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Identification of Astrocyte Secreted Proteins with a Combination of Shotgun Proteomics and Bioinformatics

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Abstract

Astrocytes are important regulators of normal brain function in mammals, including roles in synaptic signaling, synapse formation, and neuronal health and survival. Many of these functions are executed via secreted proteins. In order to analyze the astrocyte secretome, a combination of shotgun proteomics and bioinformatics was employed to analyze conditioned media from primary murine astrocyte cultures. Both two- and one-dimensional LC-MS/MS were used to analyze astrocyte secreted proteins, resulting in the identification of over 420 proteins. In order to refine our results, the intracellular protein contaminants were removed *in silico* using a cytoplasmic control. In additional rounds of refinement, putative secreted proteins were subjected to analysis by SignalP, SecretomeP, and gene ontology analysis, yielding a refined list of 187 secreted proteins. In conclusion, the use of shotgun proteomics combined with multiple rounds of data refinement produced a high quality catalog of astrocyte secreted proteins.

Introduction

Astrocytes are the most abundant cells in the human brain and play important roles in the central nervous system (CNS), including the regulation of extracellular glutamate, the coordination of synapse formation, and the maintenance of neuronal health.[1] They perform many of their physiological functions via secreted proteins, including the regulation of synapse formation by thrombospondins,[2] the promotion of axonal regeneration by extracellular metallothionein,[3] and the prevention of neuronal apoptosis by transforming growth factor-β (TGF- β).[4] A large number of bioactive proteins have been identified in astrocyte conditioned media, including trophic factors, cytokines, chemokines, proteases, and protease inhibitors.[5–8]

Astrocytes act as the sentinels of the brain and react to neuronal stress or injury by providing neuronal metabolic and trophic support.[9–11] Nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are all expressed in astrocytes[12–15]

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and are induced during reactive astrogliosis.[16, 17] The astrocytic expression of members of the transforming growth factor-β (TGF-β) super family, including glial derived neurotrophic factor (GDNF) and TGF- β, and members of the fibroblast growth factor family, including fibroblast growth factor-2 and -1 (FGF-1 and -2), are also induced upon reactive astrogliosis.[18–23]

In addition to trophic factors, astrocytes release cytokines in response to brain injury and neurodegenerative disease. A number of pro-inflammatory cytokines are induced in reactive astrogliosis, including tumor necrosis factor-β, IL-6, and IL-1β.[23–26] Among the anti-inflammatory cytokines, IL-4 and IL-10 are induced in astrocytes during multiple sclerosis (MS) pathogenesis.[27] Additionally, members of the cysteine-cysteine (CC) family of chemokines, including macrophage inflammatory protein-1 alpha (MIP-1α), regulated upon activation, normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein-1, -2, and -3 (MIP-1, -2, -3), are induced during reactive astrogliosis in MS.[28]

Beyond their role in maintaining neuronal health and survival, astrocytes also regulate synapse formation and elimination via secreted proteins. Retinal ganglion cells (RGCs) cultured with astrocytes or astrocyte conditioned media (ACM) form substantially more synapses than RGCs cultured alone in serum-free media.[29, 30] Recently, astrocyte-derived thrombospondins (TSPs), which are large extracellular matrix proteins,[31] were found to significantly modulate synapse formation in RGCs.[2] Synapse elimination also seems to be under the control of astrocyte secreted factors. In an attempt to elucidate the mechanisms of synapse elimination in the developing eye, Barres and co-workers examined the gene expression changes in RGCs co-cultured with immature astrocytes.[32] The immature astrocytes induced the expression of the complement cascade protein C1q almost 30-fold in the co-cultured RGCs. However, the astrocyte secreted factor responsible for the C1q induction remains unidentified.

In order to better understand the complex extracellular signaling mediated by astrocytes, a catalog of astrocyte secreted proteins is an essential starting point for further research. Four studies have attempted a global strategy to identify astrocyte secreted proteins.[5–8] The first two studies employed two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for protein identification.[5, 6] However, both of these studies yielded a relatively low number of total protein identifications (less than 40 total proteins) and many of the identified proteins are not known to be secreted. Two more recent studies employed shotgun proteomics with high performance liquid chromatography coupled to electrospray mass spectrometry (LC-MS/MS); a methodology which yielded a substantially larger list of putative secreted proteins.[7, 8] One study investigated secreted proteomic changes in astrocytes exposed to inflammatory cytokines.[8] The other report focused on the identifications of proteins involved in neuronal development induced by astrocyte cholinergic stimulation.[7] In contrast to these studies, we utilized a cytoplasmic control in conjunction with quantitative proteomics to identify cytoplasmic protein contaminants which are not actively secreted from cells. In addition, we applied a two-dimensional LC-MS/MS methodology to increase proteome coverage and multiple rounds of data refinement using gene ontolotgy analysis, SignalP and SecretomeP to increase the confident identification of secreted proteins.

There are numerous technical challenges in developing effective tools for global secretome analysis. In regards to cell culture technique, the optimization of protein-free media conditions, including length of serum-free incubation and cell washing procedures, is important to minimize cell death. Downstream sample processing and analytical methods are equally important and require rigorous optimization due to the low concentration of many trophic factors and cytokines which are present at low ng/ml concentrations.[33]

A number of studies have analyzed the secretomes of various cell types, including endothelial,[34] myeloid,[33] adipocytes,[35, 36], microglia,[37] retinal pigment epithelial cells,[38] and various cancer cell lines.[39–41] Many of these studies have developed novel techniques to addres the issues mentioned above, including two-dimensional LC-MS/MS shotgun proteomics.[35, 39–41] Each study employed cell washing techniques to remove serum proteins, however, some groups only used a single wash [41] while other groups found a triple wash to be optimal.[34] As for protein precipitation and extraction, two studies found a hydrophobic resin extraction to improve recovery[39, 40] while another study found the optimal precipitation conditions to be trichloroacetic acid (TCA) precipitation with sodium lauroyl sarcosinate (NLS).[33] Finally, the use of a cytoplasmic extract or whole cell lysate to control for intracellular protein contamination is an effective way to more confidently identify secreted proteins.[33, 42] In the current study, we optimized washing procedures, incubation times, employed a cytoplasmic control, and used both 1D and 2D LC-MS/MS to maximize the identification of astrocyte secreted proteins.

Experimental Method

Experimental Overview

Proteins secreted from primary astrocyte cultures were analyzed by LC-MS/MS. A cytoplasmic extract was used as a control and also analyzed by LC-MS/MS. Two different LC-MS/MS methods were used to analyze the secreted proteins: the first was a standard 1D LC-MS/MS experiment (3 technical replicates per n; n=2 biological replicates); the second was a 2D LC-MS/MS experiment in which the proteins were fractionated by off-line reverse phase high performance liquid chromatography (RPLC), the resulting fractions collected, trypsinated, and subjected to LC-MS/MS (no technical replicates; n=2 biological replicates).

