# Secretion Vesicle Proteomics: Glioblastoma Exosome and Modulation by Wild-Type p53 Genetherapy

Jeremiah D. Tipton[1], Carol L. Nilsson[2], Mark R. Emmett[1,3], Xiaoyeng Sheng[4], Charles A. Conrad[4], and Alan G. Marshall[1,3]

(1) Ion Cyclotron Resonance Program, National High Magnetic Field Lab, Tallahassee, FL; (2) Pfizer Global Research & Development, San Diego, CA;
(3) Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL; (4) M.D. Anderson Cancer Center, Houston, TX

## Overview

Two glioblastoma cell lines show phenotypic differences in response to wild-type p53 treatment (wt-p53)

Differential quantitative proteomic study of exosomes derived from the two cell lines

Protein identification with MASCOT and ProteoIQ
"Spectral Counting" for relative quantitation

Known transcriptional targets of p53 and proteins not known to be targets of p53 are quantitatively changed in wt-p53 exosomes

## Introduction

Glioma cells are resistant to chemotherapy and radiation. However, when U87 cells are transfected with wt-p53 they undergo apoptosis. U251 glioblastoma cells enter cell cycle arrest with the same treatment. The wt-p53 protein modulates the transcription of a large number of downstream targets in response to cellular stress. It has been shown that the proteins secreted from cells via exosomes may be regulated by wt-p53 expression. These vesicles are hypothesized to communicate with adjacent cells via the "bystander effect".[1] Thus, a full exosome proteomic study on empty vector and wt-p53 treated U87 glioblastoma is designed to determine which proteins may change in regulation upon treatment. Furthermore, these treatments were extended to U251 glioblastoma cells to improve functional understanding across different cell lines. Collected exosomes from different treatments and cell lines were analyzed by mass spectrometry based proteomics to provided insight into the differences in treatments and cell lines.

## Methods

**Cell Cultures** The glioma cell lines were grown in the presence of DMEM-F12 medium supplemented with 10% FBS in a humidified incubator at 5% CO . Cell cultures were grown to 90% confluency in 150 mm dishes. Cells were treated with either empty adenovirus vector (DI312) or adenovirus containing the human wt-p53 gene (Ad-p53).

**Exosome Isolation** Conditioned cell medium was collected and differentially centrifuged at 1200 rpm for 5 min and 3000 rpm for 20 min (SORVALL RT 7, rotor RHT-750) to remove cell debris. Exosomes were pelleted at 26,000 rpm (SORVALL ULTRA pro80, rotor TH-641), at 4 °C for 2 hours. The pellet was re-suspended in PBS buffer for 2 hours; the resulting pellet was harvested and stored at -80 °C. Purity of the pellets was determined by electron microscopy.

**Protein Separation and Digestion with Trypsin** Each exosomal fraction prepared from ~2 x $10^7$ cells was mixed with SDS sample buffer containing 0.5 M Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) mercaptoethanol and 0.05 % (w/v) bromophenol blue and boiled for 5 minutes. The solution was loaded onto 1.5 mm SDS gels (4-15% gradient). Lanes were cut into 20-38 equal pieces (same number for sample replicates), of either 1.5 or 5 mm each by use of a Bio-Rad Model 190 tube gel slicer. De-stained gel slices were incubated with 20 µL of 10 ng/µL trypsin at 37 °C overnight. Extracted peptide supernatant was collected and speed vacuumed to dryness and re-suspended in 12 µL of 97.5 % $H_2O$/ 2 % methanol/ 0.5 % formic acid

**Liquid Chromatography and Mass Spectrometry** Peptides were separated by nano-LC (Eksigent, Dublin, CA) with a 75 micron i.d. column packed with 5 cm of Proteoprep II packing (New Objective, Woburn, MA). The gradient was 2% B to 80% B in 30 minutes (500 nL/min; Solvent A: 97.5% $H_2O$, 2% methanol, 0.5% formic acid, Solvent B: 1.5% $H_2O$, 98% methanol, 0.5% formic acid). LC-effluent was analyzed on-line by positive-ion micro-electrospray with a modified hybrid linear ion trap (LTQ) FT-ICR MS equipped with a 14.5 T magnet. Precursor ions were measured in the ICR (500 ppb rms mass error). Data-dependent MS/MS was performed in the linear ion trap (collisional dissociation).

