Proteomics Analysis of A33 Immunoaffinity-purified Exosomes Released from the Human Colon Tumor Cell Line LIM1215 Reveals a Tissue-specific Protein Signature*

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Exosomes are 40–100-nm-diameter nanovesicles of endocytic origin that are released from diverse cell types. To better understand the biological role of exosomes and to avoid confounding data arising from proteinaceous contaminants, it is important to work with highly purified material. Here, we describe an immunoaffinity capture method using the colon epithelial cell-specific A33 antibody to purify colorectal cancer cell (LIM1215)-derived exosomes. LC-MS/MS revealed 394 unique exosomal proteins of which 112 proteins (28%) contained signal peptides and a significant enrichment of proteins containing coiled coil, RAS, and MIRO domains. A comparative protein profiling analysis of LIM1215-, murine mast cell-, and human urine-derived exosomes revealed a subset of proteins common to all exosomes such as endosomal sorting complex required for transport (ESCRT) proteins, tetraspanins, signaling, trafficking, and cytoskeletal proteins. A conspicuous finding of this comparative analysis was the presence of host cell-specific (LIM1215 exosome) proteins such as A33, cadherin-17, carcinoembryonic antigen, epithelial cell surface antigen (EpCAM), proliferating cell nuclear antigen, epidermal growth factor receptor, mucin 13, misshapen-like kinase 1, keratin 18, mitogen-activated protein kinase 4, claudins (1, 3, and 7), centrosomal protein 55 kDa, and ephrin-B1 and -B2. Furthermore, we report the presence of the enzyme phospholipid scramblase implicated in transbilayer lipid distribution membrane remodeling. The LIM1215-specific exosomal proteins identified in this study may provide insights into colon cancer biology and potential diagnostic biomarkers. Molecular & Cellular Proteomics 9: 197–208, 2010.

Exosomes represent a distinct class of membrane nanovesicles (40–100-nm diameter) of endocytic origin that are released from diverse cell types under both normal and pathological conditions (1). Although initial studies focused on exosomes released from various cell types in vitro, exosomes have also been reported in diverse body fluids such as urine (2), amniotic fluid (3, 4), malignant ascites (5–7), bronchoalveolar lavage fluid (8), synovial fluid (9), platelets (10), breast milk (11), and blood (12). Exosomes are formed through the inward budding of late endosomal membranes that give rise to intraluminal vesicles (ILVs)1 within intracellular multivesicular bodies (MVBs). MVBs have a well known intermediary function in the degradation of either proteins internalized from the cell surface (e.g. cell surface receptors) or intracellular proteins sorted from the trans-Golgi network. Proteins destined for degradation are sorted, typically in a ubiquitin-dependent manner, into the ILVs of the nascent MVBs, which then fuse with pre-existing lysosomes (13). An alternate fate for MVBs involves their fusion with the plasma membrane and ensuing release of ILVs into the extracellular environment as exosomes. The biogenesis of exosomes has been linked to the protein complex ESCRT machinery, which is required for both formation of MVBs and the recruitment of their endosome-derived cargo proteins (14).

Exosomes exhibit pleiotropic biological functions including immunomodulatory activity, mediation of cell-cell communication, and, possibly, the transport and propagation of infectious cargo such as prions and retroviruses (1, 15, 16). Despite these advances in our understanding of exosome function, the physiological significance of exosomes is still not fully understood. The observation that exosomes contains inactive RNA and microRNAs that can be transferred to another cell and be translated in the recipient suggest that exosomes may provide a novel vehicle for genetic exchange between cells (17). More recently, the finding of glioblastoma tumor cell-derived exosomes that contain mRNA mutant/vari-

1 The abbreviations used are: ILV, intraluminal vesicle; MVB, multivesicular body; ESCRT, endosomal sorting complex required for transport; CM, conditioned medium; CCM, concentrated conditioned medium; EM, electron microscopy; HPRD, Human Protein Reference Database; HIV-1, human immunodeficiency virus, type 1; RT, room temperature; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SMART, simple modular architecture research tool; TM, transmembrane-spanning domain.
ants and microRNAs characteristic of the glioma coupled with the finding of these microvesicles in serum of glioblastoma patients suggests that blood-based exosomes may provide important diagnostic information and aid in therapeutic decisions for cancer patients (18).

