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RESEARCH ARTICLE

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Proteomic Analysis for Tissues and Liquid from Bonghan Ducts on Rabbit Intestinal Surfaces

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Abstract
Research on the Bonghan system has recently prompted great interest in the theory proposed by Bong Han Kimin in the early 1960s. In order to study the biochemical characteristics of the Bonghan system, we analyzed Bonghan ducts (BHD) on the surface of rabbit intestines and characterized the liquid in the BHD at the level of the proteome. Proteomic analysis was performed using nano LC-ESI MS/MS. Using a solution digestion technique, we identified 70 different proteins in the liquid of the BHD. We used gel-based digestion to analyze the BHD itself and our results showed the presence of 207 proteins. We used these proteins to analyze gene ontology (GO) to yield insights into biological processes, molecular functions and cellular compartmentalization. Remarkably, GO clustering showed high concentrations of proteins involved in metabolism. These proteins are not usually found in blood, lymph or blood vessels, and thus can be useful for characterizing BHD. It is worth studying their association with stem cells, especially mesenchymal stem cells, cancer cells and myeloid cells.

1. Introduction

In the early 1960s, Bonghan Kim claimed to have discovered the anatomical structure corresponding to acupuncture meridians [1,2]. Despite the potential significance in both Western and Eastern medicines, his work has been ignored for many years due to the absence of verification from other researchers. Only one Japanese anatomist, Fujiwara, has ever managed to replicate his results [3].

With modern fluorescence and microscopy technologies, it has become possible to rediscover the Bonghan system and this has led to a number of new research efforts. Scientists have explored the intravascular Bonghan duct (BHD) and Bonghan corpuscles (BHC) in blood vessels [4–6] and lymphatic vessels [7–9], and organ-surface BHD and BHC [10,11]. A series of investigations to elucidate the details of BHC and BHD anatomy and morphology have been performed using confocal laser scanning

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microscopy [12], various electron microscopy techniques [13], x-ray microtomography [14], and immunohistochemical techniques [15]. Measurement of the flow speed of Bonghan liquid in BHD was performed by injecting Alcian Blue and the speed of travel was found to be 0.3 ± 0.1 mm/sec [16]. Researchers also confirmed that BHC has chromaffin cells that produce and store catecholamine, suggesting a medical significance of the BHD as a hormonal pathway [17].

Our work describes an initial step towards functionally characterizing the Bonghan system in the rabbit using proteomic analysis. In order to identify BHC proteins, we used electrospray ionization (ESI) that featured a linear ion-trap mass spectrometer coupled with nano liquid chromatography (LC). The proteins identified were clustered using GO according to their involvement in biological processes, molecular function and cellular compartmentalization. Our proteomic analysis of Bonghan liquid and BHD showed remarkably high levels in carbohydrate metabolic derivatives. We compared the chemical composition of Bonghan liquid with that observed in blood [18], lymph [19] or blood vessels [20], but found similarity in composition to that more usually associated with stem cells [21,22], cancer cells [23] and differentiated myeloid cells [24]. In particular, we identified several proteins more normally associated with mesenchymal stem cells [25–27].

2. Materials and Methods

2.1. Sample preparation

New Zealand white rabbits weighing about 1.8 kg were used for this study. The animals were housed in a temperature-controlled environment (23°C) with 60% relative humidity and a 12 hour light/dark cycle. The animals had free access to food and water and were fasted overnight before abdominal dissection. All procedures were conducted in accordance with institutional research animal care and use guidelines. The rabbits were anesthetized with intraperitoneal urethane (1.5 g/kg) and all surgical procedures were performed under general anesthesia.

We searched for BHDs on organ surfaces with the help of a stereoscopic microscope (SZX12, Olympus, Japan). The liquid in each BHD was extracted using a capillary needle and the remaining BHDs were subsequently isolated for proteomic analysis.

2.2. Tryptic digestion and LC-MS/MS analysis

The isolated BHDs were homogenized and sonicated. Then, 10 μg of tissue was loaded onto a 4–12% gradient Tris-Glycine Gel (Invitrogen, Carlsbad, CA). The PAGE-gel of the BHDs was manually segmented into 10 pieces. In-gel digestion of the gel pieces was carried out using 10 ng/μL sequencing grade modified trypsin (Promega, Madison, WI) in 50 μL of 50 mM NH_4HCO_4 buffer (pH 8.0) at 37°C overnight as described in the literature [28]. The liquid from the BHDs was in-sol digested directly under the same conditions as described above. The tryptic peptides were then loaded onto a fused silica microcapillary C18 column (75 μm × 10 cm).

LC separation was conducted under a linear gradient as follows: 0 min, 3% B; 5 min, 3% B; 75 min, 40% B; 80 min, 90% B; 90 min, 90% B; 91 min, 3% B; 110 min, 3% B. The initial solvent condition was 3% solvent B and the flow rate was 200 nL/min. Solvent A was 0.1% formic acid in H_2O and solvent B was 0.1% formic acid in acetonitrile. The separated peptides were subsequently analyzed using a linear ion-trap mass spectrometer, LTQ (ThermoFinnigan, San Jose, CA). The electrospray voltage was set at 2.0 kV, and the threshold for switching from MS to MS/MS was 250. Each full MS scan was followed by three MS/MS scans that focused on the three most pronounced peaks of the full MS scan.

