Published in final edited form as:

Mol Genet Metab. 2007 November ; 92(3): 249–257. doi:10.1016/j.ymgme.2007.07.003.

Analysis of the Liver Soluble Proteome from Bull Terriers Affected with Inherited Lethal Acrodermatitis

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Abstract

Lethal acrodermatitis (LAD) is a genetic disease affecting bull terrier dogs. The phenotype is similar to that for acrodermatitis enteropathica in humans, but is currently without treatment. The purpose of the research presented here is to determine the biochemical defects associated with LAD using proteomic methodologies. Two affected (male and female) and one unaffected (male) bull terrier pups were euthanized at 14 weeks of age, their livers dissected and prepared for two-dimensional gel electrophoresis (2DE) and densitometry. Approximately 200 protein spots were observed. The density of the spots within each gel was normalized to the total spot volume of the gel; only those soluble liver protein spots that were consistently different in both of the livers of the affected pups compared to the unaffected pup were excised manually and submitted for MALDI mass spectrometry. Thirteen proteins were identified as differentially expressed in the affected, compared to the unaffected, pups. The proteins were involved in numerous cellular physiological functions, including chaperones, calcium binding, and energy metabolism, as well as being associated with the inflammatory response. Of note were haptoglobin, glutamine synthetase, prohibitin and keratin 10 which exhibited at least a 4-fold level of differential expression. These data represent the first proteomic analysis of this mutation. The differentially expressed proteins that were identified may be key in understanding the etiology of LAD, and may lead to diagnostic tools for its identification within the bull terrier population.

Introduction

Lethal acrodermatitis (LAD) is a rare genetic disease that affects bull terrier dogs [1, 2]. There is very little research that has focused on identifying the biochemical defect associated with this disease. Identifying the defect would be beneficial in order to more rapidly identify those bull terrier pups affected by the disease, and potentially identifying those dogs which are heterozygous carriers of the mutation. Furthermore, understanding the cause of LAD may benefit the community of researchers that are involved in defining the molecular basis for cellular zinc homeostasis.

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The characteristics of LAD include growth retardation, acrodermatitis, purulent skin disease and inflammation of the folds of tissue around the nail. Affected pups can also exhibit abnormal behavior and diarrhea, though not all of these symptoms are present in all afflicted pups [1, 3]. The current diagnostic criteria for LAD includes a combination of these features in which onset occurs at an early age [3]. One study found that five affected pups exhibited low plasma zinc levels [1], though others found that zinc levels were not significantly different from dogs with atopic dermatitis [3]. The activity of alkaline phosphatase, a zinc-dependent enzyme, was also not consistently diminished in LAD pups [1, 3]. Consequently, blood zinc levels and zinc-dependent enzyme activity levels are of limited value when diagnosing this disease in pups. Nevertheless, the clinical symptoms are consistent with those observed in severely zinc-deficient animals.

The zinc deficiency symptoms are also consistent with those observed in zinc-responsive dermatosis in dogs [4, 5], acrodermatitis enteropathica in humans [6–8], and lethal trait A46 in Black Pied Danish cattle [9, 10]. These clinical symptoms include dermatitis, alopecia, anorexia, growth retardation, gastrointestinal dysfunction, defective T-lymphocyte function, and atrophy of the thymus and lymphoid tissues [1]. Both acrodermatitis enteropathica (45 mg zinc daily) [11] and lethal trait A 46 (14 g zinc acetate every other day) [12] can be treated with oral zinc supplementation, resulting in the amelioration of the clinical symptoms. However, treatment of bull terriers affected by LAD with 100 mg zinc sulfate twice-a-day was ineffective in ameliorating their symptoms. Increasing the oral dose to 880 mg zinc sulfate twice-a-day resulted in a partial improvement in the skin lesions. Parenteral treatment of the affected pups was unsuccessful as daily intravenous administration of 3 to 5 mg zinc sulfate in sterile saline caused vasculitis and intraperitoneal administration of the same solution resulted in granuloma formation [1]. Both acrodermatitis enteropathica in humans and lethal trait A 46 in cattle are caused by mutations in SLC39A4 [10, 13] whereas the cause of LAD has not been identified.