**Ingenuity Pathway Analysis** Normalized quantitative data sets were analyzed by use of Ingenuity Pathways Analysis (Ingenuity Systems, version 7.8, www.ingenuity.com). A 1.3-fold cutoff value was selected to identify proteins whose expression significantly increased or decreased. Biological functions and process were attributed to members of the data set by mapping the proteins to functions in the Ingenuity ontology and performing a right-tailed Fisher's exact test to determine the significance (p-value) of over-represented proteins to functions compared to the results expected by a random set of proteins.

## Informatics

### (A) Protein Identification

**(1) Extracted RAW Files -** *multiplierz*[2]

**(2) Search data against forward and randomized Swiss-Prot Human database (v46) with MASCOT**

20277 protein entries for both databases
2 ppm Precursor Ion Mass Tolerance
1.5 Da MS/MS Mass Tolerance
Variable Oxidized M and Acetylated C
Trypsin Specificity and 2 Missed Cleavages

**(3) Load Individual Sample Sets into ProteoIQ**

U87 p53 Vector x 3 biological replicates
U87 Empty Vector x 3 biological replicates

U251 p53 Vector x 3 biological replicates
U251 Empty Vector x 3 biological replicates

**Filter 1 Cutoff** - 1 % FDR Proteins; 0.5 Protein Probability

Resulting protein list is the full protein id list ( including mixtures of isoforms).

**Filter 2 Cutoff** - 1 % FDR Proteins; 0.5 Protein Probability and the Maximum number of protein groups per peptide = 1

Resulting protein list is the number of peptides (and Spectral Counts) uniquely associated with protein isoforms.

**Maximum # Pro Groups Per Peptide (MPGPP1) Definition**: "Specifies the number of protein groups to which a peptide can match ." Only peptides than are uniquely assigned to a single protein group are included.[3]

**Spectral Counts Definition**: "The sum of all spectra generating peptide identifications to a given protein, i.e., the number of redundant peptide spectra."[3]

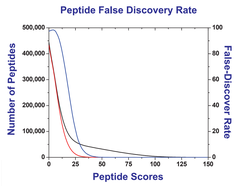



**Figure 1:** The peptide false discovery rate is determined by dividing the number of peptides observed from the forward database by the number of peptides observed from the decoy database (per binned MASCOT ion score cutoff) and multiplying by 100. The data set follows a normal distribution and excellent separation between target and decoy peptide.

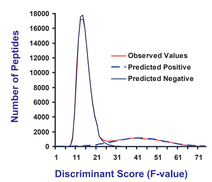



**Figure 2.** Discriminant value distributions for charge 2+ shows excellent separation of predicted negative and positive results.[4]

### (B) Relative Quantitation with Spectral Counts

**(1) Remove keratin related spectral counts**

Use the number of spectral counts from the number of peptide identified (Filter 1 above) to normalize spectral counts for each data set.

**(2) Normalize Replicate Pairs**

Normalizes sample loading across replicate pairs

| Sample Name | Number id. Peptides | Normalization Factor |
|---|---|---|
| U87 empty vector rep 1 | 2921.00 | 1.15 |
| U87 empty vector rep 2 | 1288.00 | 1.00 |
| U87 empty vector rep 3 | 2381.00 | 1.39 |
| U87 wt-p53 vector rep 1 | 2155.00 | 1.00 |
| U87 wt-p53 vector rep 2 | 1474.00 | 1.46 |
| U87 wt-p53 vector rep 3 | 2352.00 | 1.00 |

**Table 1**. Normalization factors for replicate sample pairs

**(3) Do not include proteins which have less than 9 total spectral counts for relative quantitation**

Not statistically valid to quantify on low spectral counts [5]
Perform on both Filter 1 and Filter 2 protein list

**(4) Generate Average ratio (p53 vector / empty vector treated) for each sample replicate**

Perform on both Filter 1 and Filter 2 protein lists

Final protein lists include proteins found in at least 2 replicate pairs and has greater than 9 total spectra counts

Final relative quantitation data set includes the top protein ids and spectral counts for single and isoform proteins.

