Chapter 15
Mass Spectrometry for Proteomics-Based Investigation Using the Zebrafish Vertebrate Model System

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Abstract The zebrafish (*Danio rerio*) is frequently being used to investigate the genetics of human diseases as well as resulting pathologies. Ease of both forward and reverse genetic manipulation along with conservation of vertebrate organ systems and disease causing genes has made this system a popular model. Many techniques have been developed to manipulate the genome of zebrafish producing mutants in a vast array of genes. While genetic manipulation of zebrafish has progressed, proteomics have been under-utilized. This review highlights studies that have already been performed using proteomic techniques and as well as our initial proteomic work comparing changes to the proteome between the *ascl1a*–/– and WT intestine.

Abbreviations

CID	Collision-induced dissociation
ESI-MS	Electrospray ionization mass spectrometry
LC-MS/MS	Liquid chromatography mass spectrometry
m/z	Mass/charge
MALDI-MS	MALDI mass spectrometry
MS	Mass spectrometry

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Mw	Molecular weight
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TIC	Total ion current/chromatogram

15.1 Introduction

Initially, the zebrafish model system was used extensively for investigations in early vertebrate development due to the ease of genetically manipulation [1]. In recent years, investigations utilizing zebrafish have expanded into organ development [2–6], disease modeling [7, 8], and toxicology [9]. Similarity of zebrafish in genetic identity to humans combined with homologous organ systems has demonstrated the usefulness of zebrafish as a model system for using the model system for investigation of numerous human diseases and disorders [10, 11]. Other features such as small size, production of high number of externally developing embryos, optical clarity of embryos, and fast embryogenesis make zebrafish attractive for studying these diseases.

During this time, the number of genetic tools has increased dramatically which is complemented by the sequencing and annotation of the zebrafish genome [12]. Currently, there are techniques involving both forward and reverse genetics that have increased the usefulness of the zebrafish system. One of the now classic approaches is random generation of point mutations utilizing *N*-ethyl-*N*-nitrosourea (ENU). In the mid 1990s, two large-scale ENU screens were performed which highlighted the ability of this vertebrate to be screened for novel mutations, which replicate defects in human diseases and disorders [13]. Since then there have been many additional forward genetic ENU screens including novel screens such as looking for genes that have a maternal effect on development of the embryo [14, 15]. These forward genetic screens are now being complemented by the Zebrafish Mutation Project that aims to make mutations in each gene within the zebrafish genome using TILLING (targeting induced local lesions in genomes) methods [16, 17], which have currently generated mutants in 46 % of the zebrafish protein coding genes [10]. In addition to TILLING mutations in the zebrafish genome are also created by transcription activator-like effector nucleases (TALENs) [18] and the CRISPR-Cas system [19], which can be targeted to individual genes to create double strand breaks in the DNA. Double strand breaks are then repaired inaccurately by the non-homologous end joining repair (NHEJ) system to create a variety of mutations at the break site.

Compared to development of genetic tools, proteomic analysis has not been an extensively utilized technique in the zebrafish system. Proteomic analysis can identify changes in structural and enzymatic content of organs modified by disease states, genetic changes, and differences over a developmental time course [20–29]. Thus proteomics can complement current methods of genetic modification to the zebrafish system and add another tool kit to the analysis. Already, a variety of methods have been applied to analyze the changes in the zebrafish proteome. Here we will review some of the ways that mass spectrometry (MS) is being used to investigate zebrafish proteomics and then provide an example of preliminary MS analysis that we have begun to compare differences in protein expression between wild type and mutant zebrafish embryos.

15.2 Experimental Design

ascl1a−/− embryos and WT siblings were grown to 5dpf. Intestines from mutants and WT were dissected into T-PERTissue Protein Extraction Reagent (Pierce) with Halt Protease Inhibitor Cocktail and EDTA (Pierce) with double the recommended concentration in order to prevent protein degradation. Whole protein extract was run on an 8 % SDS-PAGE gel and stained with Coomassie. In-gel trypsin digestion was performed on excised bands according to published procedures [30–32]. Samples were prepared for MS analysis and run on a nanoliquid chromatography tandem MS (nanoLC-MS/MS) using a NanoAcquity UPLC coupled to a QTOF Micro mass spectrometer (both from Waters Corp.). Raw data was processed using a ProteinLynx Global Server (PLGS version 2.5 from Waters Corp.) and the resulting pkl files were submitted to Mascot database search and proteins were identified using the UniProt database. This procedure is extensively described elsewhere [30–32].