3. Results

Figure 1 shows a stereomicroscopic image of the BHD on the surface of a rabbit intestine from which the Bonghan liquid (BHL) had previously been extracted with a capillary needle.

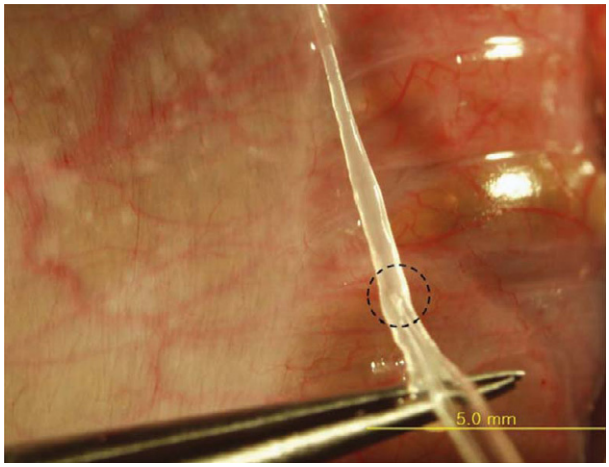


Figure 1 A glass capillary was inserted into a Bonghan duct, held by microforceps, above the large intestine of rabbit using *in situ* and *in vivo* stereomicroscopy (SZX12, Olympus, Japan). The capillary tip (dotted circle) was correctly inserted into the Bonghan duct in order to extract its liquid. The scale bar, located in the bottom right, is 5 mm.

Table 1 Protein list identified in Bonghan liquid

Accession	Name	Score	MW	Peptide
44889024	Serum albumin precursor	340.4	68,865	194
6175087	Serotransferrin precursor	230.3	76,621	33
112876	Alpha-1-antiproteinase F precursor	100.3	45,839	11
122676	Hemoglobin subunit beta-1/2	90.3	16,122	32
125307	Creatine kinase M-type	80.3	43,085	10
113996	Apolipoprotein A-I precursor	80.3	30,573	10
2494026	Histidine-rich glycoprotein precursor	80.2	58,840	9
122475	Hemoglobin subunit alpha-1/2	40.3	15,579	8
12644357	Alpha-2-HS-glycoprotein precursor	40.2	38,363	7
136066	Triosephosphate isomerase (TIM)	40.2	26,609	4
6093713	Glycogen phosphorylase, muscle form	40.2	97,228	4
136466	Transthyretin (prealbumin)	40.2	13,649	4
113608	Fructose-bisphosphate aldolase A	40.2	39,318	7
2851533	Pyruvate kinase isozymes M1/M2	40.2	58,011	4
121088	Ig gamma chain C region	30.2	35,382	6
1722804	Vitamin D-binding protein precursor	30.2	52,877	4
20141354	Beta-enolase	30.2	47,039	3
116596	Complement C3 alpha chain	30.2	81,792	3
62287932	Actin, alpha skeletal muscle	20.2	42,024	3
1169794	Glyceraldehyde-3-phosphate dehydrogenase	20.2	35,799	2
130488651	Serpin peptidase inhibitor, clade F	20.2	54,685	2
130498817	Inter-α-trypsin inhibitor heavy chain2	20.2	1,06,174	2
139654	Vitronectin precursor	20.1	53,909	2
126050	L-lactate dehydrogenase A chain	20.1	36,541	2
125138	Ig kappa-b4 chain C region	10.3	11,036	2
229506	750308A aldolase C	10.3	38,993	2
21542114	Lumican	10.2	21,820	2
122766	Hemoglobin subunit gamma	10.2	16,214	7
89242507	Ig gamma1 constant region	10.2	35,334	2
120095	Fibrinogen alpha chain	10.2	1650	1
1708184	Hemopexin precursor	10.2	51,735	1
118600944	Parvalbumin alpha	10.2	12,057	1
126723362	ATS-22	10.2	50,511	1
51703336	Ubiquitin	10.2	8560	1
552374	Alpha-globin protein	10.2	2868	1
130676	Serum paraoxonase/arylesterase 1	10.2	39,984	1
109259	Ig alpha chain C region (fragment)	10.2	35,909	1
2851405	Myosin light chain kinase, smooth muscle	10.1	1,25,641	1
1710096	Plasma retinol-binding protein precursor	10.1	23,087	1
549158	UDP-glucuronosyltransferase 2B13 precursor	10.1	60,512	1
20178272	Tropomyosin beta chain	10.1	32,817	1
126722957	Alpha-1-antiproteinase S-1 precursor	10.1	45,721	1
130483559	Farnesoid X activated receptor	10.1	55,327	1
30315907	Eukaryotic translation initiation factor 4 gamma 2	10.1	1,02,255	1
9910666	Clusterin precursor (Apolipoprotein J)	10.1	51,818	1
130502756	Ryanodine receptor	10.1	5,51,575	3
125295	Creatine kinase B-type	10.1	42,636	1
3789966	Fibrinogen A-alpha chain	10.1	41,212	1
125128	6-phosphofructokinase, muscle type	10.1	85,149	1
126723185	Soluble adenylyl cyclase	10.1	1,85,398	1
127805	Sodium/glucose cotransporter 1	10.1	73,031	1
1703316	Annexin A1 (Annexin I) (Lipocortin I)	10.1	38,711	1
126722591	Caldesmon 1	10.1	61,445	1
30315951	Probable phospholipid-transporting ATPase IF	10.1	1,33,364	1
5739088	Hensin	10.1	29,780	1
75052894	LIM and SH3 domain protein 1	10.1	29,916	1