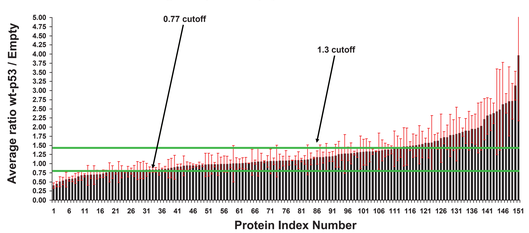



**Figure 3.** Final U251 average relative quantitation ratios with standard deviation for visualization of data. Proteins that show a 1.3-fold quantitative change were analyzed by Ingenuity Pathways to predict protein interactions in specific pathways. The data generated by these studies is being used to logically develop hypothesis-driven experiments in the study of glioblastoma motility and apoptosis. The protein index number is cross referenced to the final protein list.

## Results

### Electron Micrograph of Collected Exosome

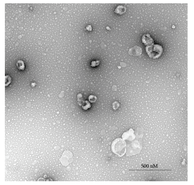


**Figure 4.** Electron micrograph of exosomes prepared from conditioned media from U87 MG cells

### SDS-PAGE (third biological replicate)

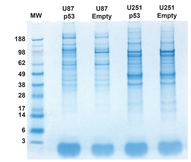
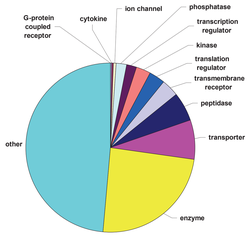



**Figure 5.** SDS-PAGE analysis: 1D gel (4-12%); separation of exosomes from U87 and U251 cell lines. Two treatments were compared in order to detect changes in exosomal proteins due to wt-p53 transfection versus control (empty) vector Di312

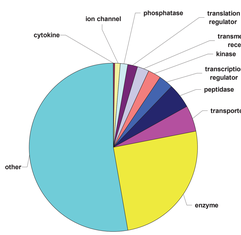
### Protein Function Assignment




U87 All proteins
Total number of peptides: 33138
Number of unique peptides: 4191
Number of protein groups: 671
Number of proteins: 730

U87 MPGPP1 proteins
Total number of peptides: 28063
Number of unique peptides: 3869
Number of protein groups: 661
Number of proteins: 706

**Figure 6.** Gene ontology functions associated with U87 exosomal proteins. Note that protein function and the number of proteins in each class are similar to those from U251 gene ontology results




U251 All proteins
Total number of peptides: 22595
Number of unique peptides: 1607
Number of protein groups: 533
Number of proteins: 596

U251 MPGPP1 proteins
Total number of peptides: 18494
Number of unique peptides: 1445
Number of protein groups: 519
Number of proteins: 557

**Figure 7.** Gene ontology functions associated with U251 exosomal proteins. Note that protein function and the number of proteins in each class are similar to those from U87 gene ontology results.

