Journal of
Proteomics & Bioinformatics

Xie et al., J Proteomics Bioinform 2013, 6:8
DOI: 10.4172/jpb.1000275

Research Article Open Access

Discovery of Novel Proteins form Injured Rat Pancreatic Extract using MALDI-TOF/MS-based Proteomics

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Abstract

Injured pancreatic tissue extract contains transcription proteins that are considered as specific soluble proteins, which contribute in promoting the trans-differentiation of stem cells into insulin-producing cells (IPCs). In this present study, 60% of the pancreatic tissues of Sprague-Dawley (SD) rats were removed, and newborn and normal pancreatic tissues were removed after 48 h to extract tissue fluid. Two-dimensional gel electrophoresis (2-DE) separation and spot analysis were conducted on differentially expressed proteins, and peptides were obtained after enzymatic digestion. Twenty two-fold or above differentially expressed proteins were identified via matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/MS), among which, five proteins were related to pancreatic development and differentiation. Moreover, the expression patterns of four proteins detected with Western blot analysis were in agreement with those detected via 2-DE. Our results and those of the bioinformatics analysis suggest that these novel proteins from injured rat pancreatic extract can be a potential source for stem cell differentiation into IPCs.

Keywords: Injured pancreatic tissue; Extract; Proteomics; Sprague-dawley rats

Introduction

Transplantation of islets from cadaver donors is a promising cell–based therapy for diabetes [1]. However, limited availability of donor's cells and immune suppression are considered major obstacles to islet transplantation [2]. Recently, several reports have demonstrated that stem cells possess broad differentiation ability. This has led many investigators to investigate the potentials of their therapeutic applications. A variety of stem cells, such as embryonic stem cells [3,4], induced pluripotent stem cells [5], and adult stem cells such as pancreatic, liver and bone marrow mesenchymal stem cells [5-8], have the ability to differentiate into insulin-producing cells (IPCs) *in vitro*, providing a new source for β-cell transplantation. Two main programs are currently used for inducing stem cell differentiation to IPCs. The first program makes use of genetic engineering technology, in which transcription factors involved in islet β-cell development are directly transferred to cells or living organs and tissues to regulate gene expression during β-cell differentiation and rewrite cell program to control blood glucose [9-14]. The other program makes use of specific soluble factors or proteins that have catalytic roles in the differentiation and proliferation of stem cells to β-cells [15-18].

However, current protocols can only induce about 10-20% of stem cells to differentiate into IPCs under *in vitro* [19]. The resulting IPCs also appear to be not fully maturated, as these cells can usually only secret low levels of insulin, which account for about one tenth of normal islet. Stem cells are not only controlled by the genetic program, but also by the microenvironment. The interaction between stem cells and the microenvironment is key to the determination of stem cells differentiation into IPCs and the maturity of IPCs [20-22]. Our preliminary studies showed that the rabbit pancreatic tissues could promote bone marrow mesenchymal stem cells into IPCs [23]. Choi et al. [24] found that the treatment of rat pancreatic extract can differentiate rat mesenchymal cells into IPCs, showing that important trans-differentiation factors and soluble proteins are key factors for promoting and inducing the differentiation of stem cells into IPCs. It is still unclear which key factors play a role in these processes of differentiation and proliferation.

Here, we removed 60% pancreatic tissues from Sprague-Dawley (SD) rats, the newborn pancreas after 48 h and normal pancreatic tissue were homogenate. Injured and normal pancreatic extracts were assessed by two-dimensional gel electrophoresis (2-DE), followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis to identify differentially expressed proteins and bioinformatics analysis, with the purpose of providing some potential proteins for stem cell differentiation into IPCs.

Materials and Methods

Animals

Sixty SD rats aged 6 weeks and weighed 120 g to 150 g of either gender were purchased from the Experimental Animal Center of Guangdong Province and fed in specified pathogen-free (SPF) animal laboratory (Experimental Animal Center, the Second Clinical Medical College of Jinan University). The local research ethics committee approved all experimental procedure.

