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Proteomic analysis of porcine pancreas development

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Porcine pancreas development is not well studied at the molecular level despite being a therapeutic resource for diabetic patients. In this study, we investigated expression of lineage markers and performed proteomic analysis. Expression of the early lineage markers Pdx1 and Ptf1a was developmentally conserved between mice and pigs, whereas expression of the islet differentiation marker Pax4 was delayed in porcine compared with murine pancreas development. Proteomic analysis found that expression levels of chymotrypsinogen were down-regulated during porcine pancreas development while those of digestive enzymes like lipases, elastase and serine protease were up-regulated. In addition, specific isoforms of protein folding assistants such as protein disulfide isomerase and prefoldin were expressed at specific stages during the maturation of digestive enzymes. Taken together, these results show that development of the porcine pancreas is regulated by a concerted interplay of pancreas lineage marker proteins and other specified proteins, resulting in a functional endocrine and exocrine organ. [BMB reports 2009; 42(10): 661-666]

INTRODUCTION

The pancreas is composed of exocrine cells, which secrete digestive enzymes such as trypsin, chymotrypsin and amylase, and endocrine cells, which produce hormones like insulin and glucagon. Development of the mammalian pancreas occurs in three steps: endoderm formation, pancreatic morphogenesis and differentiation of exocrine and endocrine cells (1). In mice, differentiation occurs in three distinct waves. The first wave of endocrine cell differentiation occurs at embryonic day 9.5 (E9.5) for the generation of pancreatic buds. The second wave starts at E14.5 and produces endocrine cells. The third wave initiates at E17 and forms exocrine cells (2).

Pancreas development requires interaction between epithelial and mesenchymal tissues (3). In early development, the fusion of dorsal and ventral buds forms endodermic epithelia located in the foregut. During the development and differentiation of the pancreas, the master regulator gene *Pdx1* is first expressed in the foregut endoderm (4). Specifically for murine development, expression of *Pdx1* in the pancreas is observed in the ventral and dorsal pancreatic buds at E9.5 and is diminished at E17 (5). Endocrine precursors differentiated from *Pdx1*- and *Ptf1a*-expressing pancreatic cells induce expression of the bHLH transcription factor *Ngn3* at E9.5. In addition, *Ptf1a* is expressed in exocrine cells at this stage (6). *NeuroD*, another bHLH transcription factor and key regulator of insulin transcription, is expressed in the pancreatic epithelia of β cells at E9.5 and in islet cells at E17 (7, 8). Expression of *Pax4*, a paired-box homeoprotein, remains exclusively in the ventral and dorsal buds of the developing pancreas, but becomes limited to β cells at birth (9). Nevertheless, the regulation of lineage markers during porcine pancreas development has not yet been examined.

The transplantation of porcine pancreatic β cells to type 1 and type 2 diabetic rats results in the recovery of glucose tolerance without host immune-suppression (10). Porcine embryonic pancreatic tissue is transplanted into human diabetic patients since transplantations of gestational stage porcine pancreas are ineffective. This indicates that porcine pancreatic tissue at the embryonic stage is less immunogenic to human diabetic patients than later gestational age tissue (11, 12). Indeed, porcine pancreatic tissue is an important resource of transplantation therapy for human diabetic patients, but little is known about its development at the molecular level. In this study, we investigated the translational patterns of porcine pancreas development in two manners: specific pancreatic lineage marker proteins and global proteomic expression. This study will provide a guideline for understanding development of the porcine pancreas.

RESULTS AND DISCUSSION

Porcine pancreas developmental analysis with lineage markers

To investigate the expression patterns of lineage markers in mice (Fig. 1A), Western blot analysis of the porcine pancreas was performed at days E69, E93, neonatal day 1 and on adults (Fig. 1B). Pdx1, an early lineage marker of pancreas specifica-

