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Proteome comparative analysis of gynogenetic haploid and diploid embryos of goldfish (*Carassius auratus*)

Recently, it was found that in the gynogenetic haploid and diploid embryos of goldfish, which have exactly the same genome, the haploid condition results in obstruction of gene expression and abnormal development while the diploid embryos have normal gene expression and development. A diploid-dependent regulatory apparatus was proposed to regulate gene expression. To study the difference at the protein expression level of the embryos of haploid and diploid in development, we extracted the total proteins of both the gynogenetic haploid and diploid embryos of goldfish in the same eye formation stage. Two-dimensional polyacrylamide gel electrophoresis was used to separate proteins. The stained gel images were analyzed with the PDQUEST software. A part of protein spots that were differentially expressed in haploid and diploid embryos were identified by matrix assisted laser desorption/ionisation-time of flight-mass spectrometry and database analysis. Sixteen protein spots that were absolutely different (only expressed in diploid embryos but not in haploid embryos or *vice versa*) and 16 protein spots that were up- and downregulated were identified unambiguously, which include some proteins that are correlative with eyes development, nerve development, developing regulation, cell differentiation, and signal transduction. The different significantly gene expression during embryos developing between diploid and haploid is demonstrated.

Keywords: Goldfish embryo / Mass spectrometry / Proteome analysis / Two-dimensional gel electrophoresis PRO 0553

1 Introduction

The precise spatial and temporal pattern of animal development is controlled by a strikingly complex genomic regulatory system. A diploid development regulatory mechanism of gene expression during embryonic morphogenesis of goldfish has recently been determined by Luo *et al.* [1]. It was found that some gynogenetic haploid embryos of the goldfish could develop to the hatching stage but none of them survived beyond the feeding stage. In general, almost all the organs showed abnormality in a gynogenetic haploid group, although some organs of a given individual of the gynogenetic group appeared normal. For example, normal and abnormal eyes were all observed in gynogenetic haploid embryos and the ratio of the embryos with normal eyes to that with abnormal eyes in every test was about 1:3. According to the statistical analysis, Luo *et al.* proposed two regulator genes in a set of chromosomes in charge of spatial pattern arrangement of eye and every copy of the two genes in a set of chromosomes had 1/2 probability to be selected for expression. Only the type in which the two genes both were selected for expression wound develop normal eyes. In haploid embryos there was only one-fourth of probability for the two genes to be selected, while in diploid embryos the probability was 100%. The further mechanism of the diploid-dependent regulatory apparatus for the goldfish embryos development is under investigation. In order to get more information at the protein expression level of the haploid and diploid embryos in development and to identify the important proteins associated with the embryonic development, we extracted the total proteins of both the gynogenetic haploid and diploid embryos of goldfish in a same eye formation stage, and the differential proteome expression analysis was performed by using 2-DE and MALDI-TOF-MS. In this work, we describe the identification of the differential expressed proteins in the development of haploid and diploid goldfish embryos.

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2 Materials and methods

2.1 Chemicals and animals

Immobilized pH gradient (IPG) DryStrips (3–10 linear), IPG buffer (3–10 linear), Cover fluid, agarose, and Silver Staining Kit were purchased from Amersham Pharmacia-Biotech (Uppsala, Sweden). DTT, iodoacetamide, potassium ferricyanide, trypsin (Proteomics sequencing grade), α-cyano-4-hydroxycinnamic acid (CHCA), and TFA were obtained from Sigma (St. Louis, MO, USA). Acrylamide, bis-acrylamide, urea, glycine, Tris, CHAPS, and SDS were from Amresco (Solon, OH, USA). ACN a is domestic product (chromatogram-grade). Other chemicals are domestic products (analytical-grade). A red cup inbred strain of goldfish, *Carassius auratus,* obtained from a goldfish breeding farm, was employed in this study. In each experiment, all the eggs used both in tests and in controls were obtained from a single spawning. Common carps, *Cyprinus carpio,* were obtained from a local fish farm.