We hypothesize that the genetic defect affects more than the intestinal zinc uptake/absorption mechanism since the affected dogs were unresponsive to zinc therapy. The results reported here indicate that the LAD mutation affects basic metabolic functions within the livers of the affected dogs. The primary defect remains obscure. Several proteins were identified that exhibited over 2-fold reduced expression in the soluble fraction of the LAD liver; many were associated with the cellular response to oxidative stress.

Materials and Methods

Three bull terrier puppies ranging from 8–12 weeks of age were referred to the Section of Medical Genetics at the Veterinary School of the University of Pennsylvania. All dogs were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. According to their owner the affected pups had a slightly lighter hair coat and were weaker and smaller than their normal littermates at birth. At weaning they had trouble nursing and later chewing and swallowing their food, which would become stuck in the high palatal arch. By 6 weeks of age, the bull terriers were smaller than their unaffected littermates and began showing a wide stance of the forelimbs and splayed toes. At about 8–10 weeks of age, the diluted color of their hair coat became more apparent and behavior changes (short-tempered, aggressiveness) became noticeable. The affected dogs, and one unaffected littermate, were euthanized at 14 weeks of age. In addition to the skin lesions, gross postmortem examination revealed thymic hypoplasia, bilateral cataracts and mild hydrocephalus. Histopathologic changes included mild bronchopneumonia, lymphoid depletion/hypoplasia in multiple organs including the spleen, intestinal Peyer's patches, the thymus and lymph nodes. Affected pups also exhibited pancreatic atrophy and mild enterocolitis. No significant inflammatory or degenerative changes were present in the liver.

The livers and other tissues (jejunum, kidney) were dissected from these dogs, flash-frozen in liquid nitrogen and stored at -80°C .

Zinc and Copper Analysis

Approximately 5 g of liver tissue was pulverized in liquid nitrogen and stored at -80°C until use. Zinc and copper concentrations of control and LAD livers were measured using flame atomic absorption spectrometry (Analytical Chemistry Laboratory, University of Georgia; Shimadzu AA6800, Columbia, MD). Pulverized liver samples were weighed and placed in acid-washed crucibles. The samples were ashed by drying in an oven 12 hr at 95°C , then placed in a muffle furnace at 450°C for 32 hr. After cooling to room temperature overnight, 0.5 mL concentrated HCl was added to the ash and incubated for 60 min at room temperature. The samples were diluted with deionized distilled water to a total volume of 5 mL, then the zinc and copper levels were assayed by flame atomic absorption.

Sample Preparation for Two-Dimensional Gel Electrophoresis (2DE)

Pulverized liver samples were processed using the Soluble/Insoluble ReadyPrep Protein Isolation Kit (BioRad, Hercules, CA) following the instruction manual included in the kit. From the stock of tissue powder 100 to 200 mg was placed separately into three microfuge tubes containing protease inhibitor cocktail (Sigma, St. Louis, MO; 1.04 μmol AEBSF, 0.8 nmol aprotinin, 20 nmol leupeptin, 40 nmol bestatin, 15 nmol pepstatin A, 14 nmol E-64) and lysis buffer. The samples were then lysed by sonication on ice using three to four 30 sec pulses, and the samples were centrifuged at $13,000 \times g$ for 30 min at 4°C . The supernatant was carefully removed and contained the soluble protein fraction; these samples were stored at -80°C . Prior to isoelectric focusing, the RC/DC protein assay (Bio Rad) was used to estimate the protein content of the samples.

Two-Dimensional Gel Electrophoresis

Immobilized pH Gradient (IPG): 2DE was performed on each liver sample in triplicate. BioRad pH 3–10 IPG strips (17 cm) were used to separate the proteins from the cell lysate according to their isoelectric point. Passive rehydration of the IPG strips was used to load the sample. The rehydration buffer contained 6 M urea, 2 M thiourea, 2% CHAPS, 0.2% tributylphosphine (TBP) and 0.5% pH 3–10 ampholytes. The sample and rehydration buffer were mixed for a final volume of 300 μL per gel. The strips were run for 60,000 volt-hours.