(*Contd*)

Table 1 Continued

Accession	Name	Score	MW	Peptide
1168847	T-cell surface glycoprotein CD4 precursor	10.1	50,854	1
26006805	Potassium voltage-gated channel subfamily H member 2	10.1	1,26,882	1
126723568	ACAP2 protein	10.1	87,846	1
547983	Myosin heavy chain, embryonic smooth muscle isoform	10.1	58,084	1
47605964	Rho-associated protein kinase 1	10.1	1,58,247	1
232178	Serine hydroxymethyltransferase, cytosolic	10.1	52,942	1
1384097	Macrophage migration inhibitory factor-related protein-8	10.1	8376	1
55976305	Sodium channel protein type 9 subunit alpha	10.1	2,25,603	1
17366976	Glucose-6-phosphate isomerase (GPI)	10.1	62,707	1
50402101	Eukaryotic peptide chain release factor subunit 1	10.1	49,000	1
115055	Bleomycin hydrolase (BLM hydrolase)	10.1	32,558	1
27373403	Antibody variable domain	10.1	12,674	1
123255522	betaB2-crystallin	10.1	23,389	1
125987	Lipopolysaccharide-binding protein precursor	10.1	53,968	1

3.1. Proteomic analysis of the Bonghan liquid

We investigated the BHL using ESI-MS/MS coupled with nano LC. The acquired MS/MS spectra were searched using SEQUEST's rabbit database, which listed 7490 proteins. Proteomic analysis of BHL identified 70 proteins (Table 1) and the criteria of the SEQUEST search were as mentioned in the Materials and Methods section.

Orthologous conversion was necessary to translate rabbit proteins into human proteins. This introduced a set of well-known limitations. The orthologous conversion scheme was as shown in Figure 2. For the orthologous conversion, we attempted a blast similarity operation that translated rabbit proteins in Table 1 into human proteins. The corresponding human proteins are listed in Table 2. Further data on the BHD are provided in the Supplementary Information on the JAMS web server.

After the conversion, the corresponding human proteins were clustered according to their involvement with biological processes, molecular functions and cellular compartmentalization in GO using DAVID and Cytoscape (Figure 3). In the cases of biological processes, the categories of 'localization,' 'response to stimuli' and 'metabolism' accounted for the majority of the proteins.

'Localization' refers to any process by which a cell or a cellular entity, such as a protein complex or organelle, is transported to and/or maintained in a specific location. Proteins in this category included annexin AI, ATPase class VI, caldesmon, vitamin D binding protein, hemoglobin, hemopexin, transferrin and more. 'Response to stimulus' refers to a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression etc.) that occurs as a result of exposure to a stimulus. The 'Response to stimulus' category

Figure 2 Protein conversion using the blast similarity operation. The process consisted of three steps: (1) obtaining the amino acid sequence of a given rabbit protein; (2) running the blast similarity operation; and (3) selecting the corresponding human protein that exhibited the lowest E-value. For the blast similarity operation, the human 'RefSeq protein' was used as the database for blast searching and 'BLASTP' was applied to compare protein sequences. The protein with the lowest E-value was selected as the best match. If two or more proteins with the same lowest E-value were identified, the upper listed protein was arbitrarily chosen.

included albumin, annexin AI, bleomycin hydrase, clusterin, crystalline and others. Some proteins, such as annexin AI, belong to two classes. We note that many proteins were related to metabolic processes such as carbohydrate, alcohol and other cellular catabolism.

In the context of molecular function, 'binding' and 'transporter activity' accounted for almost all of the BHL proteins. With respect to the cellular

Table 2 Full conversion list from Bonghan liquid proteins of rabbit onto correspondent human proteins