The molecular composition of exosomes purified from the cell culture medium from various cell types and diverse body fluids has been analyzed by proteomics as well as fluorescence-activated cell sorting, Western blot analysis, and immunohistochemistry (1, 19). In addition to displaying a protein composition that reflects their endosomal origin, these proteome profiling studies also indicate a unique protein fingerprint that reflects their cellular origin as well as possible physiological role and targeting properties. However, interpretation of exosomal proteome profiles in a biological context also highlights a cautionary note, especially if exosomes are not highly purified. For example, retroviruses such as HIV particles that bud from the cell surface using the same endocytic pathway machinery as exosomes to egress from hematopoietic cells can be a confounding factor in biochemical and physiological analyses of exosomes. Furthermore, exosomes and HIV-1 particles have similar biophysical properties such as size (40–100 and 100 nm, respectively) and buoyant density (1.13–1.21 g/liter (20) and 1.13–1.21 g/liter (21), respectively) as well as molecular composition and their ability to activate immune cells. Although earlier studies describe exosomes carrying virion cargo (22–24), recent exosome purification strategies deploying immunofluorescence capture (25) or a combination of immunoaffinity capture and density gradient centrifugation (26) demonstrate that exosomes from hematopoietic cells can be purified free of virions like HIV-1.

In-depth proteomics studies with large data sets that might contribute to the understanding of the biological function of exosomes are, to date, limited (2, 17). Moreover, strategies used to purify exosomes differ between laboratories (1) with little consensus concerning criteria of purity. Isolation strategies typically involve a combination of differential centrifugation, filtration, concentration, and flotation density gradient followed by characterization using electron microscopy, flow cytometry, and Western blotting (for a review, see Simpson et al. (1)). As a first step toward understanding the physiological role of exosomes in colon cancer biology, we describe here a robust strategy to isolate and characterize exosomes released from LIM1215 colorectal carcinoma cells (27) for the purpose of proteome analysis. This isolation strategy utilized the colon epithelial cell-specific A33 antibody (28–31) to immunofluorescence capture A33-containing exosomes using microbeads. Here, we report for the first time an in-depth proteomics analysis of A33-containing exosomes released from the LIM1215 colon carcinoma cell line. Using these data, we performed a comparative bioinformatics analysis with human urinary and mast cell-derived exosomes.
ies (1 μg/ml) for 1 h in TBST followed by incubation in horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad; 1:5,000) or IRDye 800CW goat anti-mouse IgG (LI-COR Biosciences; 1:15,000) for 1 h. All antibody incubations were carried out at RT with shaking, and blots were washed (three times) with TBST for 10 min after each incubation step. Antigen-antibody complexes were visualized by chemiluminescence (ECL Plus Western blotting detection reagents, GE Healthcare) on x-ray film or scanned using the Odyssey imager (LI-COR Biosciences).

Preparation of Immunoadfinity Capture Microbeads—Humanized A33 monoclonal antibody immunoadfinity capture microbeads (Protein G Dynabeads™, Invitrogen) were prepared as follows. Briefly, 500 μl of Dynabeads (5 × 10⁸ beads) in citrate-phosphate buffer, pH 5.0 were mixed with 100 μl of capture antibody (300 μg) and incubated for 40 min at RT with gentle rotation according to the manufacturer’s instructions. The beads were placed on a magnet for 2 min, the supernatant was discarded, and the beads were washed (two times) with citrate-phosphate buffer, pH 5.0. The Dynabeads were washed twice with 1 ml of 0.2 M triethanolamine, pH 8.2 with the use of a magnet; then suspended in 1 ml of freshly prepared 20 mM dimethyl pimelimidate in 0.2 M triethanolamine, pH 8.2; and incubated for 30 min at RT with gentle agitation. The beads were placed on a magnet for 2 min, and the supernatant was discarded. The reaction was halted by resuspending the beads in 1 ml of 50 mM Tris, pH 7.5 for 15 min with gentle mixing. The cross-linked beads were harvested with the use of a magnet and washed (three times) with PBS containing 0.05% Tween 20.