| Gene | Sequence Name | Avg Ratio p53 / Empty | Standard Deviation |
|---|---|---|---|
| S10AB | Protein S100-A11 | 0.327 | 0.053 |
| S10AA | Protein S100-A10 | 0.342 | 0.025 |
| MMP2 | 72 kDa type IV collagenase | 0.464 | 0.361 |
| TFR1 | Transferrin receptor protein 1 | 0.467 | 0.098 |
| AT2B4 | Plasma membrane calcium-transporting ATPase 4 | 0.469 | 0.320 |
| NDKA | Nucleoside diphosphate kinase A | 0.500 | 0.147 |
| CAV1 | Caveolin-1 | 0.534 | 0.263 |
| CALR | Calreticulin | 0.577 | 0.201 |
| PRDX2 | Peroxiredoxin-2 | 0.577 | 0.201 |
| RHOA | Transforming protein RhoA | 0.626 | 0.147 |
| CLIC4 | Chloride intracellular channel protein 4 | 0.628 | 0.167 |
| MOES | Moesin | 0.635 | 0.215 |
| GRP78 | 78 kDa glucose-regulated protein | 0.692 | 0.039 |
| IF5A1 | Eukaryotic translation initiation factor 5A-1 | 0.728 | 0.639 |
| EZRI | Ezrin OS=Homo sapiens | 0.760 | 0.043 |
| LEG1 | Galectin-1 | 0.769 | 0.292 |
| MFGM | Lactadherin | 1.300 | 0.014 |
| CD9 | CD9 antigen | 1.300 | 0.647 |
| TCTP | Translationally-controlled tumor protein | 1.310 | 0.203 |
| GSTP1 | Glutathione S-transferase P | 1.349 | 0.788 |
| GDIR1 | Rho GDP-dissociation inhibitor 1 | 1.405 | 0.857 |
| ALDOC | Fructose-bisphosphate aldolase C | 1.434 | 1.413 |
| RRAS | Ras-related protein R-Ras | 1.443 | 1.057 |
| ACSL4 | Long-chain-fatty-acid--CoA ligase 4 | 1.444 | 0.007 |
| ACTN4 | Alpha-actinin-4 OS=Homo sapiens | 1.505 | 0.991 |
| G6PI | Glucose-6-phosphate isomerase | 1.562 | 0.788 |
| ITAV | Integrin alpha-V OS=Homo sapiens | 1.604 | 0.351 |
| GNAQ | Guanine nucleotide-binding protein G(q) subunit alpha | 1.637 | 1.112 |
| NAMPT | Nicotinamide phosphoribosyltransferase | 1.643 | 1.386 |
| FRIH | Ferritin heavy chain | 1.737 | 1.673 |
| STIP1 | Stress-induced-phosphoprotein 1 | 1.786 | 0.461 |
| NRP1 | Neuropilin-1 | 1.841 | 1.563 |
| TCPB | T-complex protein 1 subunit beta | 1.920 | 0.829 |
| GTR1 | Solute carrier family 2, facilitated glucose transporter member 1 | 4.452 | 3.543 |
| EGFR | Epidermal growth factor receptor | 4.643 | 3.220 |
| NDRG1 | Protein NDRG1 | 5.295 | 3.224 |

**Table 2.** Subset of U87 exosome proteins that were found to be associated with cell death and apoptosis. Treatment of glioblastoma cells with wt-p53 is expected to induce cell death. Specific proteins from this list are targeted for western blot analysis.

| Gene | Sequence Name | Avg Ratio p53 / Empty | Standard Deviation |
|---|---|---|---|
| 4F2 | 4F2 cell-surface antigen heavy chain | 0.724 | 0.288 |
| ACTA | Actin, aortic smooth muscle | 2.763 | 2.038 |
| ACTN4 | Alpha-actinin-4 | 1.505 | 0.991 |
| CALR | Calreticulin | 0.577 | 0.201 |
| CD81 | CD81 antigen | 1.369 | 0.410 |
| CD9 | CD9 antigen | 1.300 | 0.647 |
| EGFR | Epidermal growth factor receptor | 4.643 | 3.220 |
| GDIR1 | Rho GDP-dissociation inhibitor 1 | 1.405 | 0.857 |
| IQGA1 | Ras GTPase-activating-like protein IQGAP1 | 1.583 | 1.214 |
| ITAV | Integrin alpha-V | 1.604 | 0.351 |
| MARCS | Myristoylated alanine-rich C-kinase substrate | 0.658 | 0.533 |
| MFGM | Lactadherin | 1.300 | 0.014 |
| MMP14 | Matrix metalloproteinase-14 | 1.303 | 0.518 |
| MMP2 | 72 kDa type IV collagenase | 0.464 | 0.361 |
| MYH9 | Myosin-9 | 1.473 | 1.314 |
| PRDX2 | Peroxiredoxin-2 | 0.577 | 0.201 |
| RHOA | Transforming protein RhoA | 0.626 | 0.147 |
| RRAS | Ras-related protein R-Ras | 1.443 | 1.057 |

**Table 3**. Subset of U87 exosome proteins that were found to be associated with cell-cell signaling and adhesion. The regulation of proteins found in this class may be associated with the "bystander effect[1]. Specific proteins from this list are targeted for western blot analysis.