Materials for Cell Culture

The Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), Neurobasal medium (NB), fetal bovine serum (FBS), penicillin/streptomyocin were obtained from Invitrogen (Carlsbad, CA). The sodium bicarbonate, HEPES, trypsin, DNase, and 70-μm cell strainer were obtained from Sigma (St. Louis, MO). 25 cm^2 culture flasks (T-25) were obtained from Nunc (Naperville, IL).

The culture medium (DMEM+FBS) consisted of Dulbecco's modified Eagle's medium (DMEM) with 4 mM glutamine and supplemented with: 1.2 mg/ml sodium bicarbonate, 3.6 mg/ml HEPES, 10% fetal bovine serum (FBS), penicillin/streptomyocin at 100 IU/ml and 100 μg/ml, respectively.

Primary Astrocyte Culture

Primary astrocyte cultures were prepared from postnatal day 1 (P1) mice as previously described with minor modifications.[43] Briefly, the cerebral cortices from individual P1 pups were removed, placed in ice-cold HBSS, minced with a scalpel blade, and then transferred to a tube with 2 ml of 0.25% trypsin at 37 °C. After 25 minutes, 1 ml of culture medium (DMEM +FBS) was added to deactivate the trypsin. DNase was added to a final concentration of 0.05 mg/ml and then the tube was spun at 300 *g* for 5 minutes. The supernatant was removed and the tissue pellet was re-suspended in 1 ml of fresh DMEM+FBS. After triturating 15–20 times with a 1 ml pipette, 4 ml of DMEM+FBS was added to the disassociated cells and then the suspension was filtered through a 70 μm cell strainer. The cells from a single pup were plated onto an uncoated, plastic T-25 flask. The DMEM+FBS was changed after the first day and then every 3 days thereafter.

Preparation of Astrocyte Conditioned Medium

After the astrocytes reached confluency (Day 7–8), the flasks were placed on a rotary shaker (200 rpm) for 24 hours. After shaking, the DMEM+FBS was removed and discarded (shaking removes the majority of microglia, oligodendrocytes, and neurons). The astrocytes were then washed three times with Hank's Balance Salt Solution (HBSS) and then 5 ml of neurobasal (NB) medium was added. After 24 hours, the astrocyte conditioned medium (ACM) was removed with a pipette and centrifuged at 3,000 *g* for 25 minutes to remove cells and debris. The conditioned medium was stored at −80 °C until processed. After thawing, the conditioned medium was concentrated 20–40X with a 3 kDa MWCO tube (Amicon,15mL) from Thermo Fisher Scientific (Waltham, MA) at 3,000 *g* in a refrigerated centrifuge (4 °C). Total protein concentration was determined by Bradford assay. Alternatively, the conditioned medium was precipitated using trichloroacetic acid (TCA) with the carrier sodium lauroyl sarcosinate (NLS) as performed by Chevallet and co-workers.[33] Briefly, NLS was added to each sample to a concentration of 0.1% followed by the addition of TCA to the concentration of 10%. Proteins were allowed to precipitate for 2 hours on ice, spun at 10,000 *g* for 15 minutes, and the supernatant discarded. The proteins pellets were washed twice with tetrahydrofuran (THF) and then re-suspended in 6 M urea.

Preparation of Cytoplasmic Protein Extract

After removal of the astrocyte conditioned media, the confluent astrocyte layer was washed with HBSS. The cells were then scraped from the flask in 1 ml of PBS and pipetted into a Dounce glass-glass homogenizer on ice. After 10 strokes with the Dounce homogenizer, the cell extract was spun at 18,000 *g* for 30 minutes in a refrigerated centrifuge (4 °C). The supernatant (cytoplasmic protein extract) was decanted and stored at −80 °C until processed.

Protein Digestion

Protein samples were diluted into 6 M urea/50 mM ammonium bicarbonate buffer (pH 8). Cysteinyl disulfides were reduced by the addition of 2 mM Tris[2-carboxyethyl] phosphine (TCEP) from Sigma for 30 minutes at 37 °C. Reduced disulfides were then alkylated by the addition of 10 mM iodoacetamide from Sigma (IAA) for 30 minutes in the dark. The urea was then diluted to <1 M with 50 mM ammonium bicarbonate and the pH was measured and adjusted to pH 7.8. Trypsin from Promega (Madison, WI) was added at a 1:20 weight-to-weight ratio and incubated for 18 hours at 37°C. After digestion, the solvent was removed by vacuum centrifugation.

Off-line Protein Separation via Reversed Phase Liquid Chromatography (RPLC)

Forty μg of undigested protein extract in 100 μl of water was delivered to a macroporous mRP-C_{18} column (2.1 × 75mm, 5μm) from Agilent (Santa Clara, CA) via a Rainin (Oakland, CA) Dynamax HPLC equipped with a 100 μl sample loop, binary pump, UV detector, and fraction collector. To increase recovery and decrease protein adsorption, the column was heated to 80 °C throughout the separation. Proteins were eluted with a linear gradient of 0.1% TFA in acetonitrile (HPLC Buffer B) from 15–55% over 60 minutes (HPLC Buffer A was 0.1% TFA in water). Fractions were collected every 5 minutes for a total of 12 fractions. Each fraction was vacuum-centrifuged to ~50 μl and then diluted to 80% acetonitrile/20% 50 mM NH_4CO_3 and digested according to the *Protein Digestion* procedure. The digested fractions were reconstituted in 20μL of 0.1% FA; 10 μl of each reconstituted fraction was analyzed by LC-MS/MS.

LC-MS/MS

Using a nanoflow HPLC from Eskigent, tryptic peptides were delivered to a trap column (Zorbax 300SB-C_{18}, 5μm, 0.3 × 50 mm, Agilent, Santa Clara, CA) via an isocratic flow of

0.1% formic acid in water (LC Buffer A) at a rate of 5 μl/min for 2 min. The flow rate was then reduced to 300 nl/min, and the peptides were flushed onto an in-house packed capillary column (Magic C_{18}, 5μm, 75 μm × 150 mm) and eluted via a 5–35% linear gradient of 0.1% formic acid in acetonitrile (LC Buffer B) over 120 minutes into a nanoelectrospray ionization (nESI) linear ion trap mass spectrometer (LTQ) from Thermo Fisher Scientific. The MS survey scan was performed in positive ion mode from *m*/*z* 400 to 2000, followed by data-dependent MS/MS acquisition up to *m*/*z* 2000. The signal threshold for switching from the survey scan to MS/MS was set at 3000. Normalized collision energy was set at 35; capillary voltage, 3000 V; capillary temperature, 200 °C. Dynamic exclusion was activated with the following parameters: repeat count was 1, repeat duration was 60 s, and the exclusion duration was 60 s.

