DATASET BRIEF

Proteome profile of the pipping muscle in broiler embryos

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This study is the first proteomics analysis of the muscularis complexus (pipping muscle) in chicken (*Gallus gallus*) broiler embryos. We used differential detergent fractionation and nano-HPLC-MS/MS analysis to identify 676 proteins from all cellular components. The identified proteins were functionally classified in accordance with their involvement in various cellular activities.

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The broiler chicken spends approximately 21 days of its life as an embryo and therefore serves as a readily accessible model for developmental biology research [1]. Hatching of the broiler embryo begins with its perforation (pipping) of the eggshell membrane and eggshell proper [2]. The pipping muscle is primarily responsible for the embryo's pipping action, through flexion and extension of the neck, which breaks the eggshell during hatching. The paired muscle is located superficially in the upper cervical region, overlying muscles of the neck: muscles spinalis, biventer, and splenius cervicis [3]. These neck muscles support the pipping muscle, to achieve the pipping action towards the end of the chick's embryonic life [4]. The pipping muscle undergoes very rapid developmental changes during embryogenesis. The muscle grows from $<0.4\%$ of body weight on Day 11 of incubation to approximately 1.93% of body weight by Day 20, after which it completely regresses by Day 3 post-hatch. This development is primarily due to rapid muscle cell hyperplasia and hypertrophy, in addition to lymph infiltration [3]. Hatching muscle development, as with most skeletal muscle development, is a highly organized process involving precise regulation of numerous developmental changes. However, these cellular processes do not occur in isolation but are usually directed by various intracellular interactions, and they involve a balance between protein synthesis and protein degradation [5]. Histological evidences have shown typical early muscle development in the pipping muscle on Day 13 of incubation, prior to a rapid increase in lymph infiltration beginning between Days 14 and 15 of incubation [6]. The proteome of the chicken pectoralis muscle has been largely profiled [7–9]. However, the proteome of the pipping muscle has not been analyzed. Here, we present the characterization of the pipping muscle proteome in the 13-day-old broiler embryo using differential detergent fractionation (DDF) and nano-HPLC-MS/MS analysis. Embryonated eggs were incubated under standard incubation conditions [10]. A pipping muscle sample was collected from each of three Ross × Ross 708 broiler embryos on Day 13 of incubation. The triplicate muscle samples were washed in physiological saline buffer, immediately frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until further processing. The frozen tissue samples were manually ground using mortar and pestle in liquid nitrogen, until a fine powder was obtained. The powder was subjected to a DDF protein extraction method as described previously [11, 12]. Four DDF extraction steps were performed on each of the triplicate tissue samples resulting in a total of 12 technical replicates. Extracted proteins (5 μg) were trypsin

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Abbreviations: DDF, differential detergent fractionation; **FA,** formic acid; **GO,** gene ontology

digested [13], and the resulting peptides were lyophilized, then stored at −80°C before being subjected to nano-HPLC-MS/MS. Samples were re-suspended, desalted, and the detergents were removed using macrotrap (Michrom) columns according to the manufacturer's instructions (see details in Supporting Information SI.1) Finally, the peptide mix was dissolved in 20 µL of 5% ACN and 0.1% formic acid (FA), and loaded on a BioBasic C18 reversed-phase column (Thermo) and washed for 20 min with 5% ACN and 0.1% FA to remove any remaining salts. Peptide separation was achieved using a Thermo Surveyor MS pump with a 110 min nano-HPLC method, consisting of a gradient from 5% ACN to 50% ACN for 75 min, followed by a 20 min wash with 95% ACN and equilibration with 5% ACN for 15 min (all solutions contain 0.1% FA). Ionization of peptides was achieved via nano-spray using a Thermo Finnigan Nanospray Source Type I operated at 1.85 kV, with 8 µm id silica tips (New Objective FS360-75-8-N-20-C12). High voltage was applied using a T-connector with a gold electrode in contact with the HPLC solvent. An online LCQ DECA XP Plus (Thermo) ion trap mass spectrometer was used to collect data over the 110 min duration of each HPLC run. Precursor mass scans were performed using repetitive MS scans, each immediately followed by three MS/MS scans of the three most intense MS peaks. Dynamic exclusion was enabled for a duration of 2 min and a repeat count of two. Both the MS and MS/MS spectra were searched against a *Gallus* subset of the NCBI database (May 2010, 18 830 entries) (http://www.ncbi.nlm.nih.gov). The protein sequences were reversed, and used as a decoy database to allow for the calculation of a false discovery rate (FDR). The spectra were presented to the search software Bioworks 3.2 EF2 (Thermo) and experimental data were matched with the target and decoy databases, and protein identification was carried out as published previously [14] (see Supporting Information SI.2 for details). All Bioworks non-filtered target and decoy result files (.srf) were uploaded to ProteoIQ 2.0.01 (Bioinquire, Athens, GA, USA) software for further validation and statistical analysis. The following program parameters were set: minimum peptide length – 5 amino acids, minimum 2 spectra per protein, minimum 1 peptide per protein, and maximum FDR – 1%. Only the ''Top'' proteins were accepted as confident identifications (within a protein group, each and every respective peptide could be matched with the top protein). Protein identifications based on a single peptide were accepted only if the peptide was detected multiple times.