Rabbit		Human		Similarities	
Accession	Name	Accession	Name	E-value	Positives
44889024	Serum albumin precursor	4502027	Albumin precursor	0	536/608 (88%)
6175087	Serotransferrin precursor	4557871	Transferrin	0	597/678 (88%)
112876	Alpha-1-antiproteinase F precursor	50363219	Serine (or cysteine) proteinase inhibitor, clade A	e-147	314/388 (80%)
122676	Hemoglobin subunit beta-1/2	4504349	Beta globin	2.00E-74	140/147 (95%)
125307	Creatine kinase M-type	21536288	Muscle creatine kinase	0	364/381 (95%)
113996	Apolipoprotein A-I precursor	4557321	Apolipoprotein A-I preproprotein	e-113	226/253 (89%)
2494026	Histidine-rich glycoprotein precursor	4504489	Histidine-rich glycoprotein precursor	e-117	248/338 (73%)
122475	Hemoglobin subunit alpha-1/2	4504345	Alpha 2 globin	9.00E-67	129/142 (90%)
12644357	Alpha-2-HS-glycoprotein precursor	156523970	Alpha-2-HS-glycoprotein	e-105	230/346 (66%)
136066	Triosephosphate isomerase (TIM)	4507645	Triosephosphate isomerase 1	e-140	247/248 (99%)
6093713	Glycogen phosphorylase, muscle form	5032009	Glycogen phosphorylase	0	816/842 (96%)
136466	Transthyretin (prealbumin)	4507725	Transthyretin	1.00E-61	123/127 (96%)
113608	Fructose-bisphosphate aldolase A	34577110	Aldolase A	0	345/364 (94%)
2851533	Pyruvate kinase isozymes M1/M2	33286422	Pyruvate kinase 3 isoform 2	0	524/531 (98%)
121088	Ig gamma chain C region	33438594	Major histocompatibility complex, class II, DQ beta 2	4.00E-08	69/149 (46%)
1722804	Vitamin D-binding protein precursor	32483410	Vitamin D-binding protein precursor	0	430/473 (90%)
20141354	Beta-enolase	153267427	Enolase 3	0	418/434 (96%)
116596	Complement C3 alpha chain	115298678	Omplement component 3 precursor	0	645/726 (88%)
62287932	Actin, alpha skeletal muscle	4501881	Alpha 1 actin precursor	0	377/377 (100%)
1169794	Glyceraldehyde-3-phosphate dehydrogenase	7669492	Glyceraldehyde-3-phosphate dehydrogenase	0	321/332 (96%)
130488651	Serpin peptidase inhibitor, clade F	115583663	Alpha-2-plasmin inhibitor	0	355/476 (74%)
130498817	Inter-α-trypsin inhibitor heavy chain2	70778918	Inter-alpha globulin inhibitor H2 polypeptide	0	849/946 (89%)
139654	Vitronectin precursor	88853069	Vitronectin precursor	0	368/483 (76%)
126050	L-lactate dehydrogenase A chain	5031857	Lactate dehydrogenase A	e-173	314/332 (94%)
125138	Ig kappa-b4 chain C region	13399298	Immunoglobulin lambda-like polypeptide 1 isoform a precursor	1.00E-11	56/100 (56%)
229506	750308A aldolase C	34577110	Aldolase A	0	334/363 (92%)
21542114	Lumican	4505047	Lumican precursor	e-102	190/192 (98%)
122766	Hemoglobin subunit gamma	4885393	Epsilon globin	1.00E-68	136/147 (92%)
89242507	Ig gamma1 constant region	13399298	Immunoglobulin lambda-like polypeptide 1 isoform a precursor	3.00E-08	56/121 (46%)
1708184	Hemopexin precursor	11321561	Hemopexin	0	401/464 (86%)
118600944	Parvalbumin alpha	4506335	Parvalbumin	8.00E-42	92/110 (83%)

(*Contd*)