Database Search

Bioworks from Thermo-Electron (version 3.0) was used to convert the *.RAW* files generated during the LC-MS/MS runs into *.dta* text files for database searching (see Figure 1). The *.dta* files from each sample were then combined into a single file using the OMSSA search engine (version 2.1.1) from the National Center for Biotechnology Information (NCBI). For the 1D LC-MS/MS samples, the technical replicate *.dta* files were all combined into a single file for database searching. Using OMSSA, the combined *.dta* files were searched against the Swiss-Prot database, with the following parameters: taxonomy was limited to *mus musculus*, parent mass tolerance was 2.0 Da, fragment mass tolerance was 0.8 Da, a maximum of two missed cleavages was allowed, and the protein expectation value (log E) was required to be < −1.3. Carbamidomethylation at cysteine residues was set as a fixed modification and oxidation of methionine was set as a variable modification. After database searching, proteins were filtered by *Top Hit per Spectrum Only* in OMSSA browser. The false discovery rate (FDR) was determined using the X!Tandem search engine (version 2.2.1).

Quantitative Analysis by Spectral Counting

From the OMSSA browser, the spectral counts for each protein, were exported to Microsoft Excel. Each protein was required to have at least two total spectral counts from the summation of the four biological replicates. To correct for differences in sample preparation and run-to-run instrument variation, the total spectral counts for individual runs were normalized to the cytoplasmic control. All zero counts were converted to 0.5 to avoid dividing by zero when calculating the enrichment factor.

Average Fold Enrichment (AFE)

After normalization, the spectral counts for each protein in a media run were compared against the spectral counts for the same protein in the cytoplasmic control. From this comparison, a fold-enrichment was determined which reflects the relative enrichment of a protein in the media versus the cytoplasmic control. The average fold enrichment (AFE) analysis across all runs was calculated and the identified proteins were then filtered based on this value. To be considered for further analysis, each protein had to be enriched by at least 2-fold relative to the cytoplasmic control.

Gene Ontology Analysis, ExPASy, SignalP, and SecretomeP

Using the online version of DAVID Bioinformatics Resources 2008 from the NIAID (http://david.abcc.ncifcrf.gov/),[44] all the identified proteins from the astrocyte conditioned media were analyzed for an extracellular localization. A functional annotation analysis was performed with the gene ontology tool (GOTERM_CC_ALL) using the UniProt Accession numbers for all identified proteins and extracellular proteins were so noted in *GO* column (see Table 1). Additionally, proteins passing the 2-fold AFE cut-off were searched against the Swiss-Prot/TrEMBL annotated database using the online ExPASy interface

(http://www.expasy.ch/). Proteins that were known to be membrane proteins, secreted proteins, or had a signal peptide were so noted in the *SecP* column (see Table 1). If, however, a protein yielded a two-fold enrichment factor but was not reported to be a secreted protein, then SignalP (http://www.cbs.dtu.dk/services/SignalP/) and SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP) were employed to determine if the protein might be secreted.[45] A SecretomeP score above 0.5 indicates a high probability of being secreted via a non-classical pathway. In order to be considered secreted, a protein had to exhibit a 2-fold enrichment factor and be known to be secreted or predicted to be secreted by SignalP or SecretomeP (see Figure 1).

Results and Discussion

Sample Preparation

Initially different sample preparation techniques were examined in order to optimize protein recovery and minimize protein contamination from both serum and dead or dying cells. In order to remove serum proteins, we examined different cell rinsing strategies, including the use of phosphate buffered saline (PBS) or Hank's Balance Salt Solution (HBSS). The use of PBS for rinsing resulted in reactive astrogliosis (data not shown). This astrogliosis subsided after a few hours after rinsing; however, reactive astrocytes can exhibit different protein secretion profiles than resting astrocytes[10, 11] so it was deemed prudent not to use PBS as a rinsing solution. Rinsing with HBSS did not result in astrogliosis and so all rinses were performed with HBSS. The number of times the cells were rinsed was also optimized to strike a balance between serum protein removal and cell stress. Three rinsings was found to remove the majority of serum proteins, as determined by the reduction of serum proteins in the LC-MS/MS analysis, while minimizing cell stress as determined by visual inspection via microscope (data not shown). Additionally, different protein concentration methods were also used, including TCA-NLS precipitation and ultrafiltration using Amicon molecular weight cut-off devices. The TCA-NLS method resulted in significant sample loss while ultrafiltration yielded good sample recovery (data not shown). Ultrafiltration was used for all experiments included in this study.

False Discovery Rate, Spectral Counting and Average Fold Enrichment (AFE)

Using the X!Tandem search engine, the false discovery rate was estimated to be 0.71%, the p-score was 75, and the valid log E cut-off <−1.3. All of the 187 proteins reported in Table 1 were above this log E cut-off. As an additional requirement for positive identification, each protein was required to have at least two total spectral counts from the summation of all four biological replicates.

By comparing the normalized spectral counts for each protein from the astrocyte conditioned media to the cytoplasmic control, we were able to determine the average fold enrichment (AFE) factor of each protein. If the protein was enriched by at least 2-fold, it was then subjected to bioinformatics analysis to determine possible secretion or residence in the extracellular compartment.

Bioinformatics Analysis

Proteins which passed the AFE filter were subjected to analysis by database search, SignalP, and SecretomeP. Employing the online ExPASy interface, the subcellular location of each protein was determined from the annotations in the Prot/TrEMBL database. Proteins that were annotated as secreted, possessing a signal peptide, or were membrane bound were included in Table 1. The remaining proteins were then subjected to SignalP and SecretomeP analysis. Those proteins which were predicted to have signal peptide by SignalP or had a significant SecretomeP score (0.5 or higher) were included in Table 1. A Gene Ontology (GO) analysis

was performed on all of the identified proteins from the astrocyte conditioned media as a confirmation of the results from the AFE and SignalP/SecretomeP analysis.