15.3 Modeling Disease

A number of proteomic studies have investigated changes between the healthy conditions compared to the diseased state. In a study by Lu et al., proteomic alterations of zebrafish gills to *Aeromonas hydrophila* bacterial infection are observed [33]. Here as would be expected, proteins regulating bacterial infection and host immunity were unregulated. Also, proteins modulating increases in energy metabolism were up regulated along with cytoskeletal proteins. The latter may be needed due to increased phagosomes to begin clearing the infection.

In another study, Chen et al. determined the proteomic response to hypoxia [34]. In this study, adult zebrafish were exposed to hypoxic conditions for 48 h followed by extraction and preparation of only white muscle. The hypoxic conditions resulted in an increase in glycolytic enzymes in skeletal muscle with down-regulation of aerobic ATP production. This study also found increases in glycolytic muscle fibers and hemoglobin alpha variants suggesting a relatively rapid shift to low oxygen conditions.

Hogl et al. used proteomic analysis of a zebrafish knockout BACE1 to identify additional membrane targets of the enzyme [35]. BACE1 cleaves the amyloid precursor protein and is therefore a drug target for Alzheimer's disease. In the BACE1 knockout proteomic analysis, the majority of proteins were unaltered but a group of 24 intrinsic membrane proteins accumulated suggesting that they were no longer cleaved. Further analysis of identified substrates can determine whether reducing the activity of BACE1 into combat Alzheimer's disease will result in additional complications.

Fleming et al. utilized proteomics to help determine the timing of when the blood–brain barrier develops [36]. Exclusion of the drug from the head but not the body suggests development of a functional barrier between the blood and brain. Lack of drug accumulation in the head coupled with functional studies suggests that the blood–brain barrier matures between 8 and 10 days post fertilization (dpf).

While this investigation primarily identifies the timing of a developmental event, the same assay might be used to characterize breakdown of the blood–brain barrier during a disease process.

15.4 Comparative Proteomics

Proteomics can also take advantage of the changes between zebrafish developmental time points and even between different species to identify proteins that may be critical for initiating these changes. For example, sex determination within zebrafish is not strictly due to chromosomal inheritance. Groh et al. has used proteomic analysis to compare differences in protein composition between the testis and the ovary. Initially a global shotgun approach to generate an overall list of differential proteins was produced [37]. Once differentially expressed proteins were identified, these investigators utilized the selected reaction monitoring (SRM) technique coupled with representational difference analysis (RDA) to more reliably detect low abundance proteins that are likely to be important in determination of either the testis or the ovary [38].

Lin et al. have used proteomics to identify evolutionary changes in the crystallins between sighted fish such as zebrafish and compare loss of diversity in these proteins to loss of diversity in nocturnal rice eel and walking catfish [39]. Both rice eel and walking catfish have degenerative eyes. Here the number of overall proteins is limited by only including the lens in this shotgun proteomic approach. While there are a number of lens proteins, a number of other high abundance proteins are excluded by selective tissue acquisition. This approach identifies the loss of alpha-crystallin in the rice eel, which may function as a chaperone protein preventing aggregation of the other crytallins.

15.5 Changes to Protein Function

As in other systems antibodies to specific proteins can be immunoprecipitated and MS can be performed on the resulting pull-down proteins [40]. With this method, interacting proteins can be identified as well as modifications to the target and associated proteins. Within the zebrafish system there has been a lack of good and reliable antibodies to specific proteins. Many of the antibodies used in the zebrafish system are specific to proteins from other systems and cross-reacting with their homologous zebrafish counterparts.

The lack of zebrafish-specific antibodies has severely limited the identification of interacting proteins by immunoprecipitation. Deflorian et al. however, has generated a host of antibodies that were then screened for unique non-overlapping patterns [41]. From the numerous antibodies generated, 9 monoclonal antibodies were selected due to interesting expression patterns while one was used for immunoprecipitation. The protein was then identified by mass spectroscopy and found to be vitellogenin 1. Even though this screening method is labor intensive, it would produce a number of good antibodies to identify interacting proteins by immunoprecipitation.

