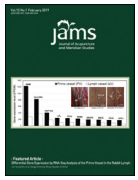
Available online at www.sciencedirect.com

Journal of Acupuncture and Meridian Studies

journal homepage: www.jams-kpi.com



Research Article

Differential Gene Expression by RNA-Seq Analysis of the Primo Vessel in the Rabbit Lymph

Jun-Young Shin, Sang-Heon Choi, Da-Woon Choi, Ye-Jin An, Jae-Hyuk Seo, Jong-Gu Choi, Min-Suk Rho, Sang-Suk Lee*

Department of Oriental Biomedical Engineering, Sangji University, Wonju, Republic of Korea
Available online 28 October 2018

Received: May 31, 2018
Revised: Jul 26, 2018
Accepted: Oct 23, 2018

KEYWORDS
gene expression level;
lymph node;
primo connectome;
RNA-Seq analysis

Abstract
For the connectome of primo vascular system, some long-type primo vessels dyed with Alcian blue injected into inguinal nodes, abdominal node, and axially nodes were visualized, which passed over around the vena cava of the rabbit. The Alcian blue dye revealed primo vessels and colored blue in the rabbit lymph vessels. The length of long-type primo vessels was 18 cm on average, of which diameters were about 20–30 μm, and the lymph vessels had diameters of 100–150 μm. Three different tissues of pure primo vessel, mixed primo + lymph vessel, and only lymph vessel were made to undergo RNA-Seq analysis by next-generation sequencing. We also analyzed differentially expressed genes (DEGs) from the RNA-Seq data, in which 30 genes of the primo vessels, primo + lymph vessels, and lymph vessels were selected for primo marker candidates. From the plot of DEG analysis, 10 genes had remarkably different expression pattern on the Group 1 (primo vessel) vs Group 3 (lymph vessel). With Fragments Per Kilobase of exon per Million the cutoff p-value for each gene was < 0.05. Fragments Per Kilobase of exon per Million of the 10 genes such as IGHM, HLA-DRA, HIST1H41, LPL, CD36, SRGN, DGAT2, SNCG, CD48, and GPD1 for primo vessels compared with those of lymph vessels increased twice or thrice. These results suggest that the selected genes could be used for the specific marker to construct primo connectome of circuit system in the rabbit.

* Corresponding author. Department of Oriental Biomedical Engineering, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea. Tel.: +82 33 730 0415; Fax: +82 33 738 7610.
E-mail: sslee@sangji.ac.kr (S.-S. Lee).

pISSN 2005-2901 eISSN 2093-8152
https://doi.org/10.1016/j.jams.2018.10.008

1. Introduction

The first circulatory system of the human body founded by William Harvey in 1628 is the cardiovascular system that plays the role of nutrition supply and maintenance function for all the cells as blood flows in the heart and through whole organ vessels. Another circulatory system is the lymphatic circulatory system discovered by Alexander Monroe in 1757, which is responsible for the body's immune system and hormones and for transporting extra body fluid called lymphatic fluid, from the tissues of the body back into the bloodstream [1,2]. And, the other circulatory system, which is called the third circulation system, is based on the reality of meridians. It will be a very important research project in the field of medicine, a core of healing and regeneration of human life.

The study of the anatomical entity of the meridian circulatory system was initiated and discovered by Dr. Kim Bong-Han, a North Korean scientist in the 1960s, who discovered a new vascular system [3–7]. In the early 2000s, the research team of Professor Soh Kwang-Sup in Seoul National University reproduced several experimental results of Bong-Han's circulatory system which was called the primo vascular system (PVS) [8,9]. It became a worldwide sensation that a new network structure in the form of micro ducts constitutes the mammalian organs. All the structures and tissues of the human body have a function corresponding to each other [3]. The isolation and morphological characterization of primo vessels in lymphatic vessels of the rabbit have been reported by various research teams [10,11]. However, it is still necessary to study tools or criteria for the identification of the primo tissue in surface of the organs and lymphatic system.

There are barriers to the entry of research into the quantitative unit (μg) of primo vessel tissue inside the lymph vessel for the production of specific antibodies and the analysis of gene and protein to identify and monitor the primo vessels [12]. Therefore, it is necessary to study the basic physical properties of primo biomolecules and the fusion of molecular biology and oriental medicine. For many years, genes related to European oyster rabbits (*Oryctolagus cuniculus*) have been studied and explored by world-renowned researchers. They are formally identified with the characteristics and functions of these genes. The condition in which a primo vessel was present in the rabbit can be established among the genetic sequences. Potential candidate genes of the rabbit's lymphatic node and vessel were used for gene expression analysis in this study [12].

The purpose of this study was to investigate the basic gene properties of the rabbit having a primo circulatory system. First, we investigated the connectome of primo vessels between two inguinal nodes and two axillary nodes in the lymphatic system. To develop monoclonal antibodies of primo vessels based on the results of RNA-Seq (sequencing) analysis and gene transcripts by distinguishing primo and lymphatic vessels, we investigated the potentially remarkable marker genes of primo vessels and compared their expression levels.