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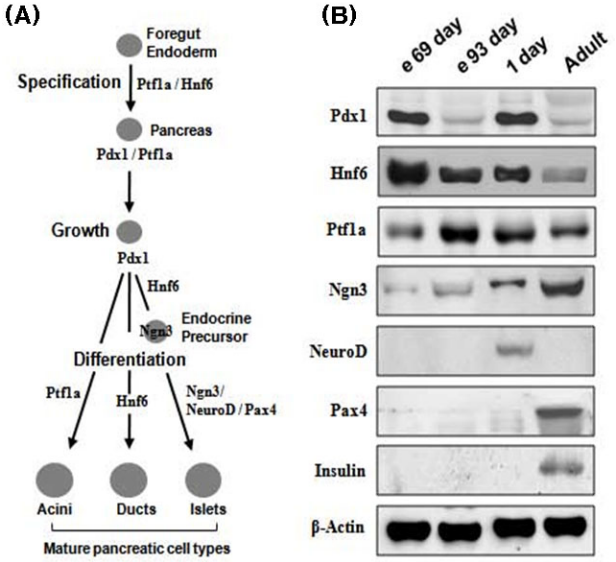


Fig. 1. Expression of lineage markers during pancreas development. (A) Pancreatic lineage markers in mice (26). (B) Expression of *Pdx1* and *Ptf1a* (pancreas specification markers), *Ngn3* (endocrine precursor marker), *NeuroD*/*Pax4* (islet differentiation markers) and Insulin (β-cell marker) in E69, E93, neonatal day 1 and adult porcine pancreases.

tion, was strongly expressed at E69 and neonatal day 1. However, it was only weakly expressed at E93 and the adult stage. Ptf1a, another early lineage marker of pancreas specification, was weakly expressed at E69 but strongly expressed at E93. Strong expression of *Pdx1* at E69 implies that pancreatic specification was already determined in fetal pigs before embryonic day E69. The high expression of *Ptf1*a in all developmental stages suggests that *Ptf1*a may have roles not only in pancreatic specification but also in other pancreatic developmental processes. *Ngn3*, an endocrine precursor lineage marker, was weakly expressed in all embryonic stages as well as at neonatal day 1, but strongly expressed in the adult pancreas. Interestingly, *NeuroD*, a lineage marker of islet differentiation, was detected exclusively at neonatal day 1. *Pax4*, another lineage marker of islet differentiation, was expressed in the adult only. These findings suggest that porcine islet differentiation is developmentally delayed compared to that of mice. The absence of insulin expression at neonatal day 1 indicates that development of islet cell clusters is completed after birth in pigs. Nevertheless, the overall expression patterns of developmental transcriptional factors in the porcine pancreas were similar with those of mouse pancreas (13).

Proteomic analysis of the porcine pancreas and classification

To examine changes in the proteomic profile during porcine pancreas development, we analyzed the pancreas proteome by high-resolution 2-DE and LC-MS. Approximately 1,000 protein