Purification of LIM1215 Cell-derived Exosomes by Immunoadfinity Capture Using A33 Antibodies—CMC from LIM1215 cells (~1.9 mg of protein in 3 ml) was preincubated with Dynabeads (5 × 10⁸ beads) for 2 h at 4 °C with gentle rotation to reduce nonspecific binding. The beads were harvested using a magnet (2 min), and the recovered supernatant solution was then incubated with A33 antibody-coated Dynabeads (300 μg of antibody/5 × 10⁸ beads) for 2 h at 4 °C with gentle rotation. The A33 antibody-coated beads were washed for 5 min at RT in 1 ml of PBS (three times) with the use of a magnet (2 min), and A33-containing exosomes were eluted from the beads with 100 μl of lithium dodecyl sulfate sample loading buffer (Invitrogen) for SDS-PAGE analysis.

LC-MS/MS—Exosome samples (16 μg) were electrophoretically separated using SDS-PAGE, and proteins were visualized by staining with Imperial protein stain (Pierce). Gel lanes were cut into 20 mm bands using a GridCutter (The Gel Co., San Francisco, CA), and proteins were trypsinized as described previously (35). Extracted tryptic peptides from each gel band were concentrated to ~10 μl by centrifugal lyophilization and analyzed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) fitted with a nanoflow reversed-phase HPLC system (Model 1200, Agilent). Reversed-phase HPLC of peptide mixtures was performed on a nanoAcquity (C₁₈) 150-mm reversed-phase HPLC column (Waters) developed using a linear 60-min gradient from 0 to 100% B (0.1% aqueous formic acid, 60% (v/v) aqueous phase UPLC column (Waters) developed using a linear 60-min gradient from 0 to 100% B (0.1% aqueous formic acid, 60% (v/v) aqueous phase UPLC column (Waters) developed using a linear 60-min gradient from 0 to 100% B (0.1% aqueous formic acid, 60% (v/v) aqueous phase UPLC column (Waters) developed using a linear 60-min gradient from 0 to 100% B (0.1% aqueous formic acid, 60% (v/v) aqueous

Automatic charge state recognition was used because of the high resolution survey scan (30,000). LC-MS/MS spectra were searched against the Ludwig non-redundant database (36) using MASCOT (v2.2.01, Matrix Science, London, UK). The search parameters used were as follows: fixed modification, carboxymethylation of cysteine (+58 Da); variable modification, oxidation of methionine (+16 Da); three missed tryptic cleavages; 20-ppm peptide mass tolerance; and 0.8-Da fragment ion mass tolerance. Peptide identifications with ion scores greater than identity scores and proteins with at least two peptide identifications were considered to generate a preliminary protein list, resulting in a 0% false discovery rate (derived from corresponding decoy database search) for both purified and crude exosome preparations. Manual interrogation of the preliminary list was done to remove FCS contaminants.

Comparison of Pure Exosome Proteomes—In-house Perl scripts were written to map protein identifiers, obtained from crude and purified exosome preparations, to NCBI Entrez gene (37) identifiers. Entrez gene identifiers were then mapped to HUGO (Human Genome Organisation) gene nomenclature committee-approved gene symbols (38) to facilitate cross-comparison at the gene product level. Perl scripts were written to compare the gene symbols.

Comparison of Pure Exosome Proteomes with Published Exosome Proteomes—Two studies that had detected more than 200 exosomal proteins were compared with the LIM1215-derived pure proteome described in this study. Psitkin et al. (2) identified 295 proteins in human urinary exosomes that were mapped to Entrez gene identifiers. In a separate study, Valadi et al. (17) identified 272 proteins in both murine and human mast cell-derived exosomes. Those 272 protein identifiers were mapped to Entrez gene and later mapped to human orthologs through Homologene (39), resulting in 266 gene identifiers. The two gene lists were then compared with the LIM1215-derived pure exosome gene identifiers using Perl scripts.

Biological Process, Molecular Function, and Subcellular Localization—Gene ontology annotations for biological process, molecular function, and subcellular localization were downloaded from the Human Protein Reference Database (HPRD) (40). In-house Perl scripts were used to map the proteins with corresponding gene ontology terms.

Domain/Motif Enrichment—Protein sequence analysis of the identified exosomal proteins was performed to identify domains/motifs that are significantly enriched. In-house Perl scripts were used to submit the protein sequences individually to the web interface of SMART (41) with an interval of 10 s between the subsequent posts. The same was done for the entire human RefSeq (42) proteome (~39,000 sequences). The y² test with Yates correction was used to detect the p value for the domain enrichment analysis. SignalP (43) was used in both the neural network and the hidden Markov model modes to predict the occurrence of signal peptides.