Identified Proteins and Data Analysis

A total of 423 proteins were identified from all the 2D- and 1D-LC-MS/MS analyses of astrocyte conditioned media. Figure 2 shows an example of a base peak chromatogram from the LC-MS/MS analysis of astrocyte conditioned media (see Fig. 2A) and a representative tandem MS/MS spectrum of a tryptic peptide (YIALEEWAGCFGIK) from secreted protein acidic and rich in cysteine (SPARC) (see Fig. 2B). A GO analysis of this data set yielded 148 proteins that were localized to the extracellular region (see Figure 3). Separately, the application of the AFE filter with a 2-fold cut-off returned 262 proteins. This protein list was refined with a manual search of the Swiss-Prot database and analysis by SignalP and SecretomeP, yielding 187 proteins passing both the AFE and ExPASy/SignalP/SecretomeP filters (see Figure 3 and Table 2). When a GO was performed on the same AFE filtered proteins, only 106 were identified as being extracellular.

The use of multiple data refinement tools, including GO, AFE, and SignalP/SecretomeP, ensures the confident and comprehensive identification of secreted proteins. These tools are highly complementary and their combined use yields high quality data sets. However, each tool has its advantages and disadvantages. The GO analysis is a very mature tool and yields a large amount of important data with minimal effort. The direct application of a GO analysis to the raw protein data yielded a total of 148 proteins identified as secreted (see Table 2). However, a manual examination of the Swiss-Prot/TrEMBL database yielded a number of secreted proteins which were not identified as such in the GO search. Out of the 262 proteins passing the AFE filter, 144 were identified as extracellular by a manual ExPASy database search while the GO analysis only yielded 106 extracellular proteins—a difference of over 25%. This difference is largely the result of some proteins lacking a GO annotation in the Swiss-Prot database. While a manual examination of the Swiss-Prot database yields a more comprehensive list of secreted proteins, this method is time consuming and cumbersome. A first pass filtering of the data through a GO analysis followed by a targeted manual database search is the most comprehensive and practical way of combining these tools. In addition, the use of AFE filtering and SignalP/SecretomeP expands the number of putative secreted proteins beyond those annotated in the Swiss-Prot database while maintaining a high degree of confidence in their identities as secreted proteins.

As mentioned in the introduction, two earlier studies employed two-dimensional gel electrophoresis to examine astrocyte secreted proteins, resulting in 40 identified proteins.[5, 6] Out of the 40 proteins identified in these studies, 38 were identified in the current study; however, out of these 38 proteins only 14 passed the criteria for enrichment and predictive secretion. Furthermore, these 14 proteins were all brefeldin A sensitive, indicating a vesicular pathway of secretion.[5] Based on our results, it would seem that only the brefeldin A sensitive proteins are actively secreted and the remaining proteins identified as `secreted' are most likely cytoplasmic proteins released from dead or dying cells.

Two more recent studies have used shotgun proteomics to identify proteins secreted by astrocytes.[7, 8] In the study by Moore and co-workers, gene ontology assignments were used to identify a total of 133 putative secreted proteins from rat primary astrocytes. When the list of secreted proteins from this study is compared against our list of secreted proteins, our study identified 85 out of the 133 proteins identified by Moore and co-workers as secreted, more than a 63% overlap in identifications. However, out of the 85 identifications common to both studies, 16 did not pass our AFE filter and 5 out of those 16 were enriched in the cytoplasmic control, indicating the possibility that these proteins are cytoplasmic contaminants.

Many of the differences between the two studies can be attributed to the differences in naming convention and gene IDs between the rat and mouse protein databases which results in errors in assessing protein overlap between the two studies. Additionally, differences in analytical strategy and instrumentation, *i.e.* the exclusive use of 1D LC-MS/MS by Moore and co-workers versus the use of both 1D and 2D LC-MS/MS in our study, and differences in the bioinformatics tools used to analyze the data set, *i.e.* the exclusive use of GO analysis by Moore and co-workers versus the combined use of GO, AFE, Swiss-Prot annotations, SignalP, and SecretomeP in our analysis, could account for the different numbers of proteins identified in two studies. Nonetheless, the data from both studies cross-validate each other while also being complementary.

Keene and co-workers also recently published a study in which they identified 169 putative astrocyte secreted proteins. They utilized Gel/LC-MS/MS, a method which is similar to 2D LC/LC-MS/MS, and a bioinformatics approach employing both a GO analysis and predictive secretion software (Protein Prowler and SecretomeP). When the list of secreted proteins from this study is compared against our list of secreted proteins, our study identified 101 out of the 155 secreted proteins identified by Keene and co-workers in their untreated control cultures, a 65% overlap in identifications. Again, this large degree of overlap between the studies strengthens the results of both studies. While the general experimental design of Keene and co-workers is very similar to ours, an important difference exists between the two studies—the length of media conditioning. Keene and co-workers collected conditioned media after both a single day and 7 days of incubation while we only collected media after a single day of conditioning. If only the protein identifications from the single day conditioned media are considered, 68 out of the 79 proteins that were identified by Keene and co-workers were also identified in our study—an 86% overlap. However, nine of the proteins identified solely by Keene and co-workers did not pass our AFE filter and thus may not be secreted. Additionally, our study identified over 100 proteins not identified in the single day conditioned media study from Keene and co-workers. This discrepancy is most likely due to the amount of starting material (we combined media from three separate T-25 flasks while they only combined media from two) and to the higher recovery and total protein identifications afforded 2D LC/LC-MS/MS separations versus 2D gel/LC-MS/MS (see Dowell et al. for a direct comparison of these techniques).[46] However, the use of longer conditioning times (7 days) by Keene and co-workers resulted in the identification of 76 more proteins than identified in their single day experiments.

Clearly, the use of a cytoplasmic control increases confidence in the identification of secreted proteins and the use of multiple filters allows for greater flexibility in the downstream analysis. By changing the degree of filtering in our AFE analysis or GO/SecretomeP analysis, there is an increased confidence in the results (see Table 2). Applying a 3-fold AFE filter, results in a reduction of protein identifications from 262 to 167 and an increase in confidence. The additional application of the SecretomeP filter results in a further refinement of the results, yielding 123 secreted proteins versus our original 187 identifications. The use of multiple filters allows for the flexibility to strike a balance between confidence and comprehensiveness in the identification of secreted proteins.

Conclusions

The present study has identified 187 putative astrocyte secreted proteins using a combination of LC-MS/MS techniques, quantitative proteomics, and bioinformatics. Use of an integrated analysis regime incorporating multiple rounds of data refinement enables a flexible and robust analysis of secreted proteins.