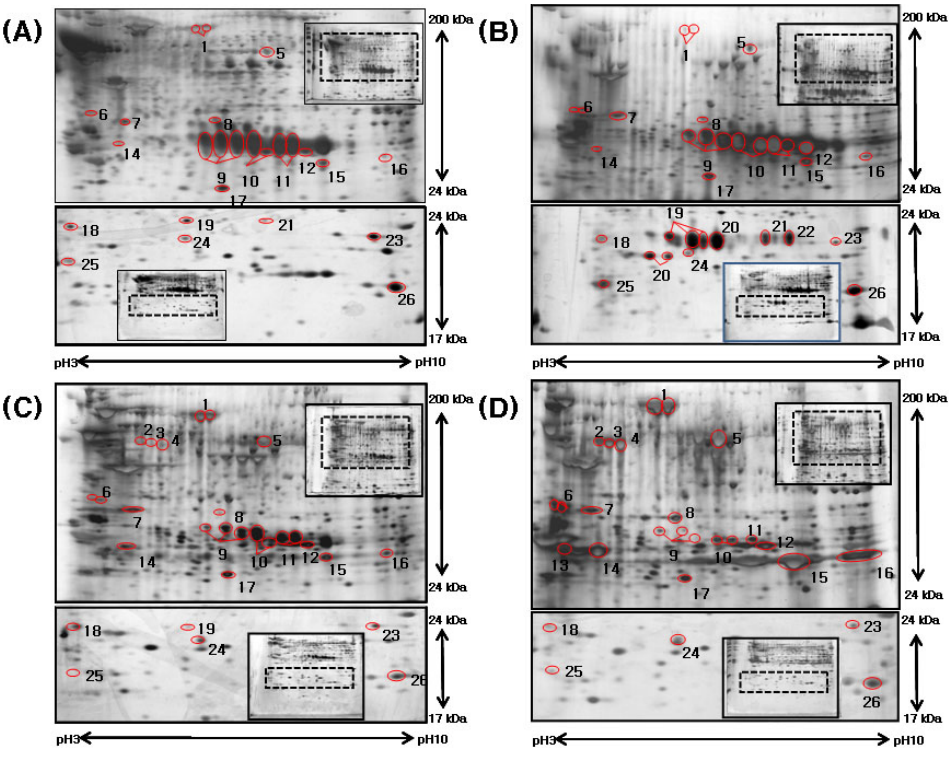


Fig. 2. Representative silver-stained 2D gel images of the different developmental stages of porcine pancreas. Three hundred micrograms of soluble proteins (A, E69; B, E93; C, neonatal day 1; D, adult pancreas) were resolved on a 2D gel (1-D, pH 3-10 IPG NL-gel; 2-D, 15% SDS-PAGE). Protein spots, which were changed by more than two-fold compared to the E69 control, were marked and identified using LCQ-MS. The enlarged 2D gel images of the upper (Mr 40-200 kDa) and lower (Mr 10-40 kDa) regions were displayed from inserted mini-2D gel images.

spots were detected in silver-stained 2-DE gels (Fig. 2). Thirty-six protein spots were changed by more than two-fold when comparing the protein spots of E93, neonatal day 1 and adult stage with those of E69. From LC-MS/MS analysis, 26 proteins were identified and assigned to 36 spots, suggesting the existence of multiple spots (#1, 6, 9, 10, 11, 19 and 20) caused

Table 1. List of 26 proteins expressed differentially during porcine pancreas development

Spot No.	Protein name	Organism	Gene name	Swiss-Prot or NCBI gi accession No.	Expression pattern	Mascot data		Mr (kDa) / pI	
						Score	% Coverage	Theoretical	Experimental
1	Serum albumin	Sus scrofa	ALB	P08835	A−	2,283 1,200	63 29	69.5/5.92	69/6.1 69/6.2
2	Pancreatic triacylglycerol lipase	Sus scrofa	PNLIP	P00591	A	1,176	71	49.9/5.63	55/5.1
3	Pancreatic lipase-like	Pan troglodytes		gi|114632954	A	237	9	51.2/6.14	54/5.3
4	Pancreatic lipase	Homo sapiens		gi|226753	A	177	11	49.4/6.29	53/5.5
5	Agmatinase-like	Bos taurus		gi|194674166	A	186	7	43.3/8.74	55/7.1
6	Protein disulfide-isomerase A6	Homo sapiens	PDIA6	Q15084	A	82 105	3 3	46.2/4.95	38/4.4 37/4.5
7	Nucleophosmin	Rattus norvegicus	Npm1	P13084	A	63	4	32.5/4.61	35/5.1
8	Glycerol-3-phosphate dehydrogenase 1-like	Rattus norvegicus		gi|109484025	D	132	10	38.0/6.61	34/6.3
9	Chymotrypsinogen B	Rattus norvegicus	Ctrb1	P07338	A	74 73 74	10 10 10	27.8/4.90	30/6.2 30/6.5 28/6.7
10	Chymotrypsinogen B	Bos taurus	Ctrb1	P00767	B−	103 70	13 11	25.7/4.99	28/7.0 26/7.2
11	Tumor necrosis factor ligand super family member 8 (CD30L)	Homo sapiens	TNFSF8	P32971	B	51 50	2 2	26.0/7.63	27/7.4 27/7.6
12	Adenylate kinase 2, mitochondria	Homo sapiens	AK2	P54819	C	95	17	26.5/7.67	25/7.9
13	Protease serine 1	Mus musculus	Prss1	Q9Z1R9	A	57	8	26.1/4.75	30/4.6
14	Elastase-3B	Macaca mulatta	ELA3B	O19023	A	122	15	27.7/5.69	25/5.0
15	Serine protease inhibitor	Sus scrofa		gi|3318722	A	987	40	23.5/8.26	23/8.0
16	Elastase-2A	Sus scrofa	ELA2A	P08419	A	153	13	28.7/8.57	25/9.0
17	Phosphatidylethanolamine-binding protein 1	Bos taurus	PEBP1	P13696	D	208	33	21.0/6.96	20/6.4
18	Pretrypsinogen I	Rattus norvegicus	Prss1	P00762	C+	78	8	25.9/4.71	24/4.1
19	EST1562859 MARC cDNA Similar to Chymotrypsinogen B1	Sus scrofa (Bos taurus)	CTRB1	gi|87234952 (gi|157428032)	C−	302 423 360	32 32 29	17.1/6.04	22/5.2 22/5.6 22/5.8
20	Prefoldin subunit 2	Mus musculus	Pfdn2	O70591	C	62 71 208	15 14 35	16.8/6.77	22/6.1 20/4.8 20/5.1
21	Peroxiredoxin-1	Homo sapiens	vPRDX1	Q06830	C−	164	22	22.1/8.16	22/7.2
22	Serine protease inhibitor	Sus scrofa		gi|2914482	C	109	17	23.5/8.26	22/7.6
23	Cofilin-1	Homo sapiens	CFL1	P23528	B−	233	42	18.5/8.22	21/8.5
24	Stathmin	Homo sapiens	STMN1	P16949	C	384	41	17.3/5.76	20/5.5
25	Trypsin-like serine protease	Sus scrofa		gi|19422351	C−	71	14	13.3/7.84	15/3.9
26	Hemoglobin subunit alpha	Sus scrofa	HBA	P01965	B	252	33	15.0/8.76	14/9.0