2.2 Production and identification of gynogenetic haploid embryos

Haploid gynogenes of the goldfish were induced using UV-irradiated sperm of common carp [2]. The goldfish has a specific double tail encoded by a recessive gene, while the common carp has a common single tail encoded by a dominant allele. The tails of all the hybrid embryos of the goldfish and common carp are all single tails just like the tail of the parent common carp (Luo, unpublished observations). With this character, the gynogenetic haploid embryos could be identified easily and unequivocally from the normal and abnormal hybrid embryos at an early developmental stage. Diploid control embryos were produced by fertilizing the eggs from the same spawning with normal sperm of the same inbred gold fish.

2.3 Protein sample preparation

Diploid and haploid embryos were collected in a same eye formation stage (26 h after fertilization). Embryos were kept in liquid nitrogen for some times immediately after weighing and were then grinded in a mortar, suspended in lysis buffer containing 8 M urea, 4% CHAPS, 60% mM DTT, 2% Pharmalyte 3–10, 1.4 mg/mL PMSF, and centrifuged for 30 min at 12 000 rpm at 4°C. The supernatant protein concentrations were determined using the Bradford assay. All samples were stored at −70°C prior to electrophoresis.

2.4 2-D electrophoresis

2-DE was performed essentially as described in [3]. The first dimension of IPG-DALT 2-DE was run on an IPG-phor isoelectric focusing system (Amersham Pharmacia Biotech). Diploid embryos and haploid embryos (approximated 400 μg proteins) were mixed, respectively, with a rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, pH 3–10L, 18 mM DTT, and a trace of bromophenol blue, to a total volume of 350 μL, and applied to IPG dry strips. (pH 3–10 L; 180 × 30 × 0.5 mm). After rehydration for 14 h, IEF was conducted automatically to a total of 36 kVh at 20°C. Following IEF separation, the gel strips were equilibrated for 2 × 15 min in an equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue. 0.2% DTT was added to the first equilibration buffer, and in the second equilibration buffer DTT was replaced with 3% iodoacetamide. The second-dimensional run was carried out on incontinuity SDS-polyacrylamide vertical slab gels with 1 mm thick, 15% separation and 4.8% stacking gels in a Bio-Rad Protein II electrophoresis apparatus. Stacking gel and separation gel were run at 12.5 and 25 mA/gel constant current, respectively. SDS marker for M_r calibration was added. After 2-DE, the gels were stained with silver nitrate.

2.5 Image acquisition and analysis

The silver-stained gels were scanned using Qinghua ultraviolet scanner in transmission model. Spot detection, quantification, and matching were performed using PDQUEST software Version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). A matchest consisting of 6 images, 3 for haploid embryos and 3 for diploid embryos was created, and one image from diploid embryos was selected as the matchset standard for spot matching. In order to correct for variability due to silver staining and reflect the quantitative variations of protein spot, the individual spot volumes were normalized by dividing their optical density OD values by the total (OD) values of all the spots present in the gel, and expressed as ppm. The significance of expression difference of protein between diploid embryos and haploid embryos was estimated by Student's *t*-test, $P < 0.05$. All statistical calculations were done with Microsoft Excel.

2.6 *In situ* digestion of proteins

The silver-stained protein spots were excised from preparative gels using a punch. Proteins were *in situ* digested as previously described [5–7]. Briefly, the spots were

washed three times with double-distilled water. The fresh solution containing 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ was used to decolor. The cysteine reduction and alkylation steps consisted of incubation first in 10 mM DTT/100 mM NH_4HCO_3 for 1 h at 57°C, then the liquid was replaced with the same volume of freshly prepared 55 mM iodoacetamide/100 mM NH_4HCO_3 solution for 30 min at room temperature in the dark. The gel pieces were dried again and rehydrated in 10 μL of 40 mM NH_4HCO_3 containing 10% acetonitrile and trypsin, for 45 min at 0°. The trypsin concentration used was 0.02 g/L. The excess liquid was removed and the pieces of gel were immersed in 40 mmol/L NH_4HCO_3 containing 10% acetonitrile at 37°C overnight. The digests were desalted with ZipTip™ (Millipore, Bedford, MA, USA) according to the manufacturer's instructions and subjected to analysis using MALDI-TOF-MS.

