
			■				■	■	■	■	■		THVTHHPVSDHEATLR
												■	**THVTHHPVFDYEATLR[b]**
		■		■	■	■							THVTHHPISDHEATLR
■				■	■	■							THMTHHPISDHEATLR
		■	■	■	■	■				■	■	■	WAAVVVPSGEEQR
								■	■	■	■	■	YFYTAVSRPGR
■									■				YFSTSVSRPGR
		■	■	■	■								YFYTAMSRPGR
■													**WEAAHVAEQQR[b]**

[a] Peptide sequences highlighted in bold represent those assigned to a single HLA identification.

[b] HLA haplotype of HT1376 cells by PCR.

[c] Positive expression confirmed by Western blot.

unique sequences were assigned to HLA-A24 (APWIEQEGPEYWDEETGK, AYLEGTCVDGLR, and WEAAHVAEQQR), HLA-C03 (GEPHFIAVGYVDDTQFVR), and HLA-G (APWVEQEGPEYWEEETR, FIAMGYVDDTQFVR, and THVTHHPVFDYEATLR). There were no unique peptides for any HLA-B allele, although of the HLA-B subtypes identified, HLA-B15 was assigned the greatest number of peptides. In conclusion, manual analysis of peptides designated as MHC Class I identifications is recommended to clarify potential confusion arising from such MASCOT results.

Validation of Exosomal Expression of Proteins Identified—It is also important to determine the validity of some MS-identified proteins by confirming their presence in the sample by other techniques. With a list as large as 353 proteins, it was not possible to do this wholesale so we restricted such validation to a set of proteins that may be of biological interest.

We performed a series of Western blot panels, analyzing up to 20 μg of HT1376 exosomes per well, to determine whether some MS-identified proteins were detectable in our exosome preparations. We stained for TSG101 as our choice marker for multivesicular bodies and hence exosomes. This protein was incidentally detected by MS by only a single peptide sequence and was therefore excluded from our data on this basis. LAMP-2, a molecule we expected to be present in exosomes, was detected in our sample by MS and was confirmed here to be strongly positive by Western blot (Fig. 4*A*). Among the MS identifications were numerous cytokeratin identifications (type I cytoskeletal keratins 1, 7, 13, 14, 16, 17, 18, and 19). We confirmed expression of cytokeratin 17 and cytokeratin 18 in the preparations, revealing abundant expression of exosomal cytokeratin 17. Cytokeratin 18, however, was only detectable with 20 μg of exosomes per well, suggesting that exosomes genuinely do express multiple cytoskeletal constituents and that the LC-MALDI MS approach is sufficiently sensitive to detect molecules such as cytokeratin 18 that are difficult to reveal by traditional Western blotting methods. Because of the anomalous issues surrounding MHC identifications, it was important to determine whether or not HLA-G was in fact expressed by HT1376 exosomes as this was not included in the PCR haplotyping of HT1376 cells. HLA-G was unequivocally confirmed to be present in exosomes by Western blot. Other membrane-associated (galectin-3, basigin, and CD73) or soluble (hnRNPK and β-catenin) molecules with documented associations in varied aspects of cancer biology were confirmed to be positively expressed by HT1376 exosomes.

Validation of Flotation Characteristics of Identified Exosomal Proteins—Although the standard exosome purification method used here is robust, it remains possible that some non-exosomal contaminating material is present in the preparations and that some of these MS identifications are not genuinely exosomally expressed proteins. To demonstrate that these proteins were exosomally expressed, we ultracentrifuged HT1376 culture medium at 70,000 $\times$ *g*, and the resuspended pellet was subjected to a second ultracentrifugation on a linear sucrose gradient. This was done to determine the capacity of the identified proteins to float at exosomal densities. Each of 15 fractions collected from the gradient was split: one-third was used for analysis by flow cytometry of exosome-coated beads, and two-thirds was used for Western blotting. The former method would reveal possible expression of candidate proteins at the exosome surface, whereas solubilizing exosomes for Western blot would allow surface and intraluminal constituents to be revealed. In the flow cytometry assay, exosome-containing fractions were identified by strong staining for tetraspanins CD9 and CD81 and for MHC Class I, which are known to be expressed on the surface of HT1376 exosomes, revealing a clear and principal peak at a density of 1.12 g/ml (Fig. 4*B*), which is within the expected exosomal density (Fig. 1*C*). This fraction, containing most of the exosomes, therefore also revealed positive surface staining for the MS-identified proteins β_1 and α_6 integrins, CD36 (lysosome membrane protein 2), CD44, CD73 (5′-nucleotidase), CD10 (neprilysin), MUC1, and basigin (CD147). 5T4, a protein not previously identified by any other exosome proteomics study, was included in this panel, demonstrating positive surface expression. The same fractions were also stained with a calnexin-specific antibody, revealing low level expression predominantly at densities greater than the exo-