*The proteins not registered in Swiss-Prots were expressed as NCBI gi accession number.**Expression patterns: A, gradual increase; A-, gradual increase but drop at E93; B, gradual decrease, B-, gradual decrease except N1 stage; C, peak at E93, C+, peak at N1, C−, peak at E93 and gradual decrease; D, trough at E93.

by possible post-translational modifications such as proteolysis, phosphorylation, acetylation and methylation (14). Trypsin-like serine protease (spot #25) showed a wide disparity between its experimental and theoretical p*I*, 3.9 and 7.84, respectively. This suggests that the nascent form of the protein encounters multiple charge-affecting modifications. Since the porcine genomic sequence remains incomplete, the interpretation of unexpected isoforms requires global prediction and experimentation of possible post-translational regulation. Eight proteins among those 26 differentially expressed were identified from the pig database (NCBI gene entries, 12,043 from *Sus scrofa*), whereas the remaining were searched for by sequence homology against human, cow, mouse and rat. The molecular functions of the 16 identified proteins were revealed as digestive enzymes and enzyme inhibitors while the other 10 proteins were structural/regulatory proteins and protein folding assistants (Table 1).

Characterization of digestive enzymes, enzyme precursors and enzyme inhibitors

To understand the function of proteins involved in porcine pancreas development, the 26 newly identified proteins were classified into six categories as shown in Fig. 3. The six categories included structural protein, regulatory protein, protein-folding assistant, digestive enzyme precursor, enzyme inhibitor and digestive enzyme.