Second Dimension SDS-PAGE: The IPG strips were equilibrated with 6 M urea, 30% glycerol, 2% SDS, 1.55 M Tris, 2 mM TBP, 0.5% bromophenol blue, for 25 min at room temperature. The strips were added to the top of 10% to 20% gradient acrylamide slab gels. The gels (22 cm $\times$ 22 cm $\times$ 1 mm) were run at 25 W/gel at 4°C until the dye front migrated within 1 cm from the bottom of the gel. The cathode buffer contained 50 mM Tris base, 384 mM glycine, 6.9 mM SDS. The anode buffer contained 25 mM Tris base, 192 mM glycine, 3.5 mM SDS. The gels were fixed in 40% methanol, 10% acetic acid. The gels were stained with colloidal Coomassie Blue (BioSafe stain, BioRad) to visualize the separated proteins. The images of the stained gels were digitized and the densities determined using Phoretix 2D software (Nonlinear Dynamics, New Castle upon Tyne, UK). Spot densities were normalized to the total spot volume within each gel from the affected or unaffected livers, and then the gels from unaffected and affected soluble liver electrophoretograms were compared. Protein spots that exhibited a 1.5-fold difference in expression were excised by hand and analyzed by mass spectrometry at the University of Georgia Proteomic Resource Facility (Athens, GA).

Mass Spectrometry

The excised protein spots were prepared for in-gel tryptic digestion [14]. Each gel plug was incubated with 150 μ l of 50 mM ammonium bicarbonate containing 12.2 M acetonitrile. The gels were dried and rehydrated with 5 μ l of 50 mM ammonium bicarbonate containing 0.5 μ g trypsin. This solution was added in 5 μ l aliquots until the gel piece regained its original size. The gel was then covered with 50 mM ammonium bicarbonate and incubated overnight at 30°C. After the addition of 1.5 μ l of 880 mM trifluoroacetic acid the peptides were extracted with 50 mM ammonium bicarbonate containing 14.6 M acetonitrile. The supernatants were dried to approximately 10 μ l. The samples were mixed with MALDI matrix solution prepared as 5 mg/ml α -cyano-4-hydroxycinnamic acid in a solution containing 1:1:1 acetonitrile, ethanol and 0.1% trifluoroacetic acid (pH 2.0). Each mixture was spotted on a MALDI target and allowed to air-dry. Peptide masses were obtained by MALDI-TOF/TOF MS (Applied Biosystems 4700 Proteomics Analyzer; Foster City, CA). Calibration was performed with the same procedure using mixtures of peptides with known molecular masses.

MS/MS ions produced by MALDI-TOF/TOF from the tryptic peptides and the results of a Mascot search (<http://www.matrixscience.com/>) were used to determine protein identification. The NCBI nr database, i.e. the non-identical nr protein database of the National Center for Biotechnology Information, was utilized for the search.

Western Blot of Prohibitin and Keratin 10

Antibodies against prohibitin (rabbit polyclonal) and keratin 10 (mouse monoclonal) were purchased from Abcam, Inc. (Cambridge, MA), and had been tested against, or were predicted to react with, their respective canine homologues. The soluble protein fraction from the liver was separated using 12.5% denaturing polyacrylamide gel electrophoresis on 7 cm $\times$ 10 cm minigels. Molecular weight markers (Rainbow high MW markers; GE Healthcare Bio-Sciences, Piscataway, NJ) were used to monitor the separation as well as the protein transfer. Following electrophoresis, the proteins were transferred to PVDF membranes for one hour at 200 mA constant current. The membranes were placed in blocking solution (3% nonfat dry milk in TBS (137 mM NaCl, 10 mM Tris, pH 7.4)). The membranes were probed separately for 24 hr with either the anti-prohibitin or anti-keratin 10 antibodies diluted 1:200 or 1:500, respectively, in 1% nonfat dry milk in TBS. The membranes were washed with TBS (4x, 5 min each at room temperature) and goat anti-rabbit or goat antimouse secondary antibody conjugated to horseradish peroxidase was added using a 1:2000 dilution. The protein bands were visualized on film with the ECL and Western Blot Detection System (GE Healthcare Bio-Sciences). The densities of the bands were analyzed with the Phoretix 2D software (Nonlinear Dynamics).