Tissue Signature Analysis of Human Colon Carcinoma (LIM1215 Cell), Urine-, and Mast Cell-derived Exosomes—Exosomal protein data with respect to the tissue from which they were derived were overlaid in a matrix. The score provided for the protein is 0 if not detected in exosome or 1 if detected. The same scoring was applied to all 759 unique protein identifiers (266 from mast cell exosomes, 295 from urine exosomes, and 394 from colorectal cancer (LIM1215-derived pure exosomes). Human protein tissue expression profiles with respect to urine, mast cells, and colorectal cancer were downloaded from the Human Proteinpedia (44), HPRD, and Human Protein Atlas (45). In addition, a urinary proteome study (46) was also used to populate the urinary proteome catalogue. The obtained tissue expression profiles were added on to the exosomal protein matrix with the same scoring pattern of 0 (not detected in a specific tissue) or 1 (detected in a specific tissue). Each cell will have a value ranging from 0 to 2 depending on its identification in exosome and corresponding

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tissue. Hierarchical clustering was performed (scaled to row) to bring protein clusters that were exclusively found in a specific tissue and its corresponding exosome using R.

Cancer Hallmarks—Biomart was used to assign gene ontology annotations to the identified proteins in LIM1215-derived pure exosomes (to get extended gene ontology annotations). The gene ontology terms were grouped to seven different classes of cancer hallmarks such as sustained angiogenesis, insensitivity to antigrowth signals, an inflammatory microenvironment, tissue invasion and metastasis, limitless replicative potential, evading apoptosis, and self-sufficiency in growth signals. Up-regulated genes in colon cancer metastasis from a few studies were downloaded from Oncomine (47). Gene symbols commonly found between colon cancer metastasis and LIM1215-derived pure exosomes were included in the metastasis category.

RESULTS AND DISCUSSION

Production and Characterization of Exosomes Derived from LIM1215 Cells—We have developed a strategy, using a combination of differential centrifugation and molecular weight cutoff membranes, for isolating crude exosomes (40–100-nm diameter) in high yield from the CM of LIM1215 cells grown for 24 h in serum-free RPMI 1640 medium supplemented with 0.8% insulin-transferrin-selenium. The recovery of crude exosomes from CCM by ultracentrifugation (100,000 × g for 1 h) was ~50 µg from 5 × 10⁸ cells. EM revealed that the crude exosomes were essentially homogeneous and 40–100 nm in diameter (Fig. 1A). Immunelectron microscopy using a NANOGOLD probe showed that A33 and the lysosomal marker (1) CD63 antibodies localized to the exosome surface (Fig. 1, B and C, respectively). Crude exosomes in the CCM were further characterized by rate zonal centrifugation through a discontinuous iodixanol (OptiPrep) gradient (Fig. 1D). Fractions of increasing density were collected, and those containing exosomes were identified by Western blotting using A33 antibodies and the common exosome molecular markers HSP70, Alix/PDCD6IP, and TSG101. It can be seen in Fig. 1D that crude exosomes released from LIM1215 cells float at a density of 1.10–1.12 g/ml (Fractions 7 and 8), consistent with the density of exosomes from other cell types (15, 17, 18).

To better characterize colon tumor cell-derived exosomes, we applied an additional purification step utilizing immunoaffinity capture with A33 antibody-coated Dynabeads. CCM containing crude exosomes, which had been pretreated with Dynabeads, was mixed with A33 antibody-coated beads (2 h at 4 °C) and recovered using a magnet. Western blot analysis using anti-A33 antibodies revealed that ~80% of the A33-containing exosomes in the CCM can be immunoaffinity captured by this approach with an overall yield of ~25 µg (i.e. ~50% compared with that achieved using ultracentrifugation at 100,000 × g for 1 h); see Fig. 1E.
Proteome Profiling of Immunoaffinity Capture-purified LIM1215 Cell-derived Exosomes—Next, we compared the proteome profiles of immune capture-purified exosomes (LIM1215-derived pure) with crude exosomes harvested directly from the CM by ultracentrifugation. Both exosome preparations were solubilized in SDS-PAGE sample buffer and analyzed using one-dimensional SDS-PAGE. Gel bands were excised and individually trypsinized, and extracted tryptic peptides were subjected to LC-MS/MS. Protein database searching of MS/MS data resulted in the identification of 285 and 394 unique proteins in the crude and pure exosome data sets, respectively (see supplemental Tables 1 and 2). As revealed in the Venn diagram (Fig. 2A), 191 proteins were common between the two exosomal data sets. 394 proteins found in the immunoaffinity capture-based purified exosomes were used in further analysis. B, subcellular localization of the 394 proteins identified in the LIM1215-derived pure exosomes is shown. 29% of the proteins have a cytoplasmic localization, whereas 19% of the proteins have plasma membrane localization. 6% of the proteins are known to be extracellular, and 20% are known to be localized to the nucleus.