Even when an appropriate zebrafish antibody is not present, the protein can be expressed with a FLAG tag in order to immunoprecipitate the protein [42]. Alternatively, Taskinen et al. expressed and purified a new avidin protein in *E. coli* to identify their native state. Here, MS demonstrated that the protein strongly binds biotin but the oligomeric state is unstable at low ionic strength [43].

15.6 Comparison of *ascl1a*−/− to WT Embryos

We have previously shown that *ascl1a*−/− embryos fail to develop intestinal epithelial secretory cells and only differentiate about 10 % of the normal number of enteric neurons by the end of embryogenesis [44]. While the Notch signaling pathway appears to play a role in formation of the intestinal secretory cells, the reason for failure of enteric neural differentiation is less clear. Mis-expressed proteins within the *ascl1a*−/− mutants may provide information about what cellular processes are affected. We have therefore begun to use a shotgun proteomic approach to identify mis-expressed proteins.

In order to limit the tissue involved in the analysis, we have dissected and pooled intestines from both *ascl1a*−/− and WT embryos at 5 dpf (workflow summary Fig. 15.1). Analysis of these extracts by SDS-PAGE demonstrates striking

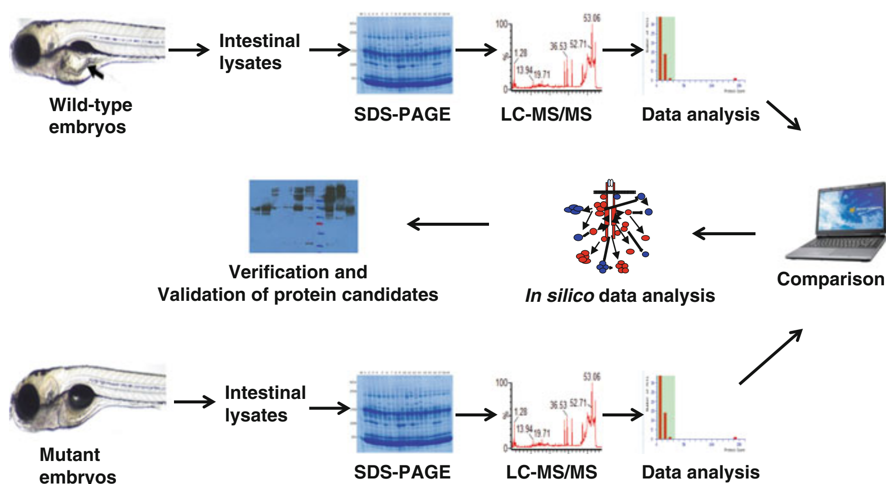

Fig. 15.1 Workflow for comparison of WT and mutant embryos. Intestinal lysates for both WT and mutant embryos 5 dpf embryos are prepared and run on SDS-PAGE gels followed by nanoliquid chromatography tandem MS (nanoLC-MS/MS) using a NanoAcquity UPLC coupled to a QTOF Micro mass spectrometer (both from Waters Corp.). Raw data is processed using a ProteinLynx Global Server (PLGS version 2.5 from Waters Corp.) and the resulting pkl files are submitted to Mascot database search and proteins are identified using the UniProt database. Proteins mis-regulated in mutant embryos are then verified by whole mount immunohistochemistry, western, or quantitative PCR

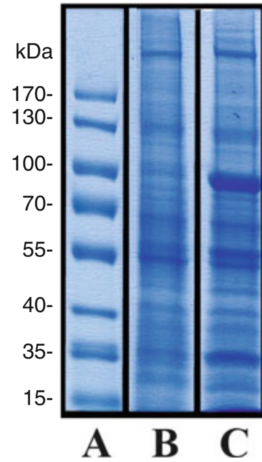

Fig. 15.2 Comparison of WT and *ascl1a*–/– 5 dpf intestine protein extract. (*A*) Molecular mass standards (in kDa). (*B*) WT intestinal protein extract. (*C*) *ascl1a*–/– protein extract

differences between WT and *ascl1a*–/– embryos (Fig. 15.2). In-gel tryptic digestion and nanoLC-MS/MS analysis identified a series of proteins that were specific to (or more abundant in) WT or *ascl1a*–/– embryos. An example of such a differentially expressed protein is Flotilin-2a (Fig. 15.3), a protein found in WT but not in the *ascl1a*–/– embryos.