Results and Discussion

The affected bull terrier pups used in this study exhibited the phenotype consistent with a lethal acrodermatitis diagnosis and with systemic zinc deficiency [1–3]. Skin lesions evolved from areas of moderate hyperkeratosis to severe hyperkeratosis with secondary bacterial and yeast infections. The rostral dorsal muzzle, lips and periocular areas had thick adherent scale with brown yellow crusts (Figures 1A and B). Similar but slightly less severe lesions were located on the distal pinna and concave pinnal surfaces. The skin of distal limbs had severe scale/crusts with brown discoloration. The dorsal carpi and tarsi were severely thickened. The claw folds were swollen, reddened and nails were discolored reddish-brown (Figure 2A). The footpads had marked villous hyperkeratosis with interdigital erythema, crusts and scaling. From 6 weeks to 12 weeks, the interdigital spaces had become so progressively swollen that the margins of the footpad and adjacent haired skin were difficult

to distinguish (Figure 2B). At 12 weeks of age computer tomography of the head was performed under general anesthesia and revealed mild hydrocephalus in the affected dogs. At the same time, two 6-mm skin biopsies were obtained from affected areas. The histopathologic findings consisted of severe and diffuse parakeratotic hyperkeratosis with epidermal hyperplasia. The thickened corneal layer formed spire-like projections at follicular ostia (Figure 3A). Multifocally, small regions of pallor (intracellular edema) were found in the stratum spinosum (Figure 3B).

The genetic defect that causes LAD has not been identified to date, but defects in the zinc and/or copper metabolic pathways are suspected. Livers from the LAD pups contained less copper than the control (control: 37.23 nmol Cu/g tissue; LAD: 11.87 nmol Cu/g tissue, 19.16 nmol Cu/g tissue). The LAD livers also contained more zinc than the control (control: 23.5 nmol Zn/g tissue; LAD: 34.43 nmol Zn/g tissue, 44.36 nmol Zn/g tissue). Previous investigators have reported both zinc and copper levels to be lower in LAD liver and serum [15]. The difference in the relative amounts of liver zinc in this current study may be associated with the age of the dogs when the tissue was acquired – Uchida et al [15] used dogs eight to 108 months of age while the age of the pups in this current study was 14 weeks of age. Increases in liver zinc occur with stress and infection, and are associated with cytokine- or hormone-induced increases in metallothionein [16, 17]. Stress also causes the efflux of liver copper associated with the synthesis and release of ceruloplasmin [17, 18]. Although the liver metallothionein and serum ceruloplasmin levels were not measured, the increased zinc and reduced copper levels in the affected livers is consistent with a normal response to stress. However, the compartmentalization of zinc in the LAD liver may also be defective, thereby increasing its susceptibility to oxidative stress.

The proteomic analysis of the soluble proteome of the liver was performed to provide insight into this disease by identifying proteins affected by the mutation. Approximately 200 proteins were visualized in the gels following staining with colloidal Coomassie blue. Thirteen differentially expressed proteins were identified by mass spectrometry (Figures 4–7). The identities of the differentially expressed proteins are shown in Table 1. Twelve proteins were lower, and two were higher, in the LAD livers. The proteins that were affected by LAD could be classified into six general biochemical functions: chaperones (calreticulin, prolyl-4-hydroxylase, 75 kDa glucose regulated protein, prohibitin), calcium binding (calreticulin, regucalcin), cellular metabolism (α -enolase, aldehyde dehydrogenase, glutamine synthetase, glutamate dehydrogenase 1), structure (prolyl-4-hydroxylase, ER-60, keratin 10), iron/hemoglobin binding (haptoglobin). The current data do not support the hypothesis that any of these proteins are the primary defect for LAD. The observed changes in the LAD liver subproteome are more likely to be associated by disease progression caused by the primary defect.

Many of the proteins that exhibited decreased expression in the LAD liver are reported to be affected by treatments that induce reactive oxygen species. Histopathology of the liver did not reveal significant inflammatory or degenerative changes; however early biochemical changes responding to oxidative stress or inflammation may have occurred as changes in LAD liver zinc and copper levels relative to the control liver were observed. The discussion that follows will focus on those proteins affected by the LAD mutation that have been reported to also be affected by oxidative stress and/or inflammation.

The 75 kDa glucose regulated protein, a mitochondrial chaperone, exhibited a two-fold decrease in the LAD livers. The protein level of this chaperone increased in human cystic fibrosis bronchial tissue [19]. It was also observed to increase in rat liver as a function of diabetes and oxidative stress [20], as well as following the initiation of apoptosis [21].