2.7 MALDI-TOF-MS of peptides

The tryptic peptide mixtures were eluted in 1 μL of a solution containing 5 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA), 60% acetonitrile, and 0.1% TFA. Then the solution was loaded on to a 96-well target plate. The samples were air-dried. Molecular weight information of peptides was obtained by using a MALDI-TOF-MS Voyager DE-STR from Applied BioSystems (Framingham, MA, USA), equipped with nitrogen laser and operating in reflector/delay extraction mode and manual acquisition control. All MALDI-MS spectra were internally calibrated using trypsin autodigestion peptides.

2.8 Database search

The peptide masses were input in software PeptIdent that can be obtained from http://www.expasy.ch. The database used for all searches was SWISS-PROT and TrEMBL. The database searches were performed using the following values: other vertebrate species, protein molecular weight range, and p*I* range, trypsin digest (two missed cleavage allowed), cysteines modified by carbamidomethylation, mass tolerance ± 50 ppm using internal calibration, The identification was based on four matching peptides and 15% coverage. Tryptic autolytic fragments and contamination were removed from the set of data used for database search.

3 Results and discussion

3.1 2-DE and the analysis of gel images

In the early experiments to investigate the 2-DE conditions we found that the relative molecular masses of the most proteins of the samples of the goldfish embryos were below 50 kDa. In order to isolate proteins more efficiently, the subsequent 2-DE was performed with 15% separation gel in the second dimension. We also compared the results of several loading amounts of the samples, which indicated that the best gel images were obtained when the amount of loaded protein was 400 μg. A typical 2-DE proteome pattern of the diploid and haploid embryos in a same eye formation stage (26 h after fertilization) is shown in Fig. 1. The resulting images were

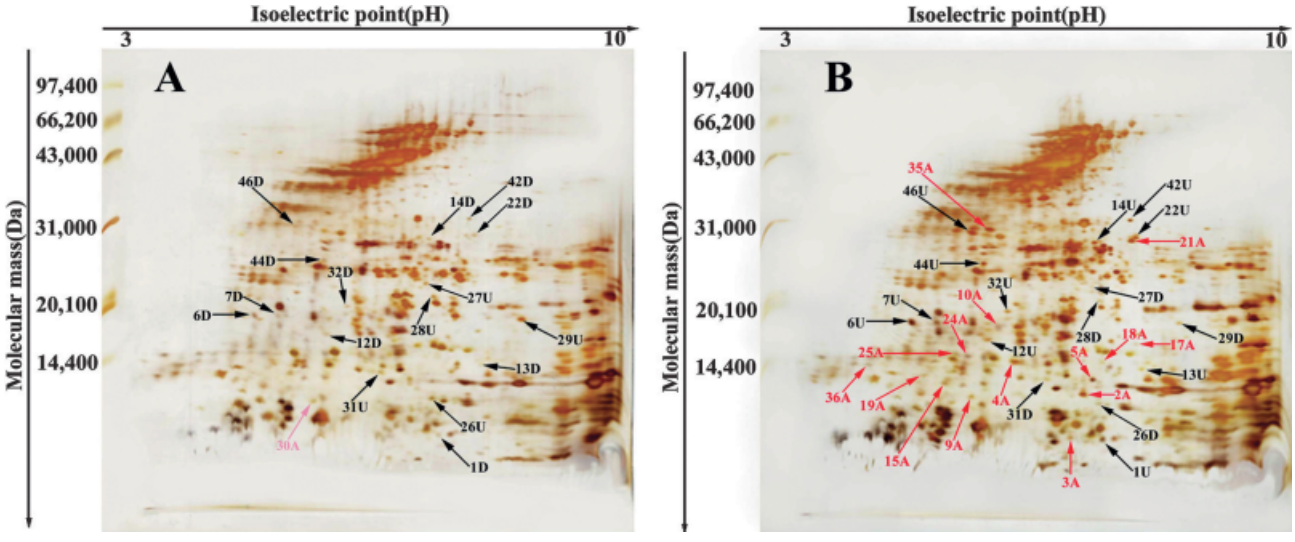

Figure 1. Silver-stained 2-D acrylamide gels of (A) gynogenetic haploid embryos and (B) diploid embryos. 400 μg of each sample was subjected to 2-DE and silver staining. Molecular weight markers are shown on the left. Blank arrows indicate the proteins that were up- and downregulated in haploid embryos, $p < 0.05$; red arrows indicate those that were detected only in diploid embryos, and the spot 30A with pink arrow was detected only in haploid embryos.