Preparation of injured and normal pancreatic extracts

Forty SD rats were anesthetized prior to the removal of 60% of their pancreatic tissues under sterile conditions, and then they were

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Received July 10, 2013; **Accepted** August 05, 2013; **Published** August 12, 2013

Citation: Xie H, Zhang H, Qi H, Wang Y, Deng CY, et al. (2013) Discovery of Novel Proteins form Injured Rat Pancreatic Extract using MALDI-TOF/MS-based Proteomics. J Proteomics Bioinform 6: 158-163. doi:10.4172/jpb.1000275

fed for 48 h and taken as the injured group. Another set of 20 SD rats was used as the normal group. Following the method of Hardikar et al. [25], the rats in both groups were sacrificed to remove their newborn pancreas, and normal pancreatic tissues were washed using cold PBS. Thereafter, the tissues were placed in PBS containing 630 U/mg of protease inhibitor solution (Roche, Basel, Switzerland), at a ratio of 10 μL:1 mL, homogenized at 4°C with a tissue homogenizer, and centrifuged at 3,000 rpm for 10 min at 4°C to separate the supernatant. The supernatant was centrifuged at 12,000 rpm for 20 min at 4°C. After centrifugation, the supernatant was filtered using a 0.22 μm membrane (Millipore, USA) to obtain the pancreatic tissue extract. The extract was treated with ultrasound in an ice bath (150 W, ultrasound 10 sec, intermittent 10 sec, for a total of 20 times), to remove the impurities using 2-D Clean Up Kit (Biosciences, USA). Then, 2D Quant Kit (Biosciences, USA) was used for the quantitative measurement of protein concentration. Finally, the sample was stored at -80°C for use.

Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing (IEF) in the first dimension: Up to 90 μg of total protein extract was placed on IPG strips of Ettan IPGphor II IEF system (GE Healthcare Amersham, UK). IEF was started at 20°C: 30 V for 12 h; 300 V for 1 h; 500 V for 1 h; 1,000 V for 1 h; 3,000 V for 1 h; and 8,000 V that was rapidly increased and finished until 45,000 V. Sodium dodecyl sulfate polyacrylamide (SDS-PA) in the second dimension: the electrophoresis was conducted using the EttanTM DALTsix vertical electrophoresis system (GE Healthcare Amersham, UK). Following the instructions in literature [26], the SDS-PA gel was prepared (0.1% SDS and 12.5% PA). Electrophoresis was performed at a constant current of 40 mA at 15°C, and stopped until bromophenol blue was 0.5 cm distant from the positive electrode. Coomassie Brilliant Blue staining was adopted, and the images were scanned.

Image analysis

The 2-DE gel stained with Coomassie brilliant blue was scanned using ImageScanner (GE Healthcare Amersham, Sweden), and spot detection, matching, database building and image analysis were performed using ImageMaster 5.0 gel image analysis software (GE Healthcare Amersham, Sweden). The mean gel in the normal group was used as reference for matching using the mean gel in the injured group, to search for spots of differentially expressed proteins. A two-fold difference was considered as the limit, and all two-fold or above protein spots were marked as screened protein spots.

MALDI-TOF/MS analysis

After Coomassie Brilliant Blue staining, interested protein spots (two-fold or above differentially expressed proteins sufficient for mass spectrometry) on the 2-DE gel image were cut by hand. The protein spots were placed in 96-well plates, rinsed twice with 50% methanol and 50 mmol/L of ammonium bicarbonate solution, rinsed once with 75% acetonitrile, dried at 40°C, followed by the addition of 10 μL of 0.02 μg/mL trypsin protease, stored at 37°C, incubated for 2 h, and then cultured with 50% acetonitrile and 0.1% trifluoroacetic acid (100 μL) for 1 h at room temperature. New extracts were repeatedly collected twice and placed into new 96-well plates for drying. Following the instructions in literature [27], MALDI-TOF/MS analysis was performed on eluted peptides in a 1.5 μL matrix.

Bioinformatics analysis

An online database, which included a variety of experimental information about the interaction and association among different proteins, has been synchronized with the establishment of high-throughput proteomics technology. Protein-protein interaction (PPI) for bioinformatics analysis has adopted PPI Spider [27], which is a free Web-based tool that explains the context of experimentally derived protein in a global PPI network. The PPI network provides the corresponding parameters for a set of uploaded standard gene symbols for protein identification [28].