Prolyl 4-hydroxylase also exhibited a two-fold decrease in protein levels in LAD livers. This enzyme functions in hypoxia-induced proline hydroxylation of collagen, the gene controlled by hypoxia-induced factor 1 [22]. This enzyme contains a tetramer that also contains protein disulfide isomerase [23]. Reactive oxygen species impairs prolyl hydroxylase activity [24], though any effects of reactive oxygen species on protein synthesis has not been reported. We propose that defective zinc compartmentalization in the LAD liver caused an increase in reactive oxygen species, including hydrogen peroxide [25], that would impair both the synthesis and activity of prolyl hydroxylase.

ER-60 protein, reduced in the LAD liver, is involved in major histocompatibility complex class I assembly [26]. Proteomic analysis of liver from an insulin-resistant hamster model indicated ER-60 and glutamate dehydrogenase protein expression decreased with the onset of insulin resistance [26]. Other investigators have observed that oxidative stress reduces the expression of α -enolase [27] and aldehyde dehydrogenase [28]. Levels of reactive oxygen species have not been measured in LAD, nor has there been a determination of insulin resistance in these animals. Nevertheless, the changes in the expression levels of the proteins reported here support the thesis that the LAD defect affects pathways involved in protection from oxidative stress.

Keratin 10 expression was reduced in the LAD liver (Figure 4; Table 1). Zinc deficiency increases keratinolytic activity in a rat buccal epithelium model [29], and may do so in the dog as well. There are several diseases in dogs and cattle that affect keratinization [30, 31]. The estimated molecular weight of the spot identified by mass spectrometry as keratin 10 was 9.9 kDa (Table 1). Since the molecular weight of keratin 10 is reported to be approximately 55 kDa, we suspect that it is a fragment. No immunoreactive bands at 55 kDa or 9.9 kDa were observed following western blotting of soluble proteins from the unaffected and affected liver (unpublished results). Its presence in the liver has not previously been reported and the western blotting results confirm this.

The increase in haptoglobin in LAD liver suggests that an acute phase response to inflammatory stimuli has occurred [32]. This result is consistent with the data from the zinc and copper analysis showing that liver copper was reduced, and liver increased, in LAD pups compared to their control. Prohibitin is reduced in the LAD liver, indicative of the presence of oxidative stress [33]. Prohibitin is located in the mitochondria, and was initially identified as an inhibitor of cellular proliferation [35]. Recently it was found to be secreted by adipocytes [36] and is present in the circulation [37]. Western blotting for prohibitin in the livers of unaffected and affected pups indicated a single band at approximately 30 kDa (Figure 8). Levels of prohibitin as measured by immunoblotting were higher in the unaffected liver, which was consistent with the results from 2DE.

This is the first report that used proteomic techniques to study the biochemical defects associated with the LAD mutation. The data have not identified the mutation for LAD; however the results suggest that the phenotype is due to defective zinc metabolism that affects the inflammatory response. As an antioxidant trace element, zinc deficiency is associated with increased susceptibility to oxidative stress [25]. Other observations that support the hypothesis that LAD is a zinc-deficiency disease include the presence of skin lesions and the postmortem examination of the organs, including the thymic hypoplasia. We acknowledge that the number of dogs used in this study is very low, and more animals will be needed to confirm the results reported here. Future experiments need to assay for prohibitin in LAD serum/plasma. If it exhibits greater amounts in the unaffected bull terrier pup serum/plasma using immunoblotting, prohibitin may provide an additional biochemical measure for identifying the disease in the bull terrier population.

Acknowledgments

This work was supported by ACORN No. 584-A from the Canine Health Foundation (A.G.) and the National Institutes of Health NCRR 0215 (M.L.C.).

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Figure 1.

1A and 1B: Bull terrier, Lethal acrodermatitis- facial features. 1A: 8-week-old puppy with coalescing thick scale and crusts on rostral muzzle and around lips. The affected dog has a dome-shaped cranium. 1B: 14-week-old-puppy. The rostral muzzle has similar lesions as 1A with mild erythema and hair loss. The nasal planum is hyperkeratotic. There is mild periocular scaling and alopecia.

Figure 2.

2A and 2B: Bull terrier 14 weeks old, Lethal acrodermatitis- severe footpad hyperkeratosis, interdigital pyoderma and paronychia. 2A: The dorsal surface of the paw had diffuse erythema with thick adherent scale and crusts. Note the marked thickening of the claw fold. The nails are discolored. 2B: The plantar surface of the foot is markedly swollen with severe hyperkeratosis and crusting of both the pawpad and interdigital skin.

Figure 3.