An inspection of the proteome data set for LIM1215-derived pure exosomes also confirmed the presence of many proteins common to exosomes studied to date. These include components of the ESCRT machinery such as Alix, a molecular marker of exosomes (20); TSG101, an endosomal marker; vacuolar protein sorting-associated proteins (VPS25, VPS28, and VPS37); charged multivesicular body proteins (CHMP2, CHMP4, and CHMP5); and tetraspanins (CD9, CD81, CD82, and tetraspanin-8) (51). Other exosome-associated proteins such as Rabs, Raps, annexins, guanine nucleotide-binding proteins, heat shock proteins, cytoskeletal proteins, meta-
bolic enzymes, HLA class I antigen, integrins, lactadherin, claudins, and clathrin were also identified. Interestingly, several kinases (LYN, MINK1, and MAPK4), proteases (ADAM10, DPEP1, and ST14), transporters (SLC1A4, SLC16A1, and CLIC1), and receptors (CD46, CD55, and NOTCH1) were also present. Cell type-specific proteins associated with the gastrointestinal tract were also present (A33 antigen, cadherin-17, carcinembryonic antigen, and ephrin-B1 and -B2) (supplemental Tables 1 and 2). Additionally, this is the first study to report the presence of phospholipid scramblase in our study. The data are available free for download from ExoCarta, a database for exosomes can induce angiogenesis via capillary sprouting (63).

Domain/Motif Enrichment Analysis of LIM1215-derived Pure Exosomal Proteins—The identified 394 proteins were searched against the SMART database for the presence of domains/motifs that are significantly overrepresented in the LIM1215-derived pure exosome data set. This study revealed that coiled coil motifs were enriched (23% occurrence) in the LIM1215-derived pure exosomes compared with 11% of proteins in the entire human proteome (p value <0.0001) (Fig. 3A). Additionally, both RAS and MIRO domains were preferentially enriched in the LIM1215-derived pure exosomes (both were observed 11% of the exosome proteins compared with 0.7% in the entire human proteome (p value <0.0001)).

Coiled coil motifs are recognized to play a role in dimerization domains of several proteins, such as GCN4 (64), Fos, and Jun (65). Interestingly, coiled coil motifs have been shown to play a vital role in vesicular transport (66) and the localization of certain proteins to the early endosomes (67). As coiled coil motifs mediate protein-protein interactions and vesicular transport, it is interesting to speculate that coiled coil motifs are required for certain proteins to be engulfed in exosomes. Likewise, RAS domains (68) are found in Ras proteins (69, 70), which are small GTPases that perform GTP binding. These small GTPases are predominantly found in the exosomes and perform functions with respect to cell growth, proliferation, and differentiation. Despite interacting with a common set of proteins, distinct signal outputs are produced by different Ras isoforms such as H-Ras, N-Ras, and K-Ras (69). Preferential enrichment of RAS domains in exosomes suggests the dominance of GTPases in these vesicles. Exosomal proteins were also preferentially enriched with MIRO domain-containing proteins. MIRO domains are found in Rho GTPases (71) that are larger than the classical small GTPases. Rho GTPases perform roles in mitochondrial homeostasis, apoptosis, and trafficking (72).