A total of 676 proteins were identified. They were deposited in the PRIDE database (http://www.ebi.ac.uk/pride; Experiment number 14845). A protein identification summary table is given in Supporting Information (Supporting Information Table 1). Detailed proteins and peptides data, including annotated spectral images for proteins identified by a single peptide, are also freely accessible online at http://lsbi.mafes.msstate.edu/support_data/gallus/Pipping_Proteome_results.html.

Gene Ontology (GO) annotations [15] were retrieved from the AgBase database [16] using the GORetriever tool. The ''Chick'' and the ''All AgBase and GO'' databases were selected to search against. Protein functions were summarized using the ''Generic'' GOSlim Set of the GOSlimViewer and GOanna tool. Proteins were classified based on the three organizing principles of GO: molecular function, cellular component, and biological process. The GO annotations were available for all identified proteins. To review the annotation details, an edited version of our GO annotation files is presented in Supporting Information (Supporting Information Table 2). When presenting the GO annotations results, we are bound with established GO categories, and readers are to consider the hierarchical structure of GO. For example, categories ''binding'' and ''protein binding'', ''cell'' and ''nucleus'', ''metabolic processes'', and ''nucleic acid metabolism'' are unique in regard to GO, but could be overlapping, respectively, in regard to protein sets they contain.

For molecular function classification, 660 pipping muscle proteins (97.6% of 676 total) had assigned 2375 GO terms belonging to 325 diverse functions, summarized into 21 GOSlim categories (Fig. 1A). The binding activity was the most abundant function (56.6% of GO terms) including unspecified binding activity (19.0%), protein binding (18.1%), nucleotide binding (12.9%), cytoskeleton protein binding (3.3%), nucleic acid binding (1.5%), lipid binding (1.1%), and carbohydrate binding (0.7%). Another major functional category was the catalytic activity (24.4% of GO terms) including unspecified catalytic activity (10.1%), hydrolase activity (5.3%), transferase activity (4.2%), kinase activity (3.2%), and peptidase activity (1.6%). A number of similar proteins involved in various molecular functions have been identified in the chicken pectoralis muscle [7]. Lipid binding protein, apoliprotein A-I, has been shown to be expressed in the skeletal muscle of chickens and acts as a local lipid transporter during development [17]. Other functions assigned to a small number of proteins are shown in Fig. 1A, while the unknown molecular function represented 0.2% of GO terms.

The distribution of pipping muscle protein based on cellular component was also determined. A total of 602 proteins (89.1%) had assigned 4665 GO terms belonging to 231 cellular compartments, summarized into 15 GOSlim categories (Fig. 1B). Most prevalent were proteins found in the cell (76.6% of GO terms), including categories of intracellular (21.7%), cell (19.4%), cytosol/cytoplasm (16.8%), mitochondria (4.1%), cytoskeleton (3.7%), nucleus (3.6%), plasma membrane (2.7%), endoplasmic reticulum (1.5%), chromosome (1.1%), ribosome (1.1%), golgi apparatus (0.8%), endosome (0.3%), and peroxisome (0.1%). The extracellular region was represented by 3.1% of GO terms. There were 20.3% annotations as unknown cellular components.

In regard to biological process, a total of 548 proteins (81.1%) had assigned 5545 GO terms from 690 cellular