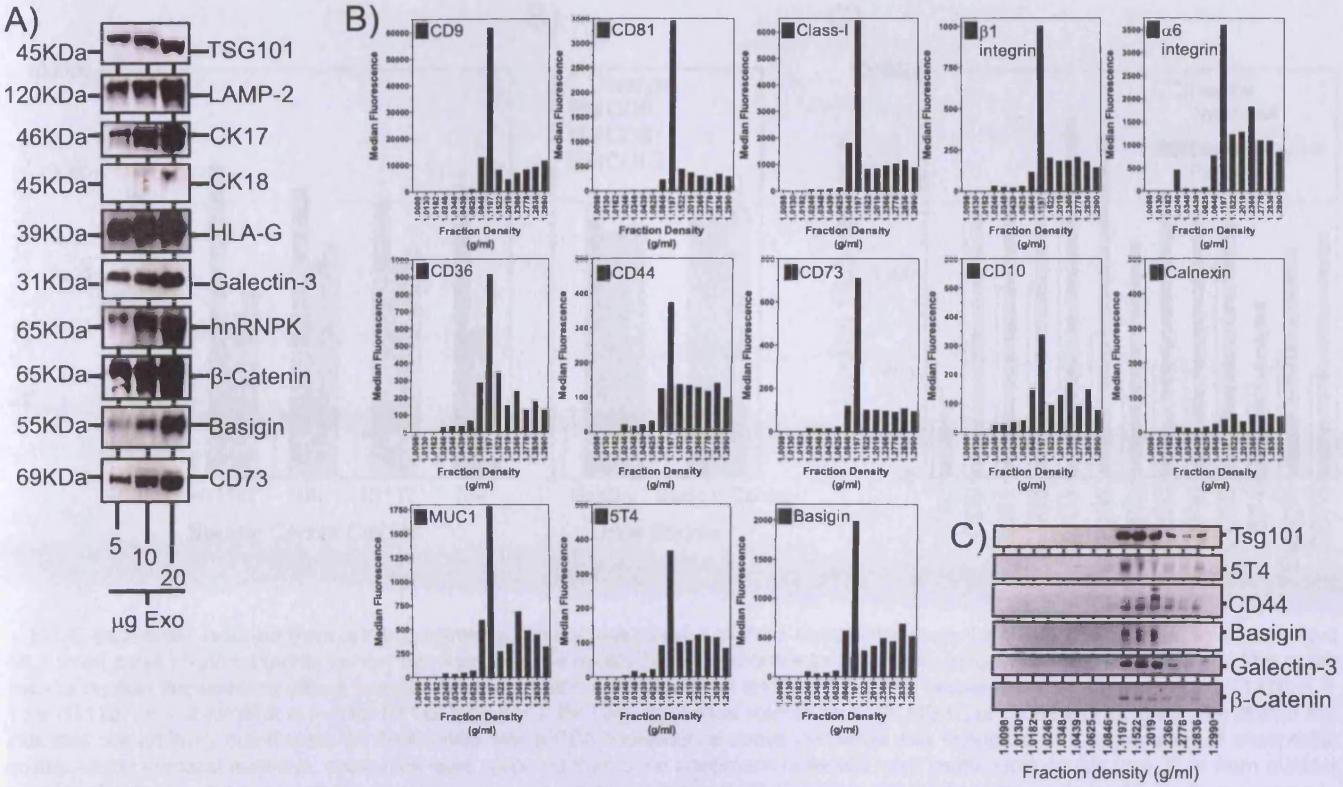


FIG. 4. **Validation of some MS-identified proteins by Western blot and flow cytometric analysis.** HT1376 exosomes (5–20 μg/well), purified by the standard sucrose cushion method, were analyzed by Western blot for expression of a range of MS identified proteins as indicated (*A*). The 70,000 × *g* pellet, obtained from HT1376 cell-conditioned medium, was subjected to fractionation by centrifugation on a linear sucrose gradient (0.2–2.5 M). Fifteen total fractions were collected, and the density was measured by refractometry. Thereafter, one-third of each fraction was coupled to latex beads followed by flow cytometric analysis for exosomal surface expression as indicated (*B*). In parallel, the remaining two-thirds of each fraction was subjected to Western blotting for proteins as indicated (*C*). The data reveal proteins floating at a recognized exosomal density range (1.12–1.2 g/ml). (The data are representative of two experiments.) *CK*, cytokeratin.

some-containing fractions. This confirms the specificity of staining for the other markers tested and the absence of calnexin in exosome-containing fractions as expected (Fig. 4*B*). To reveal relevant fractions in the Western blot panel, we stained for TSG101, highlighting densities of 1.12–1.2 g/ml as exosome-containing (Fig. 4*C*). There was some positive staining at hyperdense fractions (>1.2g/ml), but this was relatively weak and may be due to exosome or protein aggregates. The proteins 5T4, CD44, basigin, galectin-3, and β-catenin all co-localized at the same density range, consistent with their exosomal expression. Overall, these data show that 18 of the MS protein identifications achieved in this study are confirmed to be expressed by HT1376 exosomes and that membrane-associated molecules, often difficult to solubilize and identify by MS approaches, have been successfully identified and validated as localized to the exosome membrane.