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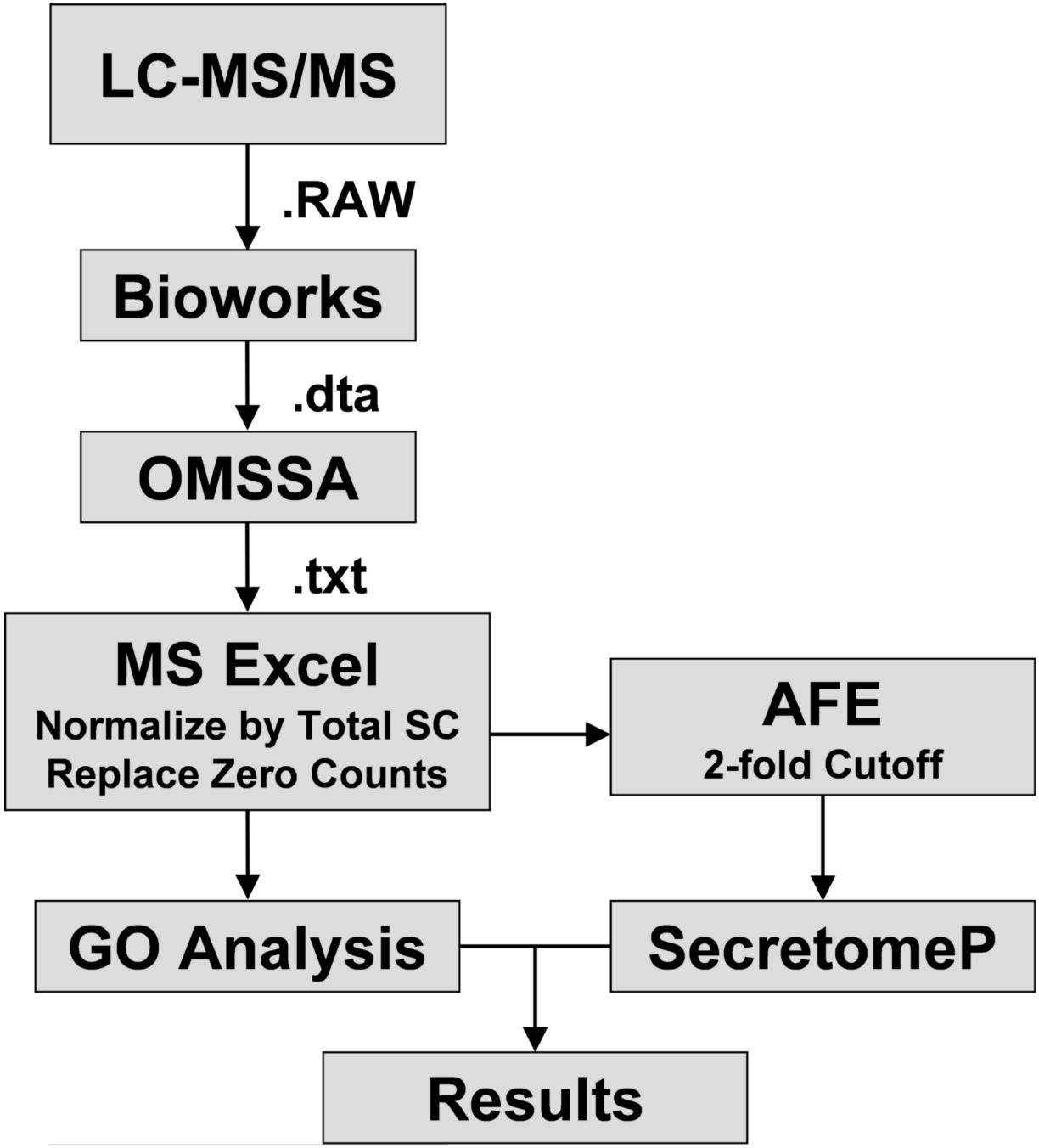

Figure 1.

Data Analysis Overview. Thermo-Finnigan LTQ .RAW files were processed into .dta files using Thermo-Finnigan Bioworks. The individual .dta files were concatenated using NCBI OMSSA and searched against the Swiss-Prot database. The search results, including the spectral counts for each protein, were exported to MS Excel. Each run was normalized against the cytoplasmic control based on the total spectral counts and zero calls were converted to 0.5. A Gene Ontology (GO) Analysis was performed to determine protein secretion. Separately, the Average Fold Enrichment (AFE) was calculated for each protein and those exhibiting at least a 2-fold enrichment versus the cytoplasmic control were then subjected to SecretomeP analysis. Finally, the GO and SecretomeP results were combined.

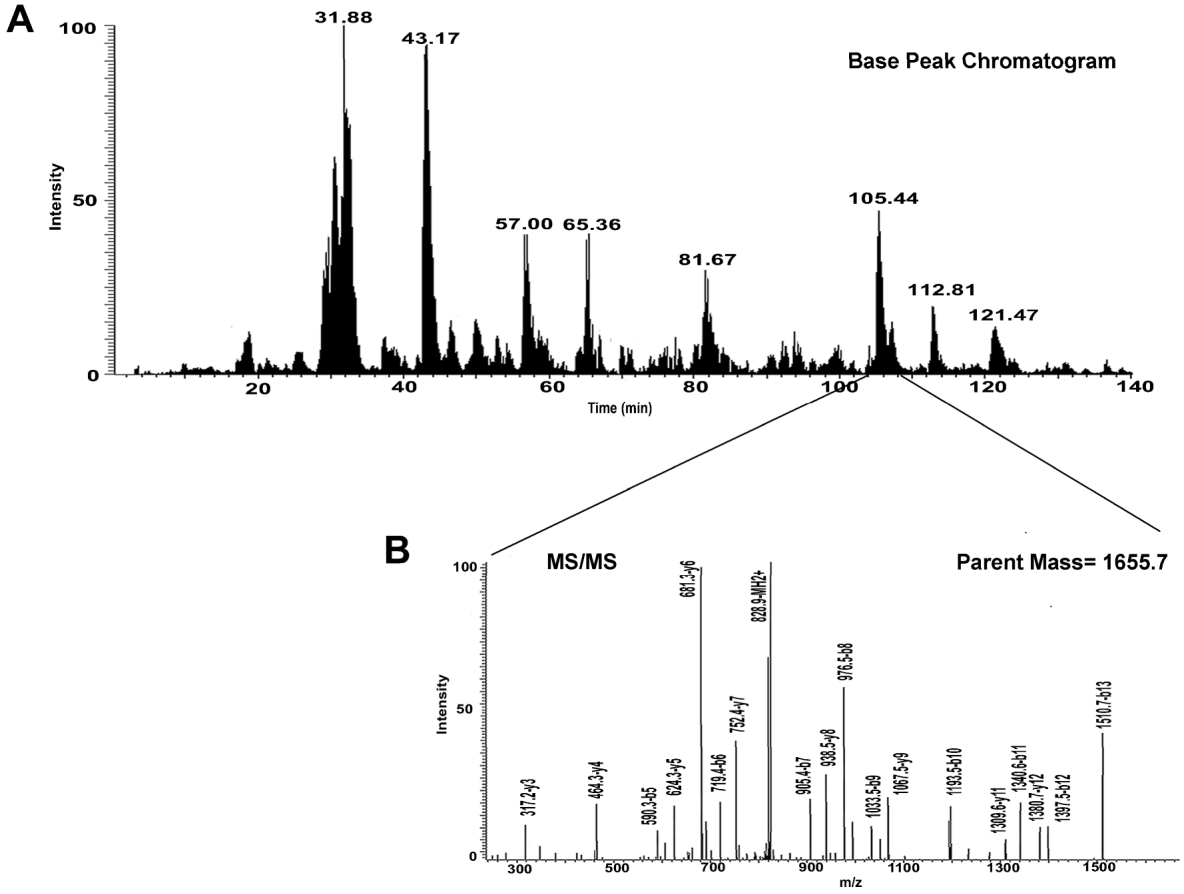

Figure 2.