Western blot identification

Total protein was extracted from the pancreatic tissue extract and quantified using the Bradford method. Up to 40 μg of the total protein sample was taken and detected *via* 10% SDSP-PA gel electrophoresis (SDSP-PAGE) analysis, and the total protein sample was then transferred to a polyvinylidene fluoride (PVDF) membrane, closed with 5% nonfat dry milk at room temperature for 2 h. Rabbit Anti-Rat IgG primary antibody cofilin1, Histidine triad nucleotide-binding protein 1 (HINT1), NDPKA, PRDX6, HTRA2, and GAPDH (Abcam, USA) were added at l:500, l:500, l:500, l:500, and l:2,000 dilutions, respectively, overnight at 4°C. The membrane was rinsed with Tris-Buffered Saline (TBST), added with horseradish peroxidase conjugated goat anti-rabbit lgG (1:6,000, Santa Cruz Bioetechnology), incubated at room temperature for l h, rinsed with TBST×3, and then stained with DAB (AP). Blots were collected using a ChemiImager System 5500 gel imaging analysis system (Alpha Innotech, USA), and the immune intensity of the images were analyzed using Image J software (National Institutes of Health, USA).

Statistical analysis

The data were expressed as mean ± SD and analyzed using Prism 4.0 software. ANOVA was used for comparison among groups and t-test was used for comparison between two groups. A value of $P<0.05$ was considered significant.

Results

Preparation and quantification of pancreatic extracts

Pancreatic extracts were separately extracted from the injured and normal groups. After removing the impurities, the protein levels were measured quantitatively, with 2.3 ± 0.17 mg/mL in the injured group and 1.8 ± 0.11 mg/mL in the normal group.

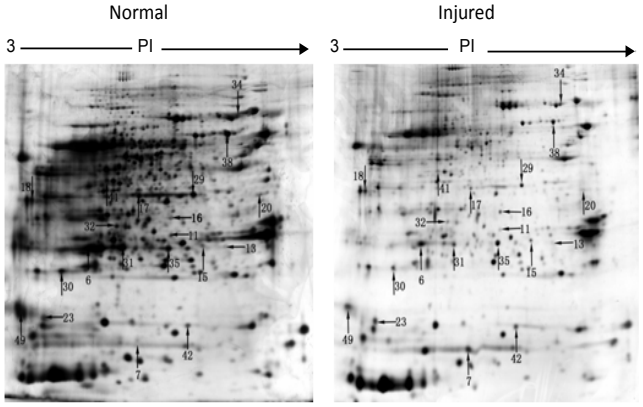

Figure 1: Representative 2-DE gel maps of pancreatic extract from injured pancreas (left) and normal pancreas (right). Total protein extracts were separated in 13 cm nonlinear IPG strips (pH 3 to pH 10) in the first dimension, followed by 13% SDS-PAGE in the second dimension, and visualized *via* silver staining. Spot numbers correspond to those listed on the first column of Table 1.

2-DE and image analysis

2-DE of pancreatic extracts was repeated thrice in the injured and normal groups (Figure 1). ImageMaster 2D Platinum 5.0 software was used for protein spot comparison in the injured and normal groups, and 1.451 ± 213 in the injured group were identified to have matched protein spots of 1.227 ± 17 (matched rate of 84.56%) and 1.707 ± 31 (n=3) in the normal group were identified to have matched protein spots of 1.507 ± 29 (matched rate of 88.28%). Fifty two-fold or above protein spots increased or decreased, among which, 13 were up-regulated and 37 were down-regulated.

Liquid chromatography-electrospray ionization (LC-ESI)-MS analysis

Thirty-two protein spots were successfully harvested from the 50 differentially expressed protein spots in the injured and normal groups. Peptides were obtained after enzymatic digestion, among which 20 protein spots were successfully identified using MALDI-TOF/MS, and clear fingerprints were obtained. Moreover, seven up-regulated spots and 13 down-regulated spots were identified (Figure 2).

Bioinformatics analysis

Using PPI, bioinformatics analysis was performed on all identified proteins, which were classified according to their different functions. Twenty differentially expressed proteins included five related to cell growth and proliferation, two related to glucose metabolism, one related to lipid metabolism, three related to protein expression regulation, six related to oxidative stress (Table 1).

Western blot analysis

Five proteins associated with cell growth and proliferation, including Cofilin-1, HINT1, NDPKA, PRDX6, and HTRA2 were detected *via* the