Preliminary Validation of Presence of Some MS-identified Proteins Present on Urinary Exosomes from Bladder Cancer Patients—Although we have previously examined exosomes present in the urine of prostate cancer patients (15), there are no studies to date specifically describing urinary exosomes of bladder cancer patients, although as we have mentioned, there is one report about urinary microparticles from this disease setting (17). Although a thorough examination of this question is outside the scope of this report, we have made preliminary efforts to ascertain the feasibility of doing such analyses of exosomes with bladder cancer patient-derived urine using the sucrose cushion purification method.

We first confirmed that the ultracentrifugation approach (sucrose cushion method) would be effective in isolating quality exosomes from other bladder cancer cell lines. We used the latex bead assay (as shown in Fig. 1) to evaluate the quality of the exosomes purified. Analysis of an additional four bladder cancer cell lines revealed it was possible to achieve purifications of quality comparable to those from HT1376 cells. The method should therefore be well suited for capturing bladder cancer exosomes irrespective of nuances of density that may differ slightly across different cells (Fig. 5*A*).

We next embarked on purifying exosomes from urine specimens using this method. Urine specimens were collected from four healthy individuals and from three patients with transitional cell carcinoma of the bladder prior to the commencement of any treatment. The latex bead assay was performed (Fig. 5*B*), revealing good levels of signals for the three