Example LTQ MS and MS/MS spectra. (A) Base peak chromatogram from a 1D LCMS/MS of a tryptic digest of astroctye secreted proteins. (B) MS/MS spectrum of tryptic peptide (YIALEEWAGCFGIK, *m/z* 828.9^{2+}) from SPARC (Secreted protein acidic and rich in cysteine).

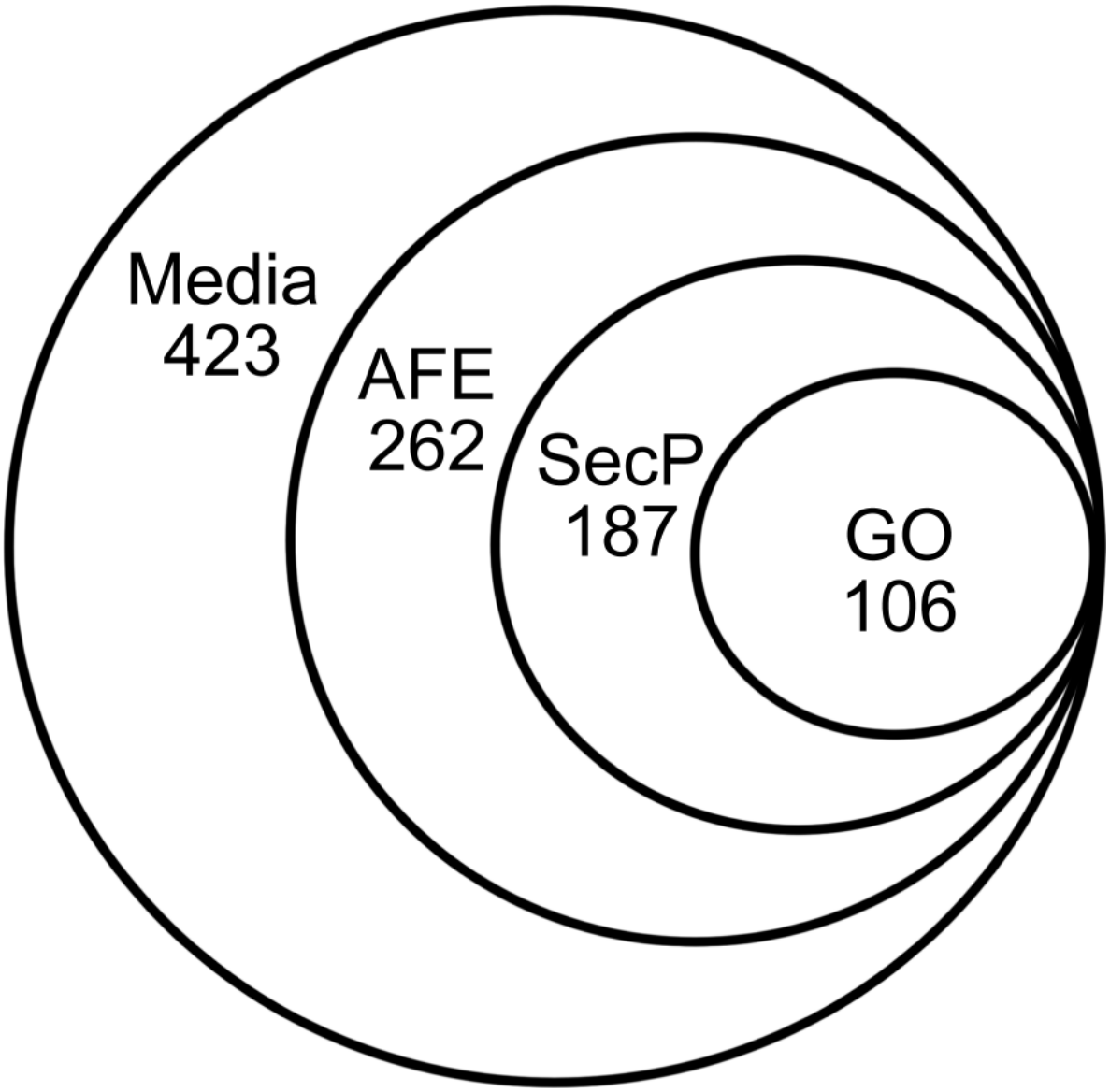

Figure 3.
Venn diagram of OMSSA protein identifications from astrocyte conditioned media. Total proteins were filtered by Average Fold Enrichment (AFE), SecretomeP (SecP), and Gene Ontology Analysis (GO).

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Table 1

Astrocyte secreted proteins identified by LC-MS/MS and bioinformatics tools

Protein Name	Acc#	SecP	GO	SUM SC	AFE
SPARC	P07214	Yes	E	447	224
Fibronectin precursor (FN)	P11276	Yes	E	330	165
Pigment epithelium-derived factor precursor (PEDF)	P97298	Yes	E	298	149
Cystatin-C	P21460	Yes	E	274	137
Complement C3	P01027	Yes	E	251	125
Clusterin	Q06890	Yes	E	241	121
Collagen alpha-1(I)	P11087	Yes	E	188	94
Cadherin-2	P15116	Yes	E	186	93
Alpha-2-macroglobulin-P	Q6GQT1	Yes		160	80
Follistatin-related protein 1	Q62356	Yes	E	147	74
Insulin-like growth factor-binding protein 5	Q07079	Yes	E	118	59
Secretogranin-3	P47867	Yes	E	110	55
Collagen alpha-2(V)	Q3U962	Yes	E	103	52
Nucleobindin-1	Q02819	signal		101	50
Collagen alpha-2(I)	Q01149	Yes	E	83	42
Calumenin	O35887	signal		83	41
Insulin-like growth factor-binding protein 2	P47877	Yes	E	469	39
Neurocan core protein	P55066	Yes	E	73	36
Dickkopf-related protein 3	Q9QUN9	Yes	E	67	33
ProSAAS	Q9QXV0	Yes	E	62	31
Latent-transforming growth factor beta-binding protein 2	O08999	Yes		44	22
Nidogen-2	O88322	Yes	E	43	21
Chitinase-3-like protein 1	Q61362	Yes	E	41	21
Apolipoprotein E	P08226	Yes	E	38	19
Neuroendocrine protein 7B2	P12961	Yes	E	37	19
Cathepsin D precursor	P18242	Yes		33	17
Macrophage colony-stimulating factor 1	P07141	Yes	E	32	16
Insulin-like growth factor-binding protein 7	Q61581	Yes	E	31	16
Serine protease inhibitor A3N	Q91WP6	Yes	E	29	14