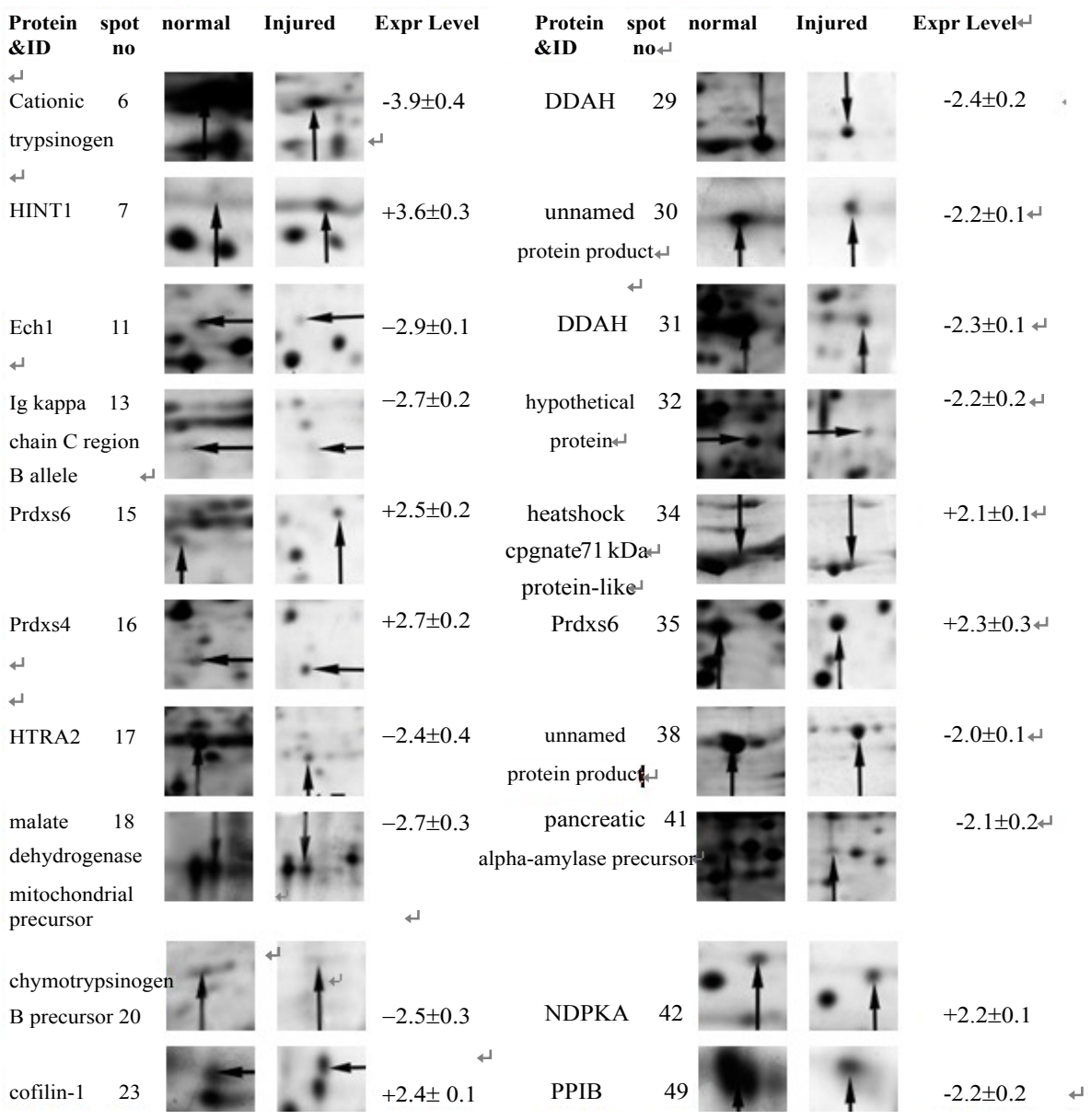

Figure 2: Detailed alteration of identified protein spots. Arrows indicate proteins with expressions altered and identified *via* peptide mass fingerprinting.

Western blot method in the injured and normal groups, and except for HINT1, the expressions of Cofilin-1, NDPKA, and PRDX6 proteins increased and HTRA2 protein expression decreased in the injured group, which were consistent with the 2-DE results (Figure 3).

Discussion

The rat partial pancreatectomy model had confirmed that the residual pancreas has a strong regenerative capacity and enables cytokine secretion to promote the regeneration of islet β-cells in a diabetic state [25]. They then injected post-pancreatectomy mouse pancreatic extract intraperitoneally into the diabetic rats, and found that the blood glucose levels of the diabetic rats could be reduced up to 190 days [29]. Kanitkar and Bhonde [30] also found that a rat pancreatic supernatant could reduce the blood glucose levels of diabetic rats *via* intraperitoneal administration, indicating that the proliferated pancreas can secrete large amounts of cytokines and promote the regeneration of islet β-cells. In the present study, we attempted to address the molecular basis of β-cell neogenesis at the tissue level *in vivo*, regardless of whether new β-cells are differentiated from stem cells. The strategy used has advantages, and should more accurately reflect the molecular regulation mechanism that the differentiation of stem cells into islet β-cells *in vitro*, and it should also reveal other important pathological changes accompanying β-cell neogenesis. Our study has identified several proteins whose expression was significantly altered in pancreatectomized rats. In the following sections, the possible functions of these proteins will be discussed.