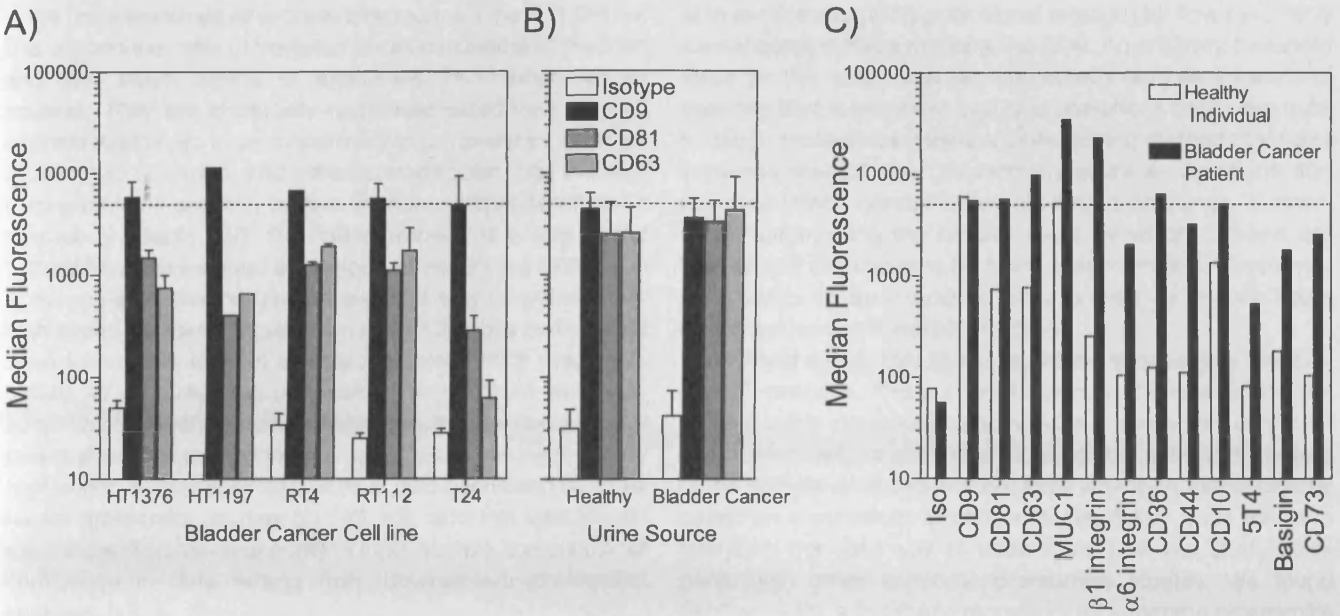


FIG. 5. **Exosomes isolated from urine specimens.** The sucrose cushion method, used in this study for HT1376 exosomes, was also tested on a small panel of other bladder cancer cell lines, and the quality of preparations was assessed using the latex bead assay (*A*). The graph depicts median fluorescence values from the flow cytometric histogram peak (mean ± S.D. of *n* preparations where $n = 5$ for HT1376, $n = 1$ for HT1197, $n = 2$ for RT4, $n = 2$ for RT112, and $n = 2$ for T24) with beads stained for CD9, CD81, or CD63 as indicated. The *dotted line* indicates our arbitrary cutoff value (of 5000 units) where CD9 fluorescence above this value was indicative of a preparation of acceptable quality. Using identical methods, exosomes were prepared from urine specimens collected from healthy individuals ($n = 4$) or from bladder cancer patients ($n = 3$) (mean ± S.D.), and exosomes were analyzed as above (*B*). Exosome preparations from one healthy individual and one bladder cancer patient, which exceeded the quality threshold, were analyzed further for surface expression of some MS-identified proteins as indicated (*C*). *Iso*, isotype.

tetraspanin molecules tested (CD9, CD81, and CD63), but only one preparation from each cohort passed our arbitrary quality threshold (over 5000 units for CD9 staining). These high quality specimens were examined further for expression of surface-oriented membrane proteins (similar to the panel in Fig. 4*B*), revealing that it is possible to detect positive expression (above isotype-stained controls) for most of these proteins tested. Importantly, some differences in the exosome profile between health and disease may be apparent using such a comparative test, such as elevated exosomal CD36, CD44, 5T4, basigin, and CD73 in cancer (Fig. 5*C*). We emphasize caution, however, in overinterpretation of these data based on the few clinical specimens that were available to us. Nevertheless, this aspect appears promising and warrants future follow-up studies.

DISCUSSION

Exosomes are highly complex nanometer-sized vesicles that are ubiquitous in biological systems. There is considerable research interest in understanding the physiological functions of exosomes in various settings, no more so than in elucidating their role in diseases like cancer. Studies of exosomes in various malignancies like prostate (8, 15, 16), breast (28), and colorectal cancers (44); melanoma (40, 45); pleural mesothelioma (5); malignancies of the central nervous system (21, 46); and others have begun. Collectively, these studies highlight expression of multiple tumor-related antigens by exosomes and perhaps general roles in immune modulation or in other aspects of cancer biology. In recent years, there has been particular interest in utilizing exosomes, isolated from patients, as tools for diagnosing disease (8, 11–14) or for discovering novel molecular markers (7). To date, however, there is only one report in the context of bladder cancer that highlights some disease-related differences in the protein constituents of urine-derived microparticles (17). Whether or not these proteins relate specifically to the exosomes present in such complex samples remains unclear.