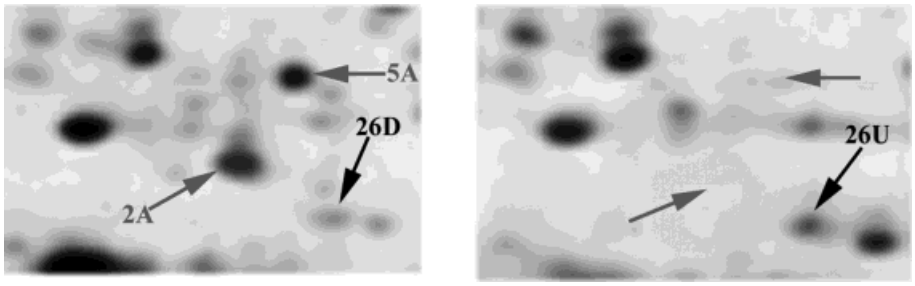

Figure 2. Magnified regions of 2-D images showing spot 2A and spot 5A, appearing in diploid embryos (left), while they were not detected at the corresponding position of haploid embryos (right); spot 26U was upregulated in haploid embryos (right) and downregulated in diploid embryos (left).

analyzed using PDQUEST software. It shows that most protein spots are distributed in the gel area of p*I* 5–10 and M_r 10–50 kDa. It also shows that there are more basic proteins than acidic ones. Under the same experimental conditions, 6 gels, 3 for diploid embryos and 3 for haploid embryos, were analyzed by the PDQUEST software. Average 577 and 655 protein spots were detected in haploid embryos and diploid embryos, respectively.

By gel matching and analysis, the proteomic profile of diploid embryos and haploid embryos are similar, as shown in Fig. 2. The average correlation coefficient between those two patterns is 0.58 by correlation analysis of gels. It is evident that there is significant difference in gene expression of haploid embryos compared to diploid embryos. In the 2-DE maps of the diploid and haploid embryos, about 50 protein spots were found different in abundance, including some up- or downregulated proteins and some absolutely differential protein spots (only expressed in diploid embryos but not in haploid embryos or *vice versa*).

3.2 MS analysis and protein identification

Forty-six protein spots, differentially expressed between diploid embryos and haploid embryos, were analyzed using MS and database searching with Internet. A representative peptide mass fingerprinting map of spot 36A that was only detected in diploid embryos is shown in Fig. 3. Thirty-two protein spots were identified unambiguously. The other 14 protein spots failed to be identified