2-DE is the most common technique used for protein separation, and it has become the core technology of proteomics separation and purification, because it can achieve a one-time separation of a large batch of proteins with high resolution and high sensitivity, and it can easily perform a computer image analysis. 60% of the pancreas was removed to find relevant proteins that promote the differentiation of stem cells into islet β cells, and regenerated and normal pancreatic extracts were used after 2 d to remove impurities. 2-DE was conducted on three independent samples in each group, and 50 two-fold or above protein spots were identified, 13 of which were increased and 37 were decreased. Thirty-two protein spots were successfully excavated and 20 out of the 30 proteins were successfully identified using LC-ESI-MS [31]. Among the 20 identified proteins, 5 were associated with cell growth and proliferation, 2 were associated with glucose metabolism, 1 was associated with lipid metabolism, 3 were associated with protein expression regulation, 6 were associated with oxidative stress and 3 were unnamed. Five proteins were involved in the regulation of cell division, proliferation, differentiation and development. Cofilin-1, NDPKA, and HINT1 were involved for the expression up-regulation, and PRDX6 and HTRA2 were involved for the expression down-regulation.

Cofilin-1 is an intracellular actin-modulating protein with a low molecular weight of 21 kDa, and it is widely distributed in a variety of non-muscle tissues, especially in the brain and liver. Intracellular signaling molecules and self-phosphorylation regulate the activity of cofilin. Ser 3 is the important spot for cofilin at the N-terminal

Spot number	Protein name	NCBI ID	MW (kDa)/pI	Reported function	Expression Level	Matched peptides
6	Cationic trypsinogen	gi|149065361	26.9/7.45	Protein expression regulation	-3.9 ± 0.4	5
7	Histidine triad nucleotide-binding protein 1(HINT1)	gi|33468857	13.8/6.36	Protein kinase C inhibitors Cell proliferation	+3.6 ± 0.3	5
11	Enoyl-CoA hydratase, mitochondrial precursor (Ech1)	gi|17530977	31.8/8.39	Fat metabolism-related	-2.9 ± 0.1	8
13	Ig kappa chain C region, B allele	gi|125144	11.7/4.97	Protein expression regulation	-2.7 ± 0.2	4
15	Peroxiredoxin-6 (Prdxs6)	gi|16758348	24.8/5.64	Oxidative stress	+2.5 ± 0.2	11
16	Peroxiredoxin-4 (Prdxs4)	gi|16758274	31.2/6.18	Oxidative stress	+2.7 ± 0.2	10
17	Serine protease HTRA2, mitochondrial	gi|157819535	49.1/9.51	Cell apoptosis	-2.4 ± 0.4	13
18	Malate dehydrogenase, mitochondrial precursor	gi|42476181	36.1/8.93	Sugar metabolism-related	-2.7 ± 0.3	13
20	Chymotrypsinogen B precursor	gi|6978717	28.4/4.9	Pancreatic protease activation	-2.5 ± 0.3	4
23	Cofilin-1	gi|8393101	18.7/8.22	Cell proliferation	+2.4 ± 0.1	5
29	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1(DDAH1)	gi|11560131	31.8/5.75	Oxidative stress	-2.4 ± 0.2	9
30	Unnamed protein product	gi|56691	24.8/8.96		-2.2 ± 0.1	6
31	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	gi|11560131	31.8/5.75	Oxidative stress	-2.3 ± 0.1	8
32	PREDICTED: hypothetical protein	gi|293349760	763.7/9.38		-2.2 ± 0.2	42
34	PREDICTED: heat shock cognate 71 kDa protein-like	gi|293347763	72.9/5.41	Oxidative stress	+2.1 ± 0.1	18
35	Peroxiredoxin-6	gi|16758348	24.8/5.64	Oxidative stress	+2.3 ± 0.3	9
38	Unnamed protein product	gi|1334284	58.0/5.35		-2.0 ± 0.1	9
41	Pancreatic alpha-amylase precursor	gi|13928684	57.8/8.34	Sugar metabolism-related	-2.2 ± 0.2	9
42	Nucleoside diphosphate kinase A	gi|19924089	17.2/5.96	Cell proliferation	+ 2.2 ± 0.1	8
49	Peptidyl-prolyl cis-trans isomerase B	gi|11968126	22.8/9.42	Cell proliferation	-2.2 ± 0.2	8

Table 1: Results of protein identifications of differentially expressed proteins using LC-ESI-MS/MS.