This is an area that calls for some attention as managing and monitoring this disease are challenging, involving highly invasive and expensive procedures. Development of non-invasive tools for bladder cancer would therefore be particularly welcome. The direct contact between bladder urothelial cells and urine presents ample opportunity for secretion of exosomes directly into the urinary space (2). Collection and analysis of urinary exosomes in this disease setting hold promise as a novel diagnostic platform. However, exploitation of urinary exosomes requires a substantive high quality proteomic description of bladder cancer exosomes to be performed. Thus, this study provides essential solid ground that will greatly facilitate future developments in this understudied disease.

We have examined exosomes isolated from the HT1376 cell line, a good example of transitional cell carcinoma of bladder, and they seem typical of exosomes from other cellular sources. They are classically nanometer-sized vesicles that express high levels of tetraspanins, MHC molecules, markers of the endocytic tract, and adhesion molecules, and the vesicles exhibit the capacity to float at characteristic densities on sucrose gradients (19). This latter property is a very useful feature that can be used as a mode of separating exosomes from non-exosomal protein material that may co-pellet under high speed ultracentrifugation, an aspect that has perhaps not been fully appreciated in all exosome proteomics studies (2, 39, 40, 47, 48), resulting in possible false positive identifications. Our choice protocol is based on the method of Lamparski *et al.* (20) that provides a good exosome yield of very high purity. Similar approaches have also been used by other recent proteomics studies (21, 43, 49), and this vital investment in deriving maximal purity of input sample is essential for confidence in data arising from downstream proteomics analyses.

A major difficulty in the field, however, is to accurately estimate levels of sample contamination. One approach used by us and others is to perform Western blots for molecules not putatively expressed by exosomes, such as markers of the endoplasmic reticulum (*e.g.* calnexin or gp96), mitochondria, and nucleus. The difficulty here is that although such compartments may be relatively poorly represented in the exosome proteome it is not clear to what degree this rule is absolutely true (*i.e.* they may be present in exosomes at low levels). Our MS approach has indeed identified several proteins that are normally located to the endoplasmic reticulum or other compartments not well represented by exosomes, and this is also true of other exosome proteomics studies (37). These identifications may reflect the higher sensitivity of MS workflows for detecting these relatively low abundance contaminants that may not be detected efficiently by Western blotting. An alternative explanation, however, may be that these constituents are genuinely expressed by exosomes. If not directly loaded into/onto exosomes during manufacture, it may be possible that some proteins may be present at low levels at the outer surface of the cell and subsequently become taken up into the endosomal system and packaged into exosomes (50). In addition, a host of poorly understood cellular alterations occurring in cancer cells may modify trafficking of some proteins, resulting in inappropriate distributions, such as hnRNPK, which may become cytoplasmically rather than nuclearly located in certain cancers (51, 52). The absolute exclusion of endoplasmic reticulum-resident proteins (or proteins related to other cellular compartments) from exosomes may be a rule that is bent or broken in cancerous cells. Our use of "sticky" latex microbeads is a simple but effective method for estimating the degree of contamination of preparations by non-exosomal soluble proteins. Preparations replete with contaminants would result in beads poorly coated with exosomes, giving poor signal strength by flow cytometry for exosome surface markers like CD9. An arbitrary threshold value for this assay was set intentionally high as a means of assuring that the highest quality preparations only were subjected to proteomics. However, developing methods that discriminate well between exosomally expressed proteins and low abundance contaminants remains a challenge. Furthermore, establishing the precise route by which proteins are loaded into exosomes is far from straightforward. These may be aspects to be examined on a protein by protein basis during subsequent validation steps.