Table 2 Continued

Rabbit		Human		Similarities	
Accession	Name	Accession	Name	E-value	Positives
126723362	ATS-22	50363219	Serine (or cysteine) proteinase inhibitor, clade A	e-146	311/388 (80%)
51703336	Ubiquitin	113423966	PREDICTED: similar to Ubiquitin-63E CG11624-PA,	1.00E-37	76/76 (100%)
552374	Alpha-globin protein	4504345	Alpha 2 globin	1.00E-07	24/25 (96%)
130676	Serum paraoxonase/arylesterase 1	19923106	Paraoxonase 1	e-176	322/355 (90%)
109259	Ig alpha chain C region (fragment)	94538335	Signal-regulatory protein gamma isoform 1 precursor	3.00E-09	74/185 (40%)
2851405	Myosin light chain kinase, smooth muscle	116008188	Myosin light chain kinase isoform 2	0	810/923 (87%)
1710096	Plasma retinol-binding protein precursor	55743122	Retinol-binding protein 4, plasma precursor	e-103	181/188 (96%)
549158	UDP-glucuronosyltransferase 2B13 precursor	4507821	UDP glucuronosyltransferase 2 family, polypeptide B17	0	426/507 (84%)
20178272	Tropomyosin beta chain	42476296	Tropomyosin 2 (beta) isoform 1	2.00E-96	200/284 (70%)
126722957	Alpha-1-antiproteinase S-1 precursor	50363219	Serine (or cysteine) proteinase inhibitor, clade A	e-146	311/388 (80%)
130483559	Farnesoid X activated receptor	4826980	Nuclear receptor subfamily 1, group H, member 4	0	435/446 (97%)
30315907	Eukaryotic translation initiation factor 4 gamma 2	4503539	Eukaryotic translation initiation factor 4 gamma	0	760/889 (85%)
9910666	Clusterin precursor (Apolipoprotein J)	42716297	Clusterin isoform 1	0	389/448 (86%)
130502756	Ryanodine receptor	126032338	Ryanodine receptor 3	0	4500/4874 (92%)
125295	Creatine kinase B-type	21536286	Brain creatine kinase	0	361/381 (94%)
3789966	Fibrinogen A-alpha chain	11761629	Fibrinogen, alpha polypeptide isoform alpha preproprotein	2.00E-96	251/446 (56%)
125128	6-phosphofructokinase, muscle type	4505749	Phosphofructokinase, muscle	0	758/780 (97%)
126723185	Soluble adenylyl cyclase	8923844	Soluble adenylyl cyclase	0	1405/1610 (87%)
127805	Sodium/glucose cotransporter 1	4507031	Solute carrier family 5 (sodium/glucose cotransporter)	0	555/647 (85%)
1703316	Annexin A1 (Annexin I) (Lipocortin I)	4502101	Annexin I	e-180	331/346 (95%)
126722591	Caldesmon 1	15149463	Caldesmon 1 isoform 4	3.00E-84	160/174 (91%)
30315951	Probable phospholipid-transporting ATPase IF	62632750	ATPase, class VI, type 11B	0	1108/1169 (94%)
5739088	Hensin	148539842	Deleted in malignant brain tumors 1 isoform b precursor	e-105	186/242 (76%)
75052894	LIM and SH3 domain protein 1	5453710	LIM and SH3 protein 1	e-131	230/264 (87%)
1168847	T-cell surface glycoprotein CD4 precursor	10835167	CD4 antigen precursor	e-136	328/460 (71%)
26006805	Potassium voltage-gated channel subfamily H	4557729	Voltage-gated potassium channel, subfamily H	0	974/1161 (83%)
126723568	ACAP2 protein	40254842	Centaurin, beta 2	0	742/778 (95%)

547983	Myosin heavy chain, embryonic smooth muscle isoform	41406064	Myosin, heavy polypeptide 10, non-muscle	0	397/500 (79%)
47605964	Rho-associated protein kinase 1	4885583	Rho-associated, coiled-coil containing protein kinase 1	0	1282/1354 (94%)
232178	Serine hydroxymethyltransferase, cytosolic	22547186	Serine hydroxymethyltransferase 1 (soluble) isoform 1	0	453/484 (93%)
1384097	Macrophage migration inhibitory factor-related protein-8	21614544	S100 calcium-binding protein A8	1.00E-24	61/74 (82%)
55976305	Sodium channel protein type 9 subunit alpha	4506813	Sodium channel, voltage-gated, type IX, alpha	0	1636/1961 (83%)
17366976	Glucose-6-phosphate isomerase (GPI)	18201905	Glucose phosphate isomerase	0	543/558 (97%)
50402101	Eukaryotic peptide chain release factor subunit 1	4759034	Eukaryotic translation termination factor 1	0	437/437 (100%)
115055	Bleomycin hydrolase (BLM hydrolase)	4557367	Bleomycin hydrolase	e-158	270/277 (97%)
27373403	Antibody variable domain	89062025	PREDICTED: similar to immunoglobulin iota chain	0.002	43/103 (41%)
123255522	BetaB2-crystallin	4503063	Crystallin, beta B2	e-108	186/205 (90%)
125987	Lipopolysaccharide-binding protein precursor	31652249	Lipopolysaccharide-binding protein precursor	e-162	350/451 (77%)

compartmentalization category, 'extracellular region' and 'protein complex' were dominant and this could be evidence that our analyses were appropriate. In Table 3, GO clustering of human BHL proteins are presented in terms of biological processes, molecular function and cellular compartmentalization.

3.2. Proteomic analysis of BHD

We also performed proteomic analyses of the BHD. The number of proteins identified in BHD was 207 (Table 4). The rabbit proteins identified in the BHD and their corresponding human proteins are shown in Table 5. GO clustering of the BHD proteins in Table 6 was achieved as in the case of BHL (Figure 4). Tables 4–6 are Supplementary Information.

For biological processes, we identified several categories: (1) 'metabolic process' (annexin AI, ATP synthase-H^+ transporting, ATPase-Na^+/K^+ transporting, carbonic anhydrase II, cytochrome b5 type A, fructose-1, 6-bisphosphatasae, lactate dehydrogenase A, etc.), (2) 'localization' (annexin AI, calreticulin, cytochrome b5 type A, vitamin D binding protein, hemoglobin, hemopexin, transferrin, vimentin, etc.), (3) 'response to stimulus' (albumin, aldo-keto reductase family 1, annexin AI, annexin AII, arginase type II, clusterin, glucose phosphate isomerase, isocitrate dehydrogenase 1, protein phosphatase 2, etc.), and (4) 'cell development' (actin alpha1, actin beta, albumin, annexin AI, apolipoprotein E, calreticulin, clusterin, protein disulfide isomerase family A, protein kinase C, protein phosphatase 2, etc.).