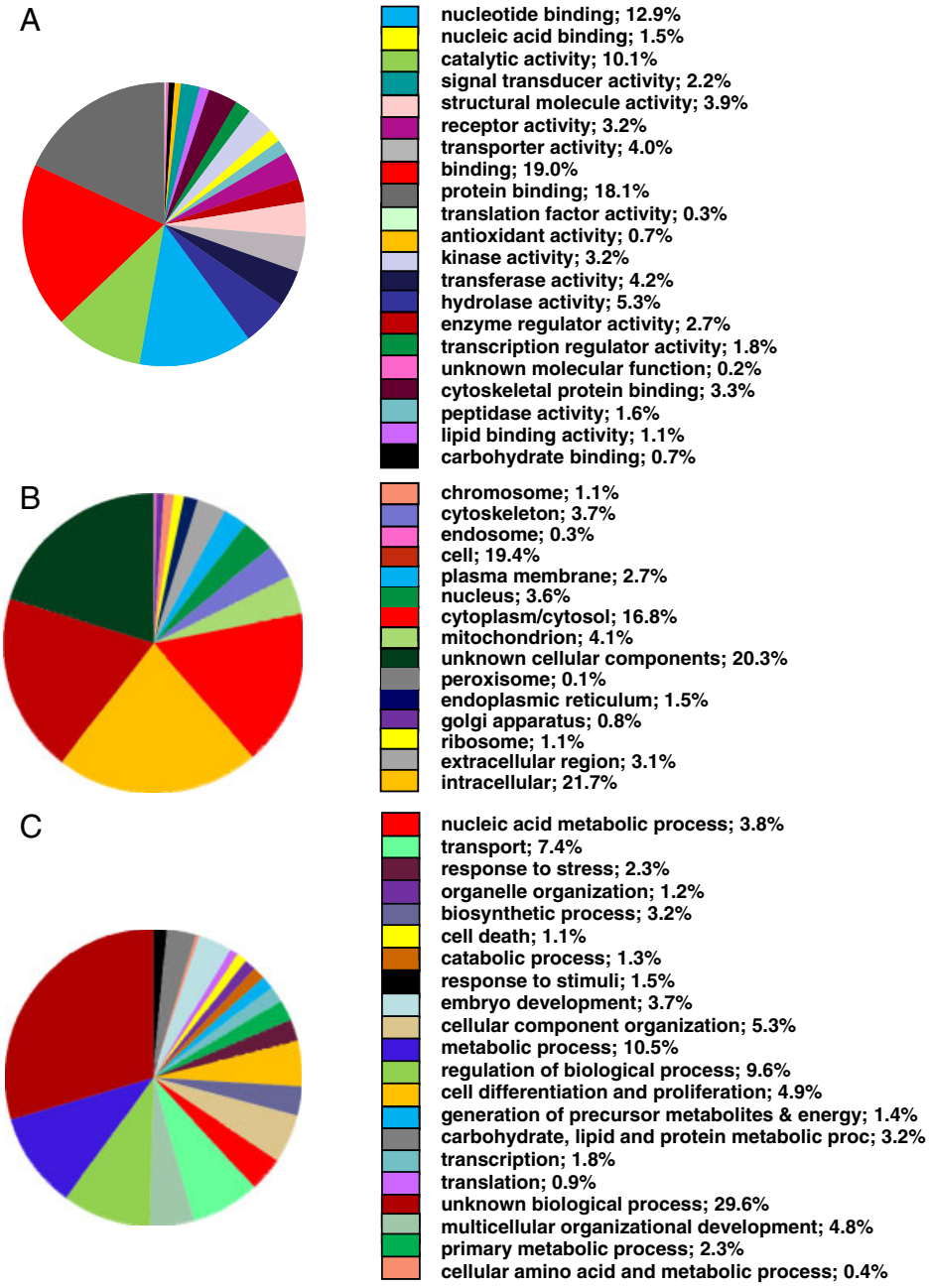

Figure 1. Classification of chicken pipping muscle proteins based on GO for molecular function (A), cellular component (B), and biological process (C). Numbers in percentages (%) correspond to the numbers of GO terms assigned for particular GO category.

functions, summarized into 21 GO categories (Fig. 1C). While 29.6% of GO terms represented unknown biological process, a large number of GO terms (21.5%) belonged to metabolism categories including metabolic process (10.5%), nucleic acid metabolism (3.8%), carbohydrate, lipid and protein metabolism (3.2%), primary metabolic process (2.3%), catabolic process (1.3%), and cellular amino acid and metabolic process (0.4%). Terms were also available for proteins associated with regulation of energy producing pathways (1.4%). These pathways include glycolysis, gluconeogenesis, tricarboxylic-acid cycle, and fatty acid oxidation. Common to most fast twitch muscles, the glycolytic pathway provides the energy for muscle growth and contraction. The rapid muscle fiber changes that occur in the pipping muscle during embryonic development suggest that the muscle possesses the mechanisms necessary for high energy production [6]. In addition, β-oxidation of fatty acids provides energy for the embryo during development [2]. Similarly, as the broiler embryo develops towards hatching, the glycogen and protein concentrations of the pipping muscle increase [10]. Other abundantly represented proteins detected by our experiment are in the category of transport

proteins (7.4%). These large protein complexes are integral membrane proteins that have multiple trans-membrane domains and facilitate transport of diverse molecules between cellular compartments. These proteins act as electron carriers and channels [14].

Transcription (1.8%) and translation (0.9%) regulation categories were present in small numbers, as well. GO terms were also available for proteins involved in regulation of biological process (9.6%), biosynthetic process (3.2%), as well as proteins associated with various developmental processes, such as multicellular organizational development (4.8%), embryo development (3.7%), cellular component organization (5.3%), organelle organization (1.2%), cell differentiation and proliferation (4.9%), and cell death (1.1%). Approximately 4% of terms were assigned to proteins responsive to stimuli (1.5%) and stress (2.3%). Heat shock proteins dominated this category. These proteins are likely expressed in the pipping muscle to enable the embryo to cope with high incubational temperature and humidity as well as the hatching process. Apoptotic changes have been described in the neck muscles of the chicken embryo [18]. This muscle cell degeneration is a part of the normal sequence of changes in muscle physiology that occurs in the chicken embryo on Days 10 through 15 of incubation [18]. Programmed cell death involves a proteolytic cascade controlled by a family of dedicated intracellular regulatory proteins [5]. The presence of the cell death proteins is suggests that apoptotic changes occur in the pipping muscle as a predictable physiological process.

Data for all identified proteins can be found in the PRIDE database (http://www.ebi.ac.uk/pride; experiment number 14845).

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