We used an LC-MALDI MS workflow, successfully identifying 353 proteins. This number of identifications is among the highest in the exosome proteomics field, and we are confident about the quality of such identifications because of the nature of the sample analyzed and because we report identifications based on a minimum of two peptides. A key issue for us in analyzing the data was to understand how our study compares with other exosome proteomics studies. We found ExoCarta (33), a database repository for exosome proteomics studies, to be a useful tool. By obtaining gene lists from these studies and our own, we were able to perform an overrepresentation analysis of the data, that is to discover whether there were more genes overlapping with ExoCarta gene sets than could be expected by chance. This statistical method, therefore, represents an unbiased approach for bioinformatics examination of our MS data. In essence, this revealed substantial data matching, particularly with high quality studies of colorectal cancer exosomes (42, 43), showing the HT1376 exosome proteome to be strongly consistent with exosomes of carcinoma origin. Similar results were evident using GeneGO MetaCore, emphasizing strong associations with carcinoma (of various sites) above other disease types. The GeneGO analysis, however, has also raised some issues that were not entirely expected, such as the predominant associations with control of the cytoskeleton. This statistically discovered association seems valid because multiple cytokeratins (cytokeratins 1, 5, 6B, 8, 13, 14, 16, 17, 18, and 19), actins (cytoplasmic 1, actin-like protein 6A, actin-related protein 2, and α cardiac muscle 1), myosin (myosin-1c, -10, and -14), tubulin (putative tubulin-like protein α-4B and tubulin β chain), and cytoskeletal linking proteins (filamin-A and -B, α-actinin-1 and -4, and plectin-1) were present in the identifications, and we confirmed expression of some by Western blot. The importance of these proteins in terms of exosome biology is unknown. Exploring exosomal cytokeratin profiles *ex vivo* may be clinically useful in bladder cancer as these can change with epithelial differentiation and with invasive properties and may help predict the outcome or assist differential diagnoses (27). An additional unexpected aspect, arising from the cell compartment GeneGO query, was the apparent strong associations with specific compartments, the melanosome and pigment granule. These tissue-specific organelles are responsible for the manufacture and storage of melanin in pigment

cells of skin and eye. One would certainly not expect bladder cancer cells to strongly resemble pigment cells, and as such, this was a surprising finding. However, the melanosome is a specialized endosomal compartment that is derived from early endosomal intermediates that strongly resemble multivesicular bodies in morphology and composition. Thus, the highly evolutionally conserved molecular machinery involved in protein chaperone functions, in membrane fusion and budding events, and in transportation of proteins to the melanosome bears significant resemblance to the generic machinery giving rise to exosomes (53), which may explain this association.

We also, albeit briefly, investigated whether exosomes could be isolated from other bladder cancer cell lines and more importantly from urine specimens using the same exosome purification method. This was highly successful, giving comparable levels of purity assessed by our latex bead assay, when using an additional four bladder cancer cell lines. When applying the techniques to freshly collected urine specimens, a source that is significantly more complex and more variable than cell culture supernatant as we described previously (15), most preparations did not reach our "exosome quality threshold." Nevertheless, preparations from healthy donors and bladder cancer patient urine stained strongly for the tetraspanin proteins CD9, CD81, and CD63. This aspect is of particular note because it may be attractive in future clinical studies to move away from the reliance on ultracentrifugation methods, which are cumbersome and impractical for large sample sets, and replace these with an affinity approach. Such tetraspanins, therefore, would be a good choice for antibody-mediated exosome capture either directly onto microtiter plates or onto microbeads (1) followed by an analysis for additional protein markers of interest (*i.e.* those within our MS identifications). In fact, we were able to demonstrate that several MS-identified exosome membrane proteins were expressed at relatively higher levels in exosomes isolated from a bladder cancer patient compared with exosomes isolated from a healthy donor. Although not exhaustive, such data suggest that urinary exosome analysis in the context of bladder cancer may prove fruitful and is certainly worthy of further attention.

In summary, we have achieved the first high quality proteomic description of bladder cancer cell-derived exosomes and have learned three key lessons that have wide applicability to other proteomics studies. First, we show the usefulness of DTT as part of a solubilization buffer. This agent increased the number of identifications more than 100-fold. Second, we highlight that care must be taken with identifications of proteins showing considerable homology (with HLA proteins being an excellent case study). Our work demonstrates that MASCOT is not capable of distinguishing between the incorrect and correct HLA molecules present in exosomes. We believe this problem has probably affected many other proteomics studies. Third, we emphasize the importance of careful sample preparation both as part of a proteomics workflow and for validation. The particular example in our study was our use of latex microbeads. This allowed us to quality control our specimens before commencing proteomics and, using this approach, also allowed us to validate, quantify, and confirm the orientation of 12 proteins in a way that is impossible with just Western blotting. Follow-up investigations, informed by this report, are now planned to identify the presence of candidate markers in the urine of bladder cancer patients with the ultimate goal of replacing highly invasive procedures currently utilized in diagnosis and monitoring with a fully non-invasive urinary exosome-based technique.

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[S] This article contains supplemental Fig. 1 and Table 1.

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