Further comparison of alteration, increase or decrease of the levels of proteins and their post-translational modifications such as phosphorylation of acetylation between *ascl1a*–/– and WT embryos will likely suggest mechanisms for the lack of differentiated enteric neurons.

15.7 Conclusions and Perspectives

While proteomic analysis in the zebrafish system has still seen limited applications, there have been a number of promising investigations. As proteomics becomes more frequently utilized in the zebrafish system, it is likely to confirm predictions based on mRNA expression. These approaches may also reveal surprising differences in protein expression that would not be predicted at the mRNA expression level. Proteomics can give more of a global picture of structural and enzymatic changes due to alterations in either the genome or the disease state that is not possible by mRNA expression alone.

While proteomic techniques will not replace genetic approaches, these techniques promise to be a valuable complement. After shotgun proteomic analysis are completed, refined predictions for what proteins are important can be made and these and other less abundant proteins can be targeted in future experiments. One approach might be to refine the input cells using a genetic approach. For example, the relative ease with which transgenic fish can be produced has increased dramatically in the past few years. This coupled with increased annotation of the genome increases the ability to create fish with specific cells labeled by fluorescent proteins.

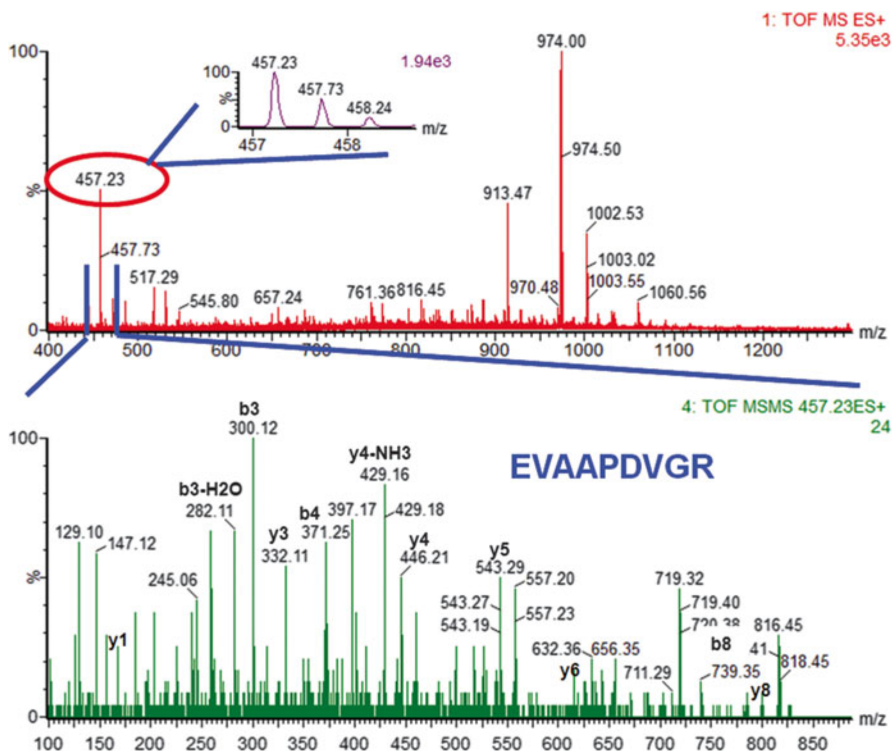

Fig. 15.3 Identification of a protein unique to *ascl1a*−/− mutants. NanoLC-MS/MS analysis led to identification of a doubly charged precursor peak with mass to charge ratio (*m*/*z*) of 457.23 (MS mode). Selection of this precursor peak for fragmentation by MS/MS (MS/MS mode) produced a series of fragment b and y ions, whose analysis led to identification of a peptide with the amino acid sequence EVAAPDVGR, which was part of the protein Flotillin-2a. This protein was found in the protein extract from WT but absent in *ascl1a*−/− 5 dpf intestine

Specific cell types from fluorescently labeled lines can then be isolated by Fluorescent Activated Cell sorting (FACs). Homogenous pools of cells can then be obtained for use in proteomic analysis. This will lessen the content of extraneous protein to add in detection of more low abundance proteins. Lessening unwanted proteins and other MS techniques to increase detection of low abundance proteins will continue to make proteomics a valuable tool for the zebrafish system.

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