| Gene | Sequence Name | Avg Ratio p53 / Empty | Standard Deviation |
|---|---|---|---|
| ACTN4 | Alpha-actinin-4 | 1.774 | 1.156 |
| B2MG | Beta-2-microglobulin | 1.719 | 0.871 |
| CD44 | CD44 antigen | 1.314 | 0.706 |
| CLUS | Clusterin | 2.307 | 0.997 |
| DESP | Desmoplakin | 0.725 | 0.215 |
| EF1D | Elongation factor 1-delta | 0.399 | 0.150 |
| FAS | Fatty acid synthase | 1.490 | 0.647 |
| GDIR1 | Rho GDP-dissociation inhibitor 1 | 0.581 | 0.153 |
| GSTP1 | Glutathione S-transferase P | 1.406 | 0.970 |
| MDHC | Malate dehydrogenase, cytoplasmic | 0.594 | 0.271 |
| MFGM | Lactadherin | 1.323 | 0.552 |
| MX1 | Interferon-induced GTP-binding protein Mx1 | 1.611 | 0.366 |
| NDKA | Nucleoside diphosphate kinase A | 0.687 | 0.186 |
| PEBP1 | Phosphatidylethanolamine-binding protein 1 | 1.302 | 0.267 |
| PRDX2 | Peroxiredoxin-2 | 1.377 | 0.283 |
| PRDX6 | Peroxiredoxin-6 | 1.897 | 0.683 |
| S10A6 | Protein S100-A6 | 1.942 | 0.744 |
| STIP1 | Stress-induced-phosphoprotein 1 | 1.808 | 1.473 |
| TCPB | T-complex protein 1 subunit beta | 3.958 | 3.573 |
| TERA | Transitional endoplasmic reticulum ATPase | 1.325 | 0.140 |
| TSP1 | Thrombospondin-1 | 1.779 | 0.850 |

**Table 4**. Subset of U251 exosome proteins that were found to be associated with cell death and apoptosis. Results differ from U87 exosomes; however, many of the identified proteins are found in the non-quantifiable U87 exosome data. The U251 exosomes are found to have increased cytokine activity. Proteins are selected for western blot analysis to verify the changes in regulation and for comparison to U87 results.

### Integrin Signaling Pathway

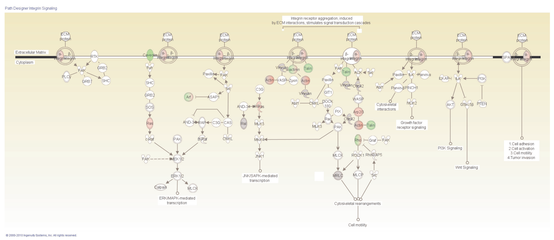



**Figure 9.** Quantitative protein changes observed in the integrin signaling pathway in U87 exosomes in response to wt-p53 treatment as compared to empty vector controls. The image was created by overlaying the quantitative proteomic data set on the integrin signaling pathway in Ingenuity Systems Analysis software. Red indicates increased levels, green indicates decreased levels and grey indicates no significant change.

## Conclusions

Triplicate analysis of exosomes harvested from U87 and U251 after treatment with wt-p53 and empty vector provide relative quantitation of proteins enriched in cell-to-cell interactions, cell death, and cancer biology.

Exosomes are believed to be involved with cell-to-cell interactions through the "bystander" effect. Enrichment of proteins associated with cell-to-cell interactions are reported by our proteomic results and previous work by other laboratories[1].

Treatment of glioblastoma with wt-p53 is known to induce apoptosis. Several proteins were identified to be differently regulated between the wt-p53 treated and empty-vector treated cells. Furthermore, differences in protein regulation are observed between U87 and U251 glioblastoma cell lines.

The data generated by these studies is being used to logically develop hypothesis-driven experiments in the study of glioblastoma motility and apoptosis.

Current protein targets for western blot analysis include: EGFR, Neuropilin-1, Integrin alpha-v, Galectin-1, and GRP-78.

## Acknowledgments

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