Non-classical Secretory Mechanism—In total, ~28% of the proteins were predicted to contain signal peptides using SignalP. Distribution of the subcellular localization of the 394 proteins revealed that 6% of the proteins are known to be extracellular (Fig. 2B). A majority of the LIM1215-derived pure exosome proteins (72%) did not contain a signal peptide. This finding suggests that exosomes might function as a non-classical secretory mechanism (2) wherein proteins are transported via the circulatory system to various tissues. It is interesting to speculate that non-classical secretory behavior of exosomes might perform a vital role in cell-cell communication either by stimulation via ligands expressed on their surface or through transfer of protein/RNA molecules. These
Exosomes might function as communicasomes wherein the molecule content of exosomes, apart from a common set of molecules, is influenced by the tissue/cell type and, indeed, the function performed.

Comparative Proteome Analysis of Urine-, Mast Cell-, and LIM1215-derived Pure Exosomes—Exosomes are likely to harbor a common set of proteins that aid in various processes including biogenesis, cell growth, cell maintenance, cell communication, and signal transduction. With the availability of 394 proteins from colorectal cancer-derived exosomes, it would be interesting to compare them with the already reported exosomal proteomes in the literature. Pisitkun et al. (2) reported 295 proteins identified in human urine exosomes, whereas Valadi et al. (17) had reported 272 proteins from murine and human mast cells. We compared the two exosomal proteomes along with the LIM1215-derived pure exosomal proteome obtained from our study. We found 31 proteins to be common between all the three exosomal proteomes obtained from urine, mast cells, and LIM1215 cells. Here, two previous exosomal studies published in the scientific literature were used to find the overlap between the exosomal proteomes. 31 proteins were found to be identified in all three exosomal proteomes, whereas 96 and 79 proteins were found to be in common between LIM1215-urine and LIM1215-mast cell exosome data sets, respectively.

![Protein domain distribution of LIM1215-derived pure exosomal proteins and overlap of urine-, mast cell-, and LIM1215-derived pure exosomal proteomes. A, distribution of protein domains that are preferentially enriched in the LIM1215-derived pure exosomes. Coiled coil domain is predicted to be present in ~23% of the exosomal proteome, whereas it is present in only ~11% of the entire human proteome (human RefSeq proteome). Likewise, RAS and MIRO domains are highly significant in the LIM1215-derived pure exosomal proteomes (11%) as compared with the entire human proteome (0.7%). B, three-way Venn diagram depicting the overlap between the exosomal proteomes derived from urine, mast cells, and LIM1215 cells. Here, two previous exosomal studies published in the scientific literature were used to find the overlap between the exosomal proteomes. 31 proteins were found to be identified in all three exosomal proteomes, whereas 96 and 79 proteins were found to be in common between LIM1215-urine and LIM1215-mast cell exosome data sets, respectively.](image-url)
been shown to be involved in endosome transport. Distribution of the molecular function for the 31 proteins revealed that 23% of them are reported to have GTPase activity (supplemental Fig. 2B). 16% of the proteins are either involved in a structural constituent of the cytoskeleton or in cytoskeletal protein binding. These observations suggest that these 31 proteins form the crux or at least a part of it in the exosomes with respect to cell communication, signal transduction, cell growth, and maintenance.