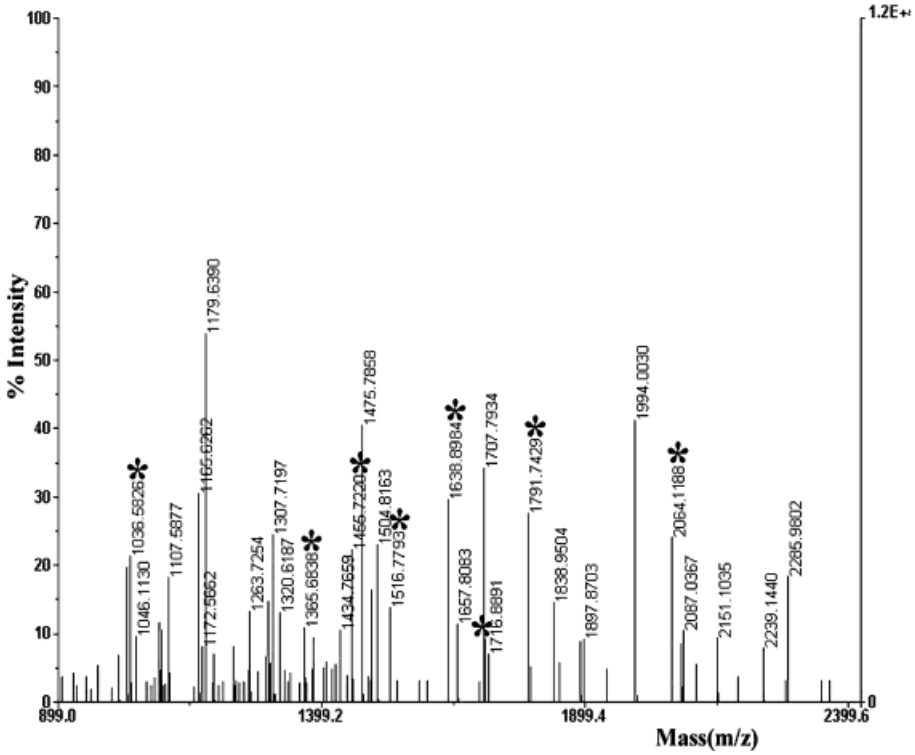

Figure 3. MALDI-TOF mass spectrum of spot 36A. The sample solution (1 μL) was deposited onto a 96-well target plate, and the solvent was removed by air-drying at room temperature. The peptide mixture was analyzed by MALDI-TOF-MS. Peaks matched in w>database search are marked with asterisks.

due to weak MALDI-TOF mass spectra or database limitation for the unknown proteins. Table 1 lists 16 identified proteins that are up- and downregulated in diploid embryos compared to that of haploid embryos ($P < 0.05$) by statistical analysis. Table 2 lists 15 proteins that were detected only in the 2-DE map of diploid embryos and 1 protein only in that of haploid embryos. All identified differential protein spots are shown in Fig. 1. The proteins detected only in diploid embryos were labeled with red arrows and the spot detected only in haploid embryos

Table 1. Up- and downregulated proteins in haploid embryos compared to diploid embryos of goldfish

Spot No.	Protein description	Accession No.	Species	M_r (kDa)	p*I*	Protein mean abundance (ppm) HE/DE[a)]	Protein alteration[b)]	*P*-value ($P < 0.05$)	Peptides matched	Coverage
1D	SC: dZ175P 12.4 (novel protein similar to immune-type receptors) (fragment)	Q8UV93	Zebrafish	Undefined	Undefined	0.70	↓	0.04	6	53.3%
6D	Vitellogenin (fragment)	Q9PTM8	Large-mouth bass	Undefined	Undefined	0.45	↓	0.01	7	44.2%
7D	ZFX type gene (fragment)	O42613	Natrix domestica	Undefined	Undefined	0.27	↓	0.02	6	50.8%
12D	Zinc finger protein zic1 (fragment)	Q9PVD6	Zebrafish	Undefined	Undefined	0.43	↓	0.03	5	34.4%
13D	ZNF6 protein (fragment)	Q42379	Zebrafish	Undefined	Undefined	0.31	↓	0.02	8	50.8%
14D	Ephrin-type A receptor 4 (EC 2.7.1.112) (tyrosine-protein kinase receptor ZEK2) (fragment)	O13148	Zebrafish	Undefined	Undefined	0.19	↓	0.01	7	41.3%
22D	Insulin-like growth factor binding protein 1	Q90YJ7	Zebrafish	28.1	7.82	0.14	↓	0.04	4	24.4%
42D	Pituitary specific transcription factor (fragment)	O42501	Common carp	Undefined	Undefined	0.35	↓	0.03	6	26.3%
44D	Wnt-1 (fragment)	Q9PT79	Medakafish	Undefined	Undefined	0.41	↓	0.02	5	32.4%
46D	G1/S-specific Cyclin D1	Q90459	Zebrafish	33.0	5.48	0.27	↓	0.01	5	29.6%
32D	γ-Crystallin M2–1	Q31555	Teleostfish	21.1	6.93	0.35	↓	0.04	4	36.0%
26U	Homeobox protein Hox-C6 (fragment)	P15862	Zebrafish	Undefined	Undefined	1.96	↑	0.01	5	53.1%
27U	One-eyed pinhead long form protein	O57516	Zebrafish	21.1	8.32	2.86	↑	0.002	7	36.8%
28U	Pbx2 (fragment)	Q9IAB9	Zebrafish	Undefined	Undefined	2.38	↑	0.03	4	24.9%
29U	Ubiquitin-conjugating enzyme 9–2	Q9DDJ0	Zebrafish	17.9	8.87	3.85	↑	0.001	5	42.7%
31U	LIM protein (fragment)	Q9W6K4	Zebrafish	Undefined	Undefined	1.72	↑	0.04	5	64.4%

a) Protein mean abundance, calculated from three gels for each sample; HE, haploid embryos; DE, diploid embryos
b) ↑, Upregulated in haploid embryos; ↓ downregulated in haploid embryos