With regard to molecular function, 'binding,' 'catalytic activity' and 'transporter activity' were prominent. In the case of cellular compartmentalization, the 'intracellular part' was dominant and other categories such as 'extracellular region,' 'macromolecular complex' and 'cell fraction' were also evidenced. The GO clustering of human BHD proteins is shown in Table 6, categorized again in terms of biological processes, molecular functions and cellular compartmentalization.

4. Discussion

Our study used proteomics to conduct molecular investigations into the BHD. The rabbit was selected to achieve our minimum required sample volume, but the database for functional clustering of proteins was incomplete. Therefore, the rabbit proteins identified in BHL and the BHD had to be associated with corresponding human proteins. A blast similarity operation was used to achieve this association. This operation uses a search strategy to match a given rabbit protein with the exact or closest amino acid sequence in a human protein

Table 3 List of gene ontology clustering of Bonghan liquid proteins in terms of biological process, molecular function and cellular compartment

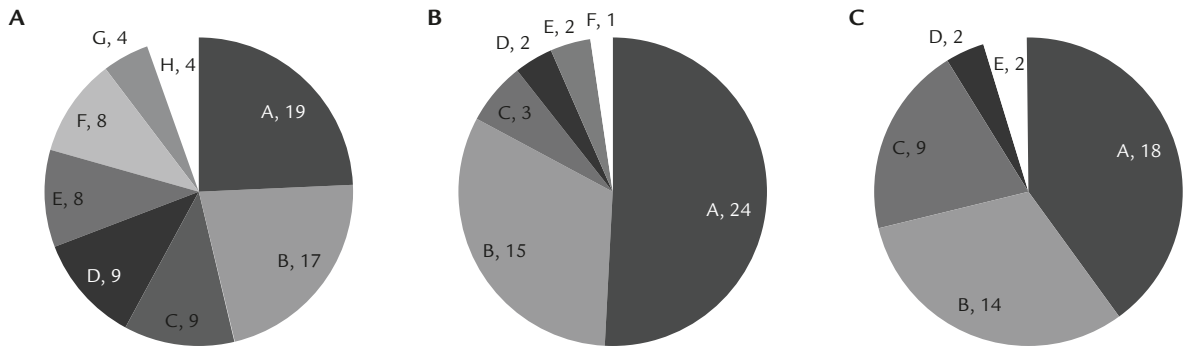
Accession	Name	GO		
		Biological process	Molecular function	Cellular component
4502027	Albumin precursor	Localization, response to stimulus	Binding	Extracellular region, protein complex
4557871	Transferrin	Localization	Binding	Extracellular region
50363219	Serine (or cysteine) proteinase inhibitor, clade A	Response to stimulus		Extracellular region
4504349	Beta globin	Localization, cellular biosynthetic process, circulation	Binding, transporter activity	Protein complex, cytosol
21536288	Muscle creatine kinase	Cellular biosynthetic process	Phosphotransferase activity, nitrogenous group as acceptor	
4557321	Apolipoprotein A-I preproprotein	Localization, alcohol metabolic process, circulation	Transporter activity	Extracellular region
4504489	Histidine-rich glycoprotein precursor			Extracellular region
4504345	Alpha 2 globin	Localization	Binding, transporter activity	Protein complex, cytosol
4507645	Triosephosphate isomerase 1	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process, cellular biosynthetic process	intramolecular oxidoreductase activity, interconverting aldoses and ketoses	
5032009	Glycogen phosphorylase	Cellular carbohydrate metabolic process, cellular catabolic process	Binding	
4507725	Transthyretin	Localization	Binding, transporter activity	Extracellular region
34577110	Aldolase A	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process, muscle contraction		
33286422	Pyruvate kinase 3 isoform 2	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process	Binding	Cytosol
33438594	Major histocompatibility complex, class II, DQ beta 2	Response to stimulus	MHC class II receptor activity	Protein complex
32483410	Vitamin D-binding protein precursor	Localization	Binding, transporter activity	Extracellular region
4501881	Alpha 1 actin precursor	Muscle contraction	Binding	Striated muscle thin filament, stress fiber
7669492	Glyceraldehyde-3-phosphate dehydrogenase	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process	Binding	