3A and 3B: Histopathology. Bull terrier 14 week old, Lethal acrodermatitis. 3A: The histopathologic changes consist of severe and diffuse parakeratotic hyperkeratosis with epidermal hyperplasia H&E 4X. 3B: Higher magnification of 3A. There is a focal area of pallor in the spinous layer due to intracellular edema. H&E 10X.

Figure 4. Differentially expressed spots 1–4 from the soluble liver protein fraction following 2DE and staining with colloidal Coomassie blue G250. The control images are from three replicates of the unaffected pup. LAD 1 and 2 columns contain replicates from each of the two affected pups. The calculated molecular weight and isoelectric points of the spots are listed in Table 1.

Figure 5.

Differentially expressed spots 5–9 from the soluble liver protein fraction following 2DE and staining with colloidal Coomassie blue G250. The control images are from three replicates of the unaffected pup. LAD 1 and 2 columns contain replicates from each of the two affected pups. The calculated molecular weight and isoelectric points of the spots are listed in Table 1.

Figure 6.

Differentially expressed spots 10–12 from the soluble liver protein fraction following 2DE and staining with colloidal Coomassie blue G250. The control images are from three replicates of the unaffected pup. LAD 1 and 2 columns contain replicates from each of the two affected pups. The calculated molecular weights and isoelectric points of the spots are listed in Table 1.

Figure 7. Differentially expressed spot 13 from the soluble liver protein fraction following 2DE and staining with colloidal Coomassie blue G250. The control images are from three replicates of the unaffected pup. LAD 1 and 2 columns contain replicates from each of the two affected pups. The calculated molecular weight and isoelectric point of the spot are listed in Table 1.

Figure 8.

Immunoblot of soluble liver protein fraction from one unaffected and LAD pup. The 12.5% acrylamide gel was loaded with 5 µg and 50 µg of protein. Following western transfer, the PVDF membrane was probed for prohibitin using a rabbit polyclonal antibody. The values under the bands represent relative normalized volume of each of the bands. The ratio of the Control vs. LAD – 5 µg: 13.7; 50 µg: 1.79.

Table 1

Differential Protein Expression from Unaffected and LAD Liver.

Gel Spot #	Protein ID	Exp MW (kDa) ^a	Exp pI ^b	MW	pI	Accession #	Control ^c	LAD ^d	Control:LAD
1	calreticulin	91.0	4.5	47.9	4.29	gi 73986468	37.9 ± 2.7	18.3 ± 2.6	2.1
2	prolyl-4-hydroxylase	79.1	5.2	57.8	4.86	gi 73964749	156.2 ± 13.2	71.4 ± 8.6	2.2
3	75 kDa glucose regulated protein	101.5	6.0	73.2	5.93	gi 73970894	83.5 ± 5.4	39.0 ± 8.2	2.1
4	ER-60 protein	86.9	6.1	57.3	5.88	gi 2245365	49.8 ± 13.1	23.4 ± 2.3	2.1
5	aldehyde dehydrogenase	76.7	6.3	57.3	6.63	gi 73995214	66.2 ± 7.4	41.6 ± 7.3	1.6
6	glutamine synthetase	60.6	6.7	42.6	6.28	gi 50950189	30.4 ± 3.4	nd	----
7	α-enolase	72.0	7.0	43.1	6.56	gi 73956736	66.8 ± 4.6	42.0 ± 7.6	1.6
8,9	glutamate dehydrogenase 1	73.1	7.3	53.7	8.06	gi 73984130	184.7 ± 16.6	82.2 ± 4.3	2.2
10	hatogloblin	20.0	5.6	36.9	5.72	gi 123511	111.0 ± 2.2	46.8 ± 9.9	0.24
11	regucalcin	46.5	5.8	38.3	6.46	gi 74007343	122.9 ± 11.7	74.9 ± 8.5	1.6
12	prohibitin	33.5	6.0	29.9	5.57	gi 49456373	56.2 ± 2.3	13.9 ± 3.7	4.0
13	keratin 10	9.9	6.5	59.1	5.09	gi 40354192	42.0 ± 3.2	9.2 ± 1.8	4.6

^aThe experimental molecular weight was estimated using high and low molecular weight standards.

^bThe experimental isoelectric point.

^cThe average normalized spot volume ± SEM from three replicates.

^dThe average normalized spot volume ± SEM from three replicates each of two LAD livers.