PROTEOMICS

Supporting Information for Proteomics

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Quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells reveal differential expression of EMT factors

SUPPLEMENTARY INFORMATION

Supplementary Materials & Methods

2.7 One-dimensional electrophoresis and Western blotting

Exosomal proteins were solubilized in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin), including protease inhibitors, for subsequent Western blot analysis. To extract cellular proteins, T24, FL3 and SLT4 cells were harvested, washed twice with ice-cold PBS, and solubilized in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin) to which complete Mini protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail (both from Roche) and 2.0 mM Pefabloc (Sigma-Aldrich, St Louis, MO, USA) was added immediately before use, on ice for 30 min. Cell lysates were centrifuged at 10,000 × g at 4 °C to remove cellular debris. Protein in the exosome and cell lysates was quantified using Bradford microassays (Bio-Rad, Hercules, CA, USA). Cell lysate and exosome samples were suspended in sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8, bromophenol blue) and subjected to denaturing electrophoresis using precast Novex 4-12% or 12% Bis–Tris NuPAGE gels (Invitrogen, Carlsbad, CA, USA) in MOPS running buffer at constant 150 V for 70 min. Proteins were electrotransferred onto Immobilon PVDF membranes (Millipore, MA, USA), and the membranes blocked with 5% (w/v) skim milk powder in PBS with 0.1% (v/v) Tween-20 (PBST) for 1 h at RT. The Western blots were visualized using ECL Prime agents (Amersham Biosciences, Buckinghamshire, UK) and imaged on a ChemiDoc-It Imaging Sytem (Bio-Rad, Hercules, CA, USA). Signals were quantified by densitometry using ImageJ 1.46r software (National Institutes of Health, Bethesda, Maryland, USA).

2.11 Bioinformatic analysis

The lists of significantly up- and downregulated proteins (>1.5 or <0.67 iTRAQ ratios, in at least two out of three replicates, respectively) in metastatic exosomes versus non-metastatic exosomes were analyzed through the use of IPA (Ingenuity® Systems, http://www.ingenuity.com). For IPA, the Core Analysis module was used to rank the proteins into biological, molecular and cellular functions. The analysis settings and parameters for IPA was as follows: (i) Reference set, Ingenuity Knowledge Base (Genes Only); (ii) Relationship to include, Direct and Indirect; (iii) Filter Summary, Consider only molecules and/or relationships where (species=Human) AND (confidence = Experimentally observed OR High (predicted)) AND (cell lines = All Cancer cell lines in ingenuity database). Student´s t-test, Fisher´s exact test with Benjamini-Hochberg correction, and spearman correlation were used to test statistical significance where indicated.

Supplementary figure legends

Supplementary Figure S1. (A) Analysis of the isogenic relationship in between the cell lines used by STR analysis. (B) Phase contrast pictures of T24 and FL3 cells grown in conventional cell culture flasks (top) and explanted cells after 6 weeks of culturing in CELLine Adhere 1000 Culture System bioreactor (bottom).

Supplementary Table S1. Data files displaying all proteins in biological replicate sample 1 detected with at least two unique peptides, and divided into separate MEMBRANE and LUMEN sheets. UNIPROT Accession, protein name, peptides identified, coverage, and further details are listed.

Supplementary Table S2. Data files displaying all proteins in biological replicate sample 2 detected with at least two unique peptides, and divided into separate MEMBRANE and LUMEN sheets. UNIPROT Accession, protein name, peptides identified, coverage, and further details are listed.

Supplementary Table S3. List of proteins with significant increased or decreased expression in exosomes from metastatic FL3 cells versus non-metastatic T24 cells. The list contains membrane and luminal proteins identified from quantitative iTRAQ proteomics that meet the following criteria: (i) Protein detected in exosomes from both FL3 and T24 cells, ii) the protein identification was made with at least two unique peptides, iii) the ratio of the protein abundance for metastatic FL3 exosomes to non-metastatic T24 exosomes was >1.5 fold or <0.67 with a variability of less than 50 % and (iv) the fold changes in abundance was observed in a second independent biological replicate that also met criteria (i) to (iii).

Supplementary Table S4. List of proteins with significant increased or decreased expression in exosomes from metastatic SLT4 cells versus non-metastatic T24 cells. The list contains membrane and luminal proteins identified from quantitative iTRAQ proteomics that meet the following criteria: (i) Protein detected in exosomes from both SLT4 and T24 cells, ii) the protein identification was made with at least two unique peptides, iii) the ratio of the protein abundance for metastatic SLT4 exosomes to non-metastatic T24 exosomes was >1.5 fold or <0.67 with a variability of less than 50 % and (iii) the fold changes in abundance was observed in a second independent biological replicate that also met criteria (i) to (iii).

Supplementary Figure S2. IPA pathway maps generated from lists of proteins with significantly altered abundance (>1.5 fold or <0.67 fold) in the top scoring networks for exosomes from metastatic FL3 (Fisher's exact test, $P = 1\times10^{-44}$) and SLT4 ($P = 1\times10^{-39}$) cells compared to non-metastatic T24 cells.