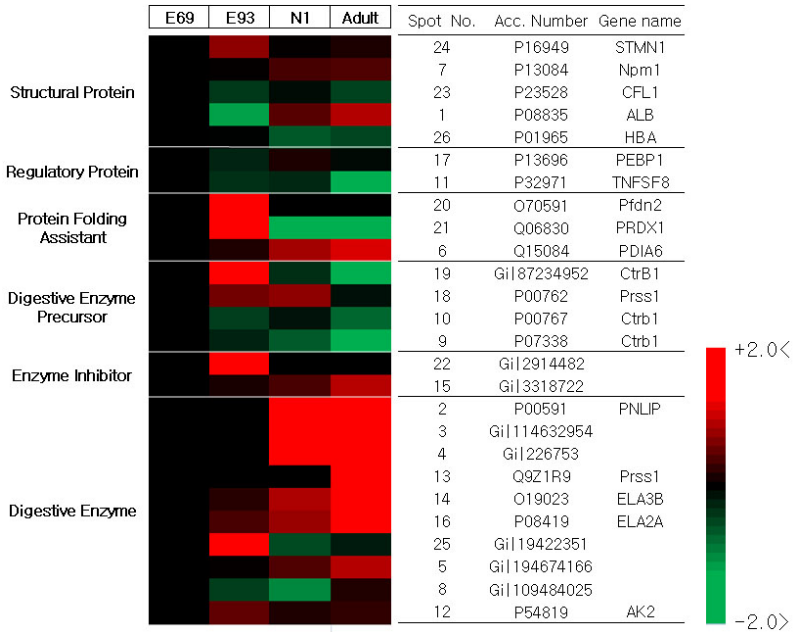
The largest difference in expression patterns was a gradual increase of digestive enzymes (type A pattern) contrasted with a gradual decrease of digestive enzyme precursors (type B pattern). This explains the maturation of pancreatic digestive enzymes and the reverse regression of precursor digestive enzymes in porcine pancreas development. For instance, digestive enzymes such as pancreatic triacylglycerol lipase (spot #2), lipase (spot #3, 4), agmatinase-like enzyme (spot #5) and elastase (spot #14, 16) had their lowest expression at E69 and gradually increased in expression during development. Serine protease (spot #13) showed basal expression up to neonatal day 1, but increased its expression level in adult. Adenylate kinase (spot #12) and trypsin-like serine protease (spot #25) displayed type C expression patterns with peaks at E93, whereas glycerol-3-phosphate dehydrogenase (spot #8) showed type D expression with a trough at E93. In a previous study, elastase II was shown as the predominant pancreatic protease during milk-feeding period while elastase I was related to weaning (15). Expression levels of pancreatic enzymes change during suckling and post-weaning after birth, as evidenced by the increase in chymotrypsin activity and decrease in lipase activity during post-weaning (16).

Chymotrypsinogen (spot #9, 10), a precursor of chymotrypsin protease, showed type B expression with gradual decreases during pancreas development. Pretrypsinogen (spot #18) peaked at neonatal day 1, suggesting that pretrypsinogen is processed to a mature digestive enzyme after birth.

Expression of serine protease inhibitor (SPI, spot #22) was decreased after birth. SPI expression with experimental p*I* 7.6 was increased gradually while SPI expression with experimental p*I* 8.0 peaked at E93 (Fig. 2, 3, Table 1). SPI isoform 1 inhibits porcine chymotrypsin while SPI isoform 2 blocks porcine pancreatic elastase (17). This suggests that SPI may inhibit the functions of digestive enzymes before birth.

Characterization of structural/regulatory proteins and protein-folding assistants

Among structural proteins, cofilin (spot #23), an actin-binding



Spot No.	Acc. Number	Gene name
24	P16949	STMN1
7	P13084	Npm1
23	P23528	CFL1
1	P08835	ALB
26	P01965	HBA
17	P13696	PEBP1
11	P32971	TNFSF8
20	O70591	Pfdn2
21	Q06830	PRDX1
6	Q15084	PDIA6
19	Gi|87234952	CtrB1
18	P00762	Prss1
10	P00767	Ctrb1
9	P07338	Ctrb1
22	Gi|2914482	
15	Gi|3318722	
2	P00591	PNLIP
3	Gi|114632954	
4	Gi|226753	
13	Q9Z1R9	Prss1
14	O19023	ELA3B
16	P08419	ELA2A
25	Gi|19422351	
5	Gi|194674166	
8	Gi|109484025	
12	P54819	AK2

Fig. 3. Functional classification of 26 differentially identified proteins displayed by relative abundance. The normalized spot intensities on 2D gels were transformed into logarithmic scale values. The individual intensities at stage E69 were set to zero (black) for the simple comparison of individual protein expression. Compared to E69 intensities, the logarithmic spot intensities at developing stages were displayed as either red or green color, corresponding to an increase or decrease, respectively. The relative up- or down-regulated expression values with more than two logarithmic scales were considered as the maximum red or green shadow intensities.

protein that plays crucial roles in cell division, cell motility and endocytosis (18, 19), showed high expression at E69 and the low at adult. Stathmin (spot #24), a microtubule-regulating protein, showed the opposite expression pattern as cofilin; it exhibited a type C expression pattern and peaked at E93. Nucleophosmin expression was (spot #7) slightly increased after neonatal day 1. Nucleophosmin is a component of chemokine receptor complex 4 (CXCR4), which functions in the development of the hematopoietic, vascular and central nervous systems (20). Serum albumin (spot #1) showed a gradual increase while hemoglobin subunit alpha (spot #26) showed a gradual decrease during pancreas development. Tumor necrosis factor (TNF) ligand CD30L (spot #11), a member of the TNF superfamily (21), displayed a type B expression pattern and was gradually decreased. Another regulatory protein, phosphatidylethanolamine-binding protein 1 (PEBP1, spot #17) was slightly decreased at E93.