Protein Name	Acc#	SecP	GO	SUM SC	AFE
Proheparin-binding EGF-like growth factor	Q06186	Yes	E	28	14
Extracellular matrix protein 1	Q61508	Yes	E	28	14
Mimecan	Q62000	Yes	E	27	13
Phospholipid transfer protein	P55065	Yes	E	26	13
Dystroglycan	Q62165	signal	E	24	12
Esophageal cancer-related gene 4	Q99LS0	Yes	E	24	12
SPARC-like protein 1	P70663	Yes	E	23	12
Biglycan	P28653	Yes	E	21	10
Insulin-like growth factor-binding protein 3	P47878	Yes	E	21	10
Protein CYR61	P18406	Yes	E	20	10
Granulins	P28798	Yes	E	19	10
Fibulin-5	Q9WVH9	Yes	E	19	10
Collagen alpha-1(IV)	P02463	Yes	E	18	9
WNT1-inducible-signaling pathway protein 2	Q9Z0G4	Yes	E	17	9
Carboxypeptidase E	Q00493	Yes	E	17	9
Lysosome-associated membrane glycoprotein 2	P17047	Yes	E	17	9
Extracellular superoxide dismutase	O09164	Yes		17	9
Complement C4-B	P01029	Yes	E	16	8
Sulfated 50 kDa glycoprotein	Q02596	Yes	E	15	7
Cathepsin L1	P06797	Yes	E	14	7
Transcobalamin-2	O88968	Yes	E	14	7
Plasma protease C1 inhibitor	P97290	Yes	E	14	7
L-lactate dehydrogenase A	P06151	0.568		54	7
Biotinidase	Q8CIF4	Yes		13	7
Insulin-like growth factor-binding protein 4	P47879	Yes	E	13	6
Tenascin	Q80YX1	Yes	E	13	6
Fibromodulin	P50608	Yes	E	13	6
Epididymal secretory protein E1	Q9ZOJ0	Yes	E	13	6
Inter-alpha-trypsin inhibitor heavy chain H2	Q61703	Yes	E	12	6
Elastin	P54320	Yes	E	12	6
Platelet-activating factor acetylhydrolase	Q60963	Yes	E	11	6
Thymosin beta-4	P20065	0.864		13	6

Protein Name	Acc#	SecP	GO	SUM SC	AFE
Transgelin	P37804	0.645		24	6
Brain acid soluble protein 1	Q91XV3	membrane		12	6
Serine protease inhibitor A3G	Q5I2A0	Yes		11	6
Metalloproteinase inhibitor 2	P25785	Yes	E	11	5
Ribonuclease pancreatic	P00683	Yes	E	10	5
Calsyntenin-1	Q9EPL2	Yes	E	10	5
EGF-containing fibulin-like extracellular matrix protein 2	Q9WVJ9	Yes	E	10	5
Calsequestrin-1	O09165	signal	E	9	5
Nuclease sensitive element-binding protein 1	P62960	0.73		11	5
Amyloid beta A4 protein	P12023	membrane		10	5
Phosphoinositide-3-kinase-interacting protein 1	Q7TMJ8	membrane		10	5
Stathmin	P54227	0.725		9	5
Serine protease HTRA1	Q9R118	Yes	E	9	4
Osteopontin	P10923	Yes	E	8	4
Insulin-like growth factor-binding protein 1	Q80W15	Yes	E	8	4
Collagen alpha-1(II)	P28481	Yes	E	8	4
FXYD domain-containing ion transport regulator 6	Q9D164	membrane	E	8	4
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	Q9R0B9	Yes	E	8	4
Transthyretin	P07309	Yes	E	7	4
Spondin-1	Q8VCC9	Yes	E	7	4
Ezrin	P26040	0.585		17	4
Myristoylated alanine-rich C-kinase substrate	P26645	membrane		8	4
Tubulin polymerization-promoting protein family member 3	Q9CRB6	0.518		8	4
Apolipoprotein A-I-binding protein	Q8K4Z3	Yes		8	4
Scotin	Q9D7I0	membrane		8	4
Thymosin beta-10	Q6ZWY8	0.752		8	4
Serine protease inhibitor A3M	Q03734	Yes		7	4
Prostaglandin-H2 D-isomerase	O09114	Yes	E	7	3
Reticulocalbin-1	Q05186	signal	E	7	3
Connective tissue growth factor	P29268	Yes	E	7	3
Serpin H1	P19324	Yes	E	7	3
Gelsolin	P13020	Yes	E	6	3

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Protein Name	Acc#	SecP	GO	SUM SC	AFE
Palmitoyl-protein thioesterase 1	O88531	Yes	E	6	3
Collagen alpha-1(V)	O88207	Yes	E	6	3
Antithrombin-III	P32261	Yes	E	6	3
Platelet-derived growth factor A chain	P20033	Yes	E	6	3
Hepatoma-derived growth factor	P51859	Yes	E	6	3
Collagen alpha-1(XI)	Q61245	Yes	E	5	3
Peptidyl-prolyl cis-trans isomerase B	P24369	Yes	E	5	3
Neuroblastoma suppressor of tumorigenicity 1	Q61477	Yes	E	5	3
45 kDa calcium-binding protein	Q61112	0.692	E	5	3
Cathepsin B	P10605	Yes		56	3
Acyltransferase-like 1-A	Q8BYI6	membrane		7	3
Coiled-coil domain-containing protein 80	Q8R2G6	Yes		7	3
Serine protease inhibitor A3C	P29621	Yes		6	3
Serine protease inhibitor A3F	Q80X76	Yes		6	3
Serine protease inhibitor A3K	P07759	Yes		6	3
Metallothionein-3	P28184	Yes		6	3
Parathymosin	Q9D0J8	0.673		6	3
CD44 antigen	P15379	Yes		6	3
Y-box-binding protein 2	Q9Z2C8	0.71		6	3
DNA-binding protein A	Q9JKB3	0.676		6	3
Moesin	P26041	0.558		12	3
Glutathione S-transferase P 1	P19157	Yes		5	3
Glutathione S-transferase P 2	P46425	Yes		5	3
PRKC apoptosis WT1 regulator protein	Q925B0	0.799		5	3
Metallothionein-2	P02798	Yes		5	3
Ubiquitin-conjugating enzyme E2 L3	P68037	0.582		5	3
Metalloproteinase inhibitor 1	P12032	Yes	E	5	2
72 kDa type IV collagenase	P33434	Yes	E	5	2
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	Q64191	signal	E	5	2
Microfibril-associated glycoprotein 4	Q9D1H9	Yes	E	5	2
FMRFamide-related peptides	Q9ESQ8	Yes	E	5	2
Fibulin-1	Q08879	Yes	E	5	2