**Exosomal Protein Content Has Tissue-associated Signatures**—As proteins are engulfed in the MVBs in the cytoplasm, the ILVs incorporate proteins present in the parent cell/tissue. To examine this, we overlaid the exosomal protein data and protein tissue expression profile data in a matrix. Hierarchical clustering was performed using R, resulting in a heat map (Fig. 4) that displays the protein clusters with respect to the tissue. As shown in Fig. 4, bright green patterns represent those proteins that are identified in the exosomal data set and also in the specific tissue. Some of the proteins are expressed exclusively in colorectal cancer and its exosome but were not known to be expressed in urine or mast cells and were not detected in their respective exosomes. A subset of these molecules can be specific to the colorectal cancer cell types, whereas the rest are proteins that could also be found in many other tissues/cell types. Investigation of these protein molecules in the scientific literature revealed a subset of proteins that are indeed involved in colorectal cancer. These molecules include A33, cadherin-17, centrosomal protein 55 kDa (CEP55), claudin 1 (CLDN1), claudin 3 (CLDN3), claudin 7 (CLDN7), epidermal growth factor receptor, ephrin receptor A2 (EPHA2), keratin 18 (KRT18), mucin 13 (MUC13), proliferating cell nuclear antigen, POLD1, PPP2R1B, carcinoembryonic antigen, epithelial cell surface antigen (EpCAM), misshapen-like kinase 1, mitogen-activated protein kinase 4, ephrin-B1, ephrin-B2, and RUVBL1. Likewise, proteins exclusively observed in the urine-derived exosomes include specific proteins found in proximal tubules (aquaporin 2 (AQP2) and Rh family, C glycoprotein (RHCG)), and the transitional epithelium of the urinary bladder (uroplakin-1 and uroplakin-2). These findings suggest that exosomes resemble the tissue/cell type from which they are derived and confirm the heterogeneous roles of tissue/cell type-specific exosomes. Given the specific tissue signatures, exosomes might harbor diagnostic markers.
Fig. 4. **Tissue-specific signature of exosomal proteins.** A heat map of exosomal proteomes along with the protein tissue expression is shown. Hierarchical clustering of the matrix plotted by the presence or absence of a specific protein in a particular exosomal data set and its corresponding protein tissue expression profiles shows proteins that are expressed in a particular tissue type as compared with the others. *Deep red* indicates that the protein is not known to be found in the specific tissue and not identified in the corresponding exosomal data set as well. *Bright green* indicates that the protein is identified in the exosomal data set and also in the specific tissue. Protein tissue profiles were downloaded from Human Proteinpedia, HPRD, Human Protein Atlas, and the urinary proteome data set. The top zoom panel displays a subset of proteins that are uniquely identified in the LIM1215 (colorectal cancer)-derived pure exosomes and are also known to be expressed in colorectal cancer tissues. The bottom zoom panel shows subset of proteins that are uniquely identified in urine-derived exosomes.
LIM1215-derived Pure Exosomes Have Attributes of Cancer—Cancer can be attributed by six categories (73) such as sustained angiogenesis, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential, evading apoptosis, and self-sufficiency in growth signals. Recently, the inflammatory microenvironment was also attributed to cancer (74) and included as the seventh hallmark of cancer (75). In this study, ~50% (199 of 394 proteins) of the LIM1215-derived pure exosome proteins could be categorized in at least one of the seven cancer hallmark attributes (supplemental Fig. 3). 36% of these proteins belong to the category of self-sufficiency in growth signals, whereas 24% had limitless replicative potential. 17% of the proteins are up-regulated in colon cancer metastasis, whereas 6% evade apoptosis. Given the known cell-cell communication of exosomes, the presence of LIM1215-derived pure exosomes with cancer hallmark attributes suggests their importance in cancer biology.

Conclusions—We have shown that highly purified human colon tumor exosomes can be obtained by immunoaffinity capture using the colon epithelial cell-specific A33 antibody. Comparative proteomics analysis of LIM1215-derived pure exosomes with human urine- and murine mast cell-derived exosomes revealed the existence of proteins common between exosomes derived from different cell types. Interestingly, exosomes display tissue-associated protein signatures as well. Domain/motif enrichment analysis revealed the significant over-representation of coiled coil, RAS, and MIRO domains in the LIM1215-derived pure exosome data set. Our study indicates that more robust and purified exosome proteome profiles need to be obtained to clearly catalogue the common molecular signatures of exosomes in general and to better understand their underlying biological functions. The colon cancer-associated exosomal proteins identified in this study may provide diagnostic information and aid in the development of a blood-based test for colorectal cancer patients, especially when the exosomal fraction of the blood is targeted.

Acknowledgments—We thank Cong Dacchi Ho and Anna for assistance with the electron microscopy imaging at the Department of Pathology, University of Melbourne. We thank the Australian Proteomics Computational Facility for providing us with the computer resources for our research.

* This work was supported by National Health and Medical Research Council Program Grant 487922 (to R. J. S.) and a University of Melbourne Australian postgraduate award (to J. W. E. L.). [5] The on-line version of this article (available at http://www.mcponline.org) contains supplemental Figs. 1–3 and Tables 1–4. † To whom correspondence should be addressed: Ludwig Inst. for Cancer Research, P. O. Box 208, Royal Melbourne Hospital, Royal Parade, Parkville, Victoria 3050, Australia. Tel.: 61-3-9341-3155; Fax: 61-3-9341-3192; E-mail: Richard.Simpson@ludwig.edu.au.

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