Table 2. Absolutely different proteins (only expressed in diploid embryos but not in haploid embryos or *vice versa*)

Spot No.	Protein description	Accession No.	Species	M_r (kDa)	p*I*	Peptides matched	Coverage
2A	Vsx1 transcription factor (fragment)	Q9I9A4	Medakafish	Undefined	Undefined	7	59.1%
3A	Chain1:glycoprotein hormones α-chain	P27794	European freshwater eel	10.6	8.57	6	57%
4A	Vitellogenin (fragment)	Q8UWG2	Atlantic salmon	Undefined	Undefined	8	62.9%
5A	Vitellogenin receptor (fragment)	Q8UWF5	Atlantic salmon	Undefined	Undefined	4	87.9%
9A	Splice short isoform of gene enhance protein ISL-2A	P53408	King salmon	12.6	6.77	9	70.4%
10A	Chain 1: ovulatory protein-2	Q98988	Brook trout	25.1	6.97	6	38.8%
15A	Chain 1: gonadotropin β-chain	P27767	European freshwater eel	12.8	5.55	4	30.2%
17A	Gonadotropin α-subunit (fragment)	Q90287	Goldfish	Undefined	Undefined	4	24.7%
18A	β-crystallin S (γ-crystallin S)	P10112	Common carp	20.6	6.49	7	58.4%
19A	Gonadotropin I β-subunit 2	Q9YIB3	Goldfish	14.4	4.88	5	52.3%
21A	Chain1: insulin-like growth factor binding protein 2	Q8UVU5	Gilthead sea bream	29.5	8.27	5	29.5%
24A	DMRT2 protein (fragment)	Q9DG18	Salmo gairdneri	Undefined	Undefined	4	67.4%
25A	Granulin 1	Q90ZD0	Zebrafish	16.1	5.43	5	28.6%
	Vitellogenin receptor (fragment)	Q8UWF5	Atlantic salmon	Undefined	Undefined	4	74.2%
30A	chain1: LH-BETA subunit	Q90W19	Atlantic halibut	13.0	5.00	4	56.5%
35A	Odorant receptor 10.8 (fragment)	Q9PW84	Goldfish	Undefined	Undefined	8	43.0%
36A	WNT-8like (fragment)	O42121	Medakafish	Undefined	Undefined	8	46.5%

Protein spot 30A was detected only in haploid embryos and others were detected only in diploid embryos.

was labeled with pink arrows. The other up- and downregulated spots were marked with black arrows. Obviously, there were more proteins which are expressed or upregulated in the diploid embryos than that of haploid embryos.

3.3 Analysis of the identified differential proteins

Identified proteins listed in Table 1 were differentially expressed between diploid embryos and haploid embryos. According to their function, these proteins can be classified into six categories (Table 3). β-Crystallin S and γ-crystallin M2–1 belong to the β/γ-crystallin family, in which β-crystalline S was only detected in diploid embryos and γ-crystallin M2–1 was downregulated in haploid embryos. Crystalline is the dominant structural components of the vertebrate eye lens. It is proposed that downregulation of β-crystallin S and γ-crystallin M2–1 in haploid embryos may contribute to the abnormality of eyes of the haploid embryos. Vsx1 transcription factor also is correlated with eye development. Vsx1 is a paired-like homebox-containing gene dynamically expressed in subsets of hindbrain and spinal cord neurons and most prevalent in retina during fish development. Homebox-containing genes encode transcription factors that control a variety of cell fate decisions during the development. The paired-like family of homebox genes including Vsx1 appears to play a particular role in craniofacial and ocular development [7, 8]. Expression of Vsx1 influences retinal progenitor cell proliferation, cell specification, and the maintenance of specific cell types [9].