Among protein folding assistants, protein disulfide-isomerase (PDI, spot #6), which improves the folding rate of active proteins (22), showed a similar expression pattern as digestive enzymes. This suggests that PDI may help the folding and maturation of pancreatic digestive enzymes during pancreatic development. However, prefoldin (spot #20), a microtubule chaperone, was down-regulated after birth.

MATERIALS AND METHODS

Pig pancreas protein extraction

Porcine pancreas samples were extracted at E69 (n = 9), E93 (n = 8), neonatal day 1 (n = 2) and adult stage (n = 2). Porcine E69 and E93 are comparable to mouse E12.5 and E17, respectively. The pancreas was dissected and homogenized in 5 mM phosphate buffer (pH 7.0) and Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea). Total protein was prepared according to the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea).

Western blot analysis

Western blotting was performed as previously described (23). Antibodies in the present study were purchased from Chemicon (CA, USA), SantaCruz (CA, USA) and Sigma Company (MO, USA). The relative quantification of protein levels was determined by LAS3000 (Fuji Photo Film, Tokyo, Japan) with β-actin as the internal control.

Two-dimensional gel electrophoresis and protein staining

Two-dimensional gel electrophoresis was performed as previously described (24). In brief, 300 μg of pancreas proteins were rehydrated in a 300 μl solution containing 7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 50 mM DTT and a trace of bromophenol blue. The sample mixture was incubated for 12 h at room temperature. Isoelectrofocusing of proteins was performed at 50 mA and 250 V for 15 min and 1000 V for 2 h using IPG strip holder channels (Bio-Rad, Hercules, CA). The gel strips were equilibrated in buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (wt/vol) glycerol and 2% (wt/vol) SDS. After equilibration, second dimensional separation was performed by 15% SDS-PAGE. The two-dimensional electrophoresis (2-DE) gels were stained with a Silver Staining Kit (Amersham Biosciences, Sweden)

Protein identification by LC-MS/MS

Protein spots of interest were digested in-gel with trypsin (Promega, USA). Tryptic peptides dissolved in 0.5% TFA solution were objects for MS analysis. Peptides concentrated in an MGU30-C_{18} trapping column (LC Packings) were directed onto a C_{18} reverse-phase column (10 cm × 5 μm, PROXEON, Denmark) at a flow rate of 120 nl/min. Peptides were eluted by a gradient of 0-65% acetonitrile for 80 min. All MS and MS/MS spectra provided by the LCQ-Deca ESI ion trap mass spectrometer (ThermoFinnigan, USA) were acquired in data-dependent mode. Each full MS scan (*m/z* range, 400-2,000) was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum with dynamic exclusion. MS/MS spectra were searched for by Mascot software version 2.2 (http://www.matrixscience.com) using the mammalian genome database of nrNCBI. Peptide and fragment mass tolerance were 2.0Da and 0.8Da, respectively. Carbamidomethylation of cysteine, oxidation of methionine and propionamide of cysteine were considered as variable and fixed protein modifications. Protein harboring at least two significant peptides and a Mascot probability score at $P < 0.05$ was considered as the final, identified protein.

Image analysis and bioinformatics

Silver-stained 2D gel images were scanned using a flatbed scanner and analyzed by Progenesis workstation version 2002 (Non-linear Dynamics, UK). From a sequential comparison, the intensities of protein spot volumes were normalized by background subtraction, gel matching and warping. Subsequently, the expression levels based on spot intensity were calculated by statistical analysis. The numerical data were further evaluated for expression pattern analysis. For the visual presentation of expression patterns during porcine pancreas development, 26 differentially identified proteins were classified into six categories of molecular function, and heat map analysis for their relative abundance was performed using Excel 2007 and DAnTE version 1.0, R-based bioinformatics tool (25).

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