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Protein Name	Acc#	SecP	GO	SUM SC	AFE
Delta-like protein	Q09163	membrane	E	5	2
Chordin-like protein 1	Q920C1	Yes	E	4	2
Lysyl oxidase homolog 3	Q9Z175	Yes	E	4	2
Galectin-3	P16110	Yes	E	4	2
Cell adhesion molecule 4	Q8R464	membrane	E	4	2
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Q9R0E2	Yes	E	4	2
Amyloid-like protein 2	Q06335	membrane	E	4	2
Cathepsin Z	Q9WUU7	Yes	E	4	2
Collagen alpha-2(IV)	P08122	Yes	E	4	2
Glucose-6-phosphate isomerase	P06745	Yes	E	21	2
Peroxiredoxin-4	O08807	Yes	E	3	2
Tyrosine-protein phosphatase non-receptor type substrate 1	P97797	Yes	E	3	2
Gamma-glutamyl hydrolase	Q9Z0L8	Yes	E	3	2
Fractalkine	O35188	Yes	E	3	2
Glypican-4	P51655	Yes	E	3	2
Prolargin	Q9JK53	Yes	E	3	2
WAP four-disulfide core domain protein 2	Q9DAU7	Yes	E	3	2
Thioredoxin domain-containing protein 4	Q9D1Q6	signal	E	3	2
Brevican core protein	Q61361	Yes	E	3	2
Inter-alpha-trypsin inhibitor heavy chain H3	Q61704	Yes	E	3	2
Translationally-controlled tumor protein	P63028	Yes	E	3	2
Beta-hexosaminidase beta chain	P20060	signal		5	2
Legumain	O89017	signal		5	2
Keratinocytes-associated transmembrane protein 2	Q8K201	membrane		5	2
Polyadenylate-binding protein-interacting protein 2	Q9D6V8	0.816		5	2
Sterile alpha motif domain-containing protein 3	Q8C4H2	0.594		4	2
Lysosomal protective protein	P16675	signal		4	2
Ubiquitin	P62991	0.693		84	2
Acid ceramidase	Q9WV54	0.781		4	2
Drebrin	Q9QXS6	0.72		4	2
Acylphosphatase-2	P56375	0.712		4	2
Plasma glutamate carboxypeptidase	Q9WVJ3	Yes		4	2

Protein Name	Acc#	SecP	GO	SUM SC	AFE
Protein DJ-1	Q99LX0	0.537		4	2
Chromobox protein homolog 3	P23198	0.836		4	2
Putative RNA-binding protein 3	O89086	0.745		4	2
Eukaryotic initiation factor 4A-III	Q91VC3	0.634		4	2
Eukaryotic initiation factor 4A-I	P60843	0.631		4	2
Stathmin-2	P55821	membrane		4	2
Syndecan-4	O35988	membrane		4	2
PDZ and LIM domain protein 5	Q8CI51	0.522		4	2
Profilin-1	P62962	0.56		16	2
Secernin-1	Q9CZC8	0.516		14	2
Protein kinase C and casein kinase substrate	Q9WVE8	0.608		4	2
Isocitrate dehydrogenase	O88844	0.576		4	2
Chloride intracellular channel protein 4	Q9QYB1	0.563		4	2
Sulfhydryl oxidase 1	Q8BND5	Yes		4	2
BolA-like protein 1	Q9D8S9	0.864		3	2
LIM and SH3 domain protein 1	Q61792	0.722		3	2
3'(2'),5'-bisphosphate nucleotidase 1	Q9Z0S1	0.652		3	2
Probable ATP-dependent RNA helicase DDX56	Q9D0R4	0.534		3	2
SH3 domain-binding glutamic acid-rich-like protein 3	Q91VW3	0.758		3	2
Corneodesmosin	Q7TPC1	Yes		3	2
Placenta-specific protein 9	Q8K262	Yes		3	2
Methylcrotonoyl-CoA carboxylase beta chain	Q3ULD5	signal		3	2
Sortilin-related receptor	O88307	membrane		3	2
Chromobox protein homolog 1	P83917	0.872		3	2
Probable ATP-dependent RNA helicase DDX5	Q61656	0.502		3	2
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q9R0P9	0.54		6	2
Thioredoxin domain-containing protein 16	Q7TN22	Yes		3	2
Cysteine-rich protein 2	Q9DCT8	0.807		3	2
WD repeat-containing protein 1	O88342	0.518		3	2
Opioid growth factor receptor	Q99PG2	membrane		3	2

Note: *Protein Name* is the Swiss-Prot name for the identified protein. *Acc #* is the Swiss-Prot accession number for the identified protein. *Sum SC* is the total normalized spectral counts from all LC-MS/MS runs. The *SecP* column indicates the results from the ExPasy, SignalP, and SecretomeP analysis: `Yes' indicates known secretion from Swiss-Prot database; `signal' indicates a known or predicted signal peptide

from the Swiss-Prot database or SignalP analysis;,a number in the *SecP* column indicates the numerical score returned by SecretomeP (greater than 0.5 indicates high probability of non-classical secretion).
`E' in the *GO* column indicates an extracellular protein as determined by Gene Ontology Analysis.

Table 2

Summary of identified proteins passing successive filtering by Average Fold Enrichment (AFE) and SecretomeP (SecP).

	Total number of proteins	Number of extracellular proteins[A]	Number of secreted proteins[B]
Media	423	148	112
AFE^2	262	106	88
AFE^2 +SecP	187	106	88
AFE^3	167	80	71
AFE^3+SecP	123	80	71

AFE^2 and AFE^3 are a two- and three-fold cutoff, respectively.

(A) Number of proteins identified as residing in the `Extracellular Region' by Gene Ontology Analysis.

(B) Number of proteins identified as being `Secreted' by Gene Ontology Analysis.