Six protein spots involved in development regulation were identified, in which two proteins (homeobox protein Hox-C6, ubiquitin-conjugating enzyme 9–2) were upregulated

Table 3. Identification of proteins significantly different between haploid embryos and diploid embryos, sorted by function

Spot No.	Protein description	Protein expression[a)]	
		Haploid embryos	Diploid embryos
Eye development			
2A	Vsx1 transcription factor (fragment)	ND	OD
18A	β-crystallin S (γ-cristallin S)	ND	OD
32D	γ-Crystallin M2–1	↓	↓
Hormone protein			
3A	Chain 1: glycoprotein hormones α-chain	ND	OD
15A	Chain 1: gonadotropin β-chain	ND	OD
17A	Gonadotropin α-subunit (fragment)	ND	OD
19A	Gonadotropin | β-subunit 2	ND	OD
Regulation protein			
12D	Zinc finger protein zic1 (fragment)	↓	↑
21A	Chain1: insulin-like growth factor binding protein 2	ND	OD
22D	Insulin-like growth factor binding protein 1	↓	↑
26U	Homeobox protein Hox-C6 (fragment)	↑	↓
29U	Ubiquitin-conjugating enzyme 9–2	↑	↓
42D	Pituitary specific Transcription factor (fragment)	↓	↑
Cell differentiation			
46D	G1/S-specific cyclin D1	↓	↑
26U	Homeobox protein Hox-C6 (fragment)	↑	↓
Signal transduction			
44D	Wnt-1 (fragment)	↓	↑
36A	WNT-8like (fragment)	ND	OD
Nervous development			
9A	Splice short isoform of gene enhanced protein ISL-2A	ND	OD
14D	Ephrin-type A receptor 4 (EC 2.7.1.112) (tyrosine-protein kinase receptor ZEK2) (fragment)	↓	↑
27U	One-eyed pinhead long form protein	↑	↓

a) ND, not detected; OD, only detected; ↓, downregulated; ↑, upregulated

in haploid embryos, three proteins (pituitary specific transcription factor, insulin-like growth factor binding protein 1, zinc finger protein zic1) were downregulated in haploid embryos, and one protein (insulin-like growth factor binding protein 2) was only detected in diploid embryos. The biological activity and availability of IGFs are regulated by a group of secreted proteins that belong to the IGF-binding protein (IGFBP) gene family. Insulin-like growth factor binding proteins (IGFBPs) are a family of proteins that specifically bind IGF-I and IGF-II receptors. Six distinct IGFBPs, designated as IGFBP-1 to IGFBP-6, have been isolated and characterized in mammalian to date [10, 11]. These proteins act as carrier proteins in the bloodstream and control the efflux of IGFs from the vascular space. The expression of IGFBPs can alter IGF biological activity by modulating their interaction with the IGF receptor. Duan *et al.* [12] reported that the zebra fish IGFBP-2 mRNA was expressed in many embryonic tissues and when IGFBP-2 was overexpressed or added to cultured zebra fish and mammalian cell, IGFBP-2 significantly inhibited IGF-I-stimulated cell proliferation and DNA synthesis.

The pituitary specific transcription factor can regulate the rainbow trout growth hormone (GH) gene promoter [13]. Hox-C6 is a sequence-specific transcription factor, which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis, and its overexpression can inhibit the differentiation of cell [14]. The ubiquitin/proteasome degradative pathway operates *via* two separate enzymatic pathways in which substrate proteins are covalently modified by a chain of ubiquitin molecules and then degraded. Ubiquitin-conjugating enzymes (conjugating-conjugating enzyme 9–2) catalyze the formation of an isopeptide bond between the *C*-terminal glycine of ubiquitin and the ε-amino group of one or more specific lysine residues of a substrate protein [15]. Kurtzman *et al.* [9] investigated that Vsx 1 was a substrate of ubiquitin/proteasome pathway and ubiquitin-dependent proteolysis regulated Vsx 1 during zebra fish retinal development. This indicated that overexpression of ubiquitin-conjugating enzymes 9–2 may influence Vsx 1-dependent retinal progenitor cell proliferation, cell specification, and the maintenance of specific cell types during retinogenesis.

Zic genes encode zinc finger proteins homologous that are generally expressed in the dorsal neural tube in vertebrate neural development. Zic1 controls the expansion of neuronal precursors by inhibiting the progression of neuronal differentiation. Aruga *et al.* [16, 17] described that Zic1 regulated the patterning of vertebral arches in cooperation with Gli3. In Zic1-deficient mice, multiple abnormalities are shown in the axial skeleton. The deformities are severe in the dorsal parts of the vertebrae, vertebral arches but less in the vertebral bodies. When Zic1 is misexpressed on the ventral side of the spinal cord, neuronal differentiation is inhibited irrespective of the dorsoventral position.