70778918	Inter-alpha globulin inhibitor H2 polypeptide	Cellular carbohydrate metabolic process		Extracellular region
88853069	Vitronectin precursor	Response to stimulus		Extracellular region
5031857	Lactate dehydrogenase A	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process		Cytosol
13399298	Immunoglobulin lambda-like polypeptide 1 isoform a precursor	Response to stimulus		
34577110	Aldolase A	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process, muscle contraction		
4505047	Lumican precursor			Extracellular region
4885393	Epsilon globin	Localization	Binding, transporter activity	Protein complex, cytosol
13399298	Immunoglobulin lambda-like polypeptide 1 isoform a precursor	Response to stimulus		
11321561	Hemopexin	Localization	Binding, transporter activity	Extracellular region
50363219	Serine (or cysteine) proteinase inhibitor, clade A	Response to stimulus		Extracellular region
4504345	Alpha 2 globin	Localization	Binding, transporter activity	Protein complex, cytosol
19923106	Paraoxonase 1	Response to stimulus	Transporter activity	Extracellular region
55743122	Retinol-binding protein 4, plasma precursor	Localization, response to stimulus	Binding, transporter activity	Extracellular region
42476296	Tropomyosin 2 (beta) isoform 1		Binding	Striated muscle thin filament
50363219	Serine (or cysteine) proteinase inhibitor, clade A	Response to stimulus		Extracellular region
4503539	Eukaryotic translation initiation factor 4 gamma, 2 isoform 1	Cellular biosynthetic process		Protein complex
42716297	Clusterin isoform 1	Response to stimulus		Extracellular region
21536286	Brain creatine kinase		Phosphotransferase activity, nitrogenous group as acceptor	
11761629	Fibrinogen, alpha polypeptide isoform alpha preproprotein	Response to stimulus, circulation	Binding	Extracellular region, protein complex
4505749	Phosphofructokinase, muscle	Cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process		Protein complex, cytosol
8923844	Soluble adenylyl cyclase	Cellular biosynthetic process		Cytosol
4507031	Solute carrier family 5 (sodium/glucose cotransporter), member 1	Localization	Binding, transporter activity	

(*Contd*)

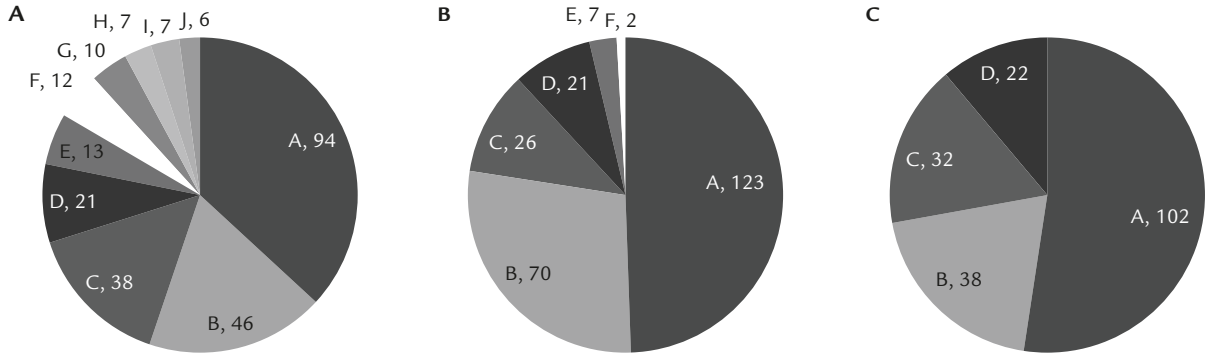
Table 3 Continued

Accession	Name	GO		
		Biological process	Molecular function	Cellular component
4502101	Annexin I	Localization, response to stimulus	Binding	
15149463	Caldesmon 1 isoform 4	Localization, muscle contraction	Binding	
62632750	ATPase, class VI, type 11B	Localization	Transporter activity	
5453710	LIM and SH3 protein 1	Localization	Binding, transporter activity	
10835167	CD4 antigen precursor	Response to stimulus, cellular biosynthetic process	Protein complex	
4557729	Voltage-gated potassium channel, subfamily H, member 2 isoform a precursor	Localization, circulation, muscle contraction	Binding, transporter activity, phosphotransferase activity, nitrogenous group as acceptor	Protein complex
41406064	Myosin, heavy polypeptide 10, non-muscle		Binding	Protein complex, stress fiber
22547186	Serine hydroxymethyltransferase 1 (soluble) isoform 1	Cellular catabolic process	Binding	Cytosol
21614544	S100 calcium-binding protein A8	Response to stimulus		Extracellular region
4506813	Sodium channel, voltage-gated, type IX, alpha	Localization	Binding, transporter activity	Protein complex
18201905	Glucose phosphate isomerase	Response to stimulus, cellular carbohydrate metabolic process, cellular catabolic process, alcohol metabolic process, cellular biosynthetic process	Intramolecular oxidoreductase activity, interconverting aldoses and ketoses	Extracellular region
4759034	Eukaryotic translation termination factor 1	Cellular biosynthetic process		
4557367	Bleomycin hydrolase	Response to stimulus	Bleomycin hydrolase activity	
4503063	Crystallin, beta B2	Response to stimulus		
31652249	Lipopolysaccharide-binding protein precursor	Localization, response to stimulus		Extracellular region



Biological process	Molecular function	Cellular component
A. Localization B. Response to stimulus C. Cellular carbohydrate metabolic process D. Cellular catabolic process E. Alcohol metabolic process F. Cellular biosynthetic process G. Circulation H. Muscle contraction	A. Binding B. Transporter activity C. Phosphotransferase activity, nitrogenous group as acceptor D. Intramolecular oxidoreductase activity, interconverting aldoses and ketoses E. MHC class II receptor activity F. Bleomycin hydrolase activity	A. Extracellular region B. Protein complex C. Cytosol D. Striated muscle thin filament E. Stress fiber

Figure 3 Clustering of proteins identified in Bonghan liquid. The clustering was executed consistent with the three GO domains of biological processes (A), molecular functions (B) and cellular compartmentalization (C). The pie chart values indicate the numbers of proteins included in each category. Certain proteins belonged to more than two categories and as a result the total number of proteins in the pie chart is different from the number of proteins identified in Table 1.