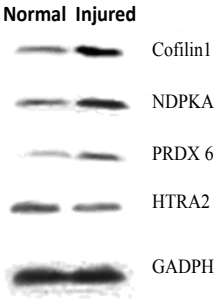

Figure 3: Validation of pancreatic Cofilin-1, NDPKA, PRDX6, and TRA2 levels using Western blot analysis. (A) Western blots of Cofilin-1, NDPKA, PRDX6, and HTRA2. β-actin was used to control equal loading. (B) The expression pattern of Cofilin-1, NDPKA, PRDX6, and TRA2 via Western blot was in agreement with that via 2-DE.

end, which is the only phosphorylated spot. Cytokines regulate cofilin activity *via* Ser 3-phosphorylation and dephosphorylation. Studies have demonstrated that high glucose can strengthen cofilin-1 expression via the protein kinase C signaling pathway [32], and that cofilin overexpression can promote cell proliferation and improve anti-injury ability [33].

NDPKA, which is a polypeptide that consists of 152 amino acids, has a molecular weight of approximately 17 kDa. NDPK can regulate the G-protein activity by directly binding G proteins and transferring high-energy phosphate bond, and it plays a negative regulatory function, such as growth inhibition [34]. The pluripotency and regulatory mechanisms of NDPKA are significant in the clarification of the biological functions of NDPKA [35].

HINT1, which contains 126 amino acid sequences, has a molecular weight of 13.8 kDa, and it belongs to the histidine trimeric protein superfamily, along with nucleotide transferase and hydrolase activity. HINT1 proteins interact with microphthalmia-associated transcription factor and cyclin-dependent kinase 7 (CDK7) for transcriptional regulation and growth regulation [36].

PPIB is a unique peptidyl-prolyl cis-trans isomerase that highly and specifically catalyzes phosphorylated Ser/Thr-Prolinamide phthalate cis/trans isomers into one other, resulting in functional changes. This configuration change is the regulatory mechanism following the phosphorylation of newly discovered proteins [37]. PPIB proteins can bind with cyclin D1 to play a role in the G1 phase, promote cells from G1 phase to S phase, and accelerate cell proliferation. PRDX6 expression increases and participates in cell proliferation, differentiation, and apoptosis signal transduction when the level of hydrogen peroxide is adjusted [38].

HTRA2, which is a member of the mammalian HTRA serine protease family, consists of 458 amino acid residues. Moreover, HTRA2 has a relative molecular weight of 51 kDA, and is a protease that functions as mitochondria in apoptosis regulation. By inhibiting the activity of caspases, HRTA2 can inhibit the occurrence of cell apoptosis [39,40].

The proteome profiling technique used in the present study provided a broad-based and effective approach for the rapid assimilation and identification of adaptive protein changes during pancreatic regeneration induced by pancreatectomy. In the current study, pancreatic development and differentiation-associated proteins were screened, and four proteins were verified using the Western blot method. The results are consistent with the 2-DE findings. Among the four proteins, the expressions of cofilin-1, NDPKA, and PRDX6 increased, and the expression of HTRA2 decreased, indicating that these proteins may be the key trans-differentiation factors for promoting the differentiation of stem cells into IPCs.

In summary, 2-DE was used for the separation and analysis of differentially expressed proteins from injured and normal pancreatic extracts, and MALDI-TOF/MS was employed for the identification of differentially expressed proteins. Moreover, cofilin-1, NDPKA, PRDX6, and HTRA2 proteins were found to be associated with pancreatic proliferation and differentiation, which is very important and will lead to a better understanding of the regulation mechanism of pancreatic regeneration, and provide new key transcription factors and soluble proteins for the differentiation of stem cells into IPCs in vitro

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgements

This work was supported by the national 973 special plan of China (No.2007CB516811and 2004CCA01500), the National Natural Science Foundation of China (No. 30772042), the Natural Science Foundation of Guangdong (No.37415 and No.6027540) and The Science and Technology Project of Shenzhen (No. 201001005,201001002,201102163).

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