Splice short isoform of gene enhancer protein expresses in pancreatic cells, many neurons in the brain spinal cord and the peripheral nervous system, and in some endocrine cells in the pituitary and thyroid. It can bind one of the *cis*-acting domains of the insulin gene enhancer and may be involved in both differentiation and maintenance of many neuron cells and endocrine cells during embryogenesis [18]. Ephrin-type A receptor 4 is a receptor for members of the Ephrin-B family and may play a role in early pattern formation within the development nervous system [19]. One-eyed pinhead is required for the formation of the ventral neuroectoderm, endoderm, and prechordal plate [20]. One-eyed pinhead mutation disrupts embryonic development, resulting in cyclopia and defects in endoderm, prechordal plate, and ventral neuroectoderm formation [21].

We also identified two protein spots (Wnt-1, Wnt-8like) that are involved in signal transduction, in which Wnt-1 was downregulated in haploid embryos and Wnt 8-like was only detected in diploid. The Wnts are a family of secreted glycoproteins involved in cell-cell signaling and pattern formation during development. Dorsky *et al.* [22] determined the role of Wnt signals and concluded that endogenous Wnt signalling normally promoted pigment-cell formation by medial crest cells and thereby contributed to the diversity of neural crest cell fates. Wnt-1 was originally identified as a proto-oncogene, but its normal function is embryogenesis. The gene is the vertebrate homologue of the *Drosophila* segment polarity gene wingless, and encodes a secretary protein, which plays an important role in the early morphogenesis of neural tissues. Wnt-1 expression is necessary for proper development of the midbrain and anterior hindbrain [23–25].

The cyclin D1 gene is a single copy gene within the zebrafish genome. The initial expression of cyclin D1 transcription occurs at the presumed onset of G1 phase in the developing embryo. G1/S-specific cyclin D1 is essential for the control the cell cycle G1/S transition and plays an important role in cell differentiation and transformation [26]. The vertebrate olfactory system utilizes odorant receptors to receive and discriminate thousands of different chemical stimuli [27]. We found only a few other cyclins as differentially expressed proteins. In our opinion, perhaps two factors, have caused this result. (i) cyclins are believed to be involved in inducing mitosis and accumulate in interphase of cell cycle but are destroyed at the conclusion of mitosis. Perhaps not all cyclins exist in sufficient amounts to be detectable in 2-D gels. (ii) Although the development way is different for the haploid and diploid embryos, both of them could develop to the hatching stage, so the scales of the mitosis in the two kinds of embryos are probably about the same at the eye formation stage. If so, the expressing level of some cyclins could be about the same and could not be found in the list of the differentially expressed proteins.

We believe that there are two categories for the proteins totally failed to be expressed in haploid embryos. The first category is the leading proteins, which are critical for the development, such as Vsx1 transcription factor, and Zic1 protein. The reason for these leading proteins to be failed to express in haploid is possibly due to their genes not selected by the diploid-dependent regulatory apparatus proposed by Luo *et al.* [1]. The second category contains the genes that are expressed following the leading genes, such as growth factor binding protein and gonadotropin.

4 Concluding remarks

Reference maps of haploid embryos and diploid embryos using 2-DE were obtained and comparative analysis of gel images was performed. Some differential expressed proteins were found between diploid embryos and haploid embryos and 32 differentially expressed proteins were identified using MS, in which some proteins are related with eye development, developing regulation, nerve development, cell differentiation, signal transduction, and morphogenesis. The results are the basis for proteome comparative analysis of other stage embryos of gynogenetic haploid and diploid of goldfish and provide the method for study gene expression during embryo development of goldfishes. Gene expression is significantly different during the development of embryos between diploids and haploids. This difference may cause the abnormality of haploid embryos.

This work was supported by the State Key Basic Research Program of China (No. 2001 CB5102) and by the "863" program of China (No. 2001 AA233031).

Received April 17, 2003
Revised June 28, 2003
Accepted June 30, 2003

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