Biological process	Molecular function	Cellular component
A. Metabolic process B. Localization C. Response to stimulus D. Cell development E. Regulation of biological quality F. Cellular component assembly G. Regulation of multicellular organismal process H. Circulation I. Muscle contraction J. Actin cytoskeleton organization and biogenesis	A. Binding B. Catalytic activity C. Transporter activity D. Structural molecule activity E. Enzyme inhibitor activity F. MHC class II receptor activity	A. Intracellular part B. Extracellular region C. Macromolecular complex D. Cell fraction

Figure 4 Clustering of proteins identified in Bonghan duct. Note the similarity with Figure 3.

database, as described in Figure 2. We note that this process is limited because of the possibility of protein conversions that do not maintain cross-species functionality. However, orthologous conversions have been successfully used in other cases where species-specific protein databases were unavailable [29]. In this study, almost all the corresponding human proteins were identical or similar to the original rabbit proteins when we conducted a manual survey of their functionalities.

The human proteins used in the blast similarity search were clustered into three domains, namely, biological processes, molecular functions and cellular compartmentalization. In this search we used Cytoscape, a free software program (www.cytoscape.org). In the "biological processes" category, it was clear that metabolic processes, especially carbohydrate-based ones, were very prominent categories in both BHL and the BHD. Other scientists have reported proteomic analyses of blood and lymph that do not show these remarkable carbohydrate-based processes [18,19]. Another study concluded that the proteome of blood vessels also included few carbohydrate-based processes [20]. Our findings imply that either BHL or the BHD must require an efficient energy supply. Proteomic analyses of certain cell types such as stem cells, cancer cells and differentiated myeloid cells, all of which show vigorous proliferation or differentiation, have shown a similar abundance of carbohydrate- or energy-related processes [21–24].

We note the existence of proteins related to (1) the recruitment of mesenchymal stem cells (MSC) [25], (2) the cell processes in MSCs (Ezrin, Actinin, myosin) [26], and (3) the differentiation of MSC/myofibroblasts (alpha-smooth muscle actin, CD147) [27]. These protein profiles suggest that BHDs located on the organ surface has a role as a temporary depot and point of differentiation of stem cells for tissue regeneration.

In conclusion, our proteomic analysis of rabbit BHL and BHDs suggests that proteins can be categorized in terms of their involvement with biological processes, molecular functions and cellular compartmentalization following orthologous conversion to human proteins. The abundance of carbohydrate-based processes was surprising. This fact distinguished the proteomic fingerprint of the Bonghan system from that of blood, lymph or blood vessel physiology, but was similar to that of stem cells, especially mesenchymal stem cells, cancer cells and differentiated myeloid cells.

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Tables of Data

Database analysis

All MS/MS spectra recorded were searched on rabbit database, obtained from the taxonomy site in NCBI (www.ncbi.nlm.nih.gov/sites/entrez?db= taxonomy), by using the SEQUEST algorithm. Dynamic modifications were permitted for oxidized methionine (+16Da), carboxyamidomethylated cysteine (+57Da). SEQUEST criteria for peptide selection were *Xcorr*, which must be greater than 1.8, 2.3 and 3.5 for +1, +2 and +3 charge state respectively, and delta Cn above 0.1. The parameter for selection of identified proteins was a protein consensus score which was above 10.1. Due to the poorness of the functional DB for rabbits, it was needed to orthologously convert the rabbit proteins into corresponding human proteins. Blast similarity operation, provided by NCBI, was used for this purpose. The resulting human proteins were clustered according to biological process, molecular function and cellular compartment with the help of Cytoscape (www.cytoscape.org) which was freely obtained from the web.

Table 1 List of proteins identified in Bonghan liquid. The accession column refers to GI accession number, the score refers to the consensus score from SEQUEST, MW is molecular weight and peptide is the number of peptides identified by proteomic analysis. The X correlation value (greater than 1.8, 2.3 and 3.5 for +1, +2 and +3 charges, respectively), delta Cn (greater than 0.1), and number of top matches (only 1) were used as criteria for peptide selection. The consensus score was utilized for filtering proteins. Proteomic analysis of Bonghan liquid was conducted twice and this table shows results from just one experiment. Only part of the protein conversion results are listed in this table. The total protein conversion list is shown in Table 2, in the supplementary material.

Table 2 Conversion of Bonghan liquid proteins from rabbit into corresponding human proteins. E-value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower E-value indicates the more valid result. 'Positive' means positive matrix score which is expressed as the ratio of the number of identified or conserved amino acids to the total number of amino acids of each protein. In the process of protein conversion, one protein from rabbits (part of fibrinogen alpha chain; GI number, **120095**) was missed because the short peptide consists of only 16 amino acids.