2. Materials and methods

2.1. Sample preparation

For the laboratory animals, New Zealand white female rabbits (approximately 1.8 kg) were purchased from Dae Han Bio Link Co., Ltd (Eumsung, Chungcheongnam-do, Republic of Korea). All procedures conformed to the ethical regulations for animal experiments constituted by the Institutional Regulation Board of Sangji University (approval number 2017-19) [10,11]. Two rabbits were sacrificed for anatomy experiments in the first week, and the other 8 rabbits were sacrificed in one month. Each rabbit was kept under constant temperature and humidity conditions (23°C, relative humidity 60%), with a 12-hour light–dark cycle. All rabbits were deprived of food and water for 1 day before sacrificing.

The rabbits used in the anatomical experiment were sacrificed by injecting 3.0 mL of a mixture of Zoletil (2.5 mL) and Rompun (0.5 mL) into the leg muscle. Especially because the Zoletil reagent is classified as a controlled substance, we obtained approval from the Ministry of Food and Drug Safety, Republic of Korea. The adipose tissues surrounding the inferior vena cava, inguinal region of two legs, and axial region of two arms were then separated and removed. Then the inguinal and axial primo vessels, inside lymph vessels near the inferior vena cava, which were stained blue were visualized. Images of the PVS under a microscope image analysis system (JSZ-7XT; Samwon, Seoul, Republic of Korea) were captured using a charge-coupled device camera (DP70; Olympus, Tokyo, Japan). Other processes of dissection were performed under general anesthesia [10–13].

Alcian blue (AB) solution was prepared by dissolving 0.1 g of AB (Sigma, St. Louis, MO, USA) in 10 mL of phosphate-buffered saline (pH 7.4) which was filtered by using a 0.45-μm membrane filter (Merck Millipore, Darmstadt, Germany) using a syringe (BD, Franklin Lakes, NJ, USA). After the sides of the rabbit's inguinal region in the two legs and axial region in the two arms had been incised, AB solution, preheated to 37°C in a water bath, was injected into two inguinal lymph nodes, two axial lymph nodes, and one vena cava lymph node [11,13]. All samples consisting of either pure primo vessels or mixed with primo vessels and lymph vessels were immersed in 0.3 mL of TRIzol and stored in the liquid nitrogen dewar.

2.2. Experimental groups and combinations

This study completed the basis for the invention by examining the genes in the primo vasculature. The following three experimental groups were compared in this study, as shown in Table 1. The first experimental group (Group 1) is a sample containing only the primo vessel. Although the primo vessel in purity was organized as a nonvisible vessel, observation is possible through the spread of blue reagents of AB within 20 minutes. Primo vessels are carefully collected using tweezers. The second experimental group (Group 2) is a sample (lymph vessel + primo vessel) in which a primo vessel and a lymph vessel are mixed after the staining of AB. The

Table 1 The different combinations of three experimental groups, the number of rabbits, and the wet weight of the primo vascular tissue samples in each group.

Experimental groups	Sample contents	No. of rabbits	Volume (μL)	Quantity (μg)	Combinations		
					C-1	C-2	C-3
Group 1	Primo vessel	10	17	1.384	-	Group 1 vs Group 2	Group 1 vs Group 3
Group 2	Primo + lymph vessel	10	17	1.564	Group 1 vs Group 2	-	Group 3 vs Group 2
Group 3	Lymph vessel	10	17	0.934	Group 1 vs Group 3	Group 3 vs Group 2	-

third experimental group (Group 3) is a sample containing only the lymph vessel. Group 3 obtained the nondyeing pure lymph vessel after the staining of AB. The number of rabbits for each experimental group is 10. The different combinations of three experimental groups to analyze the differentially expressed gene (DEGs) analysis for the all genes obtained from RNA-Seq data are C-1, C-2, and C-3 corresponding to Group 1 vs Group 2 and Group 1 vs Group 3, Group 1 vs Group 2 and Group 3 vs Group 2, and Group 1 vs Group 3 and Group 3 vs Group 2, respectively, as noticed in Table 1.

2.3. RNA-Seq (sequencing) experiments

The passed samples of three experimental groups on RNA-quality control (QC) proceed to the library construction stage automatically [15]. Total RNA was extracted from 10 samples, including the pure primo vessels and the composited lymphatic vessels with primo vessels, using the TRIzol LS reagent (Ambion, TX, USA), which satisfied all criteria of sample requirements. In the next stage, the purified mRNAs were disrupted into short fragments, and the double-stranded cDNAs were immediately synthesized [16,17]. The cDNAs were subjected to end repair and poly (A) addition and then connected with sequencing adapters using the TruSeq RNA Sample Prep Kit (for eukaryotes) (Theragen Etex Co., Ltd., Suwon, Republic of Korea). Suitable fragments, automatically purified using a BluePippin 2% agarose gel cassette (Sage Science, MA, USA), were selected as templates for polymerase chain reaction amplification. The final library sizes and qualities were evaluated electrophoretically using an Agilent High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA); the fragment size range was 350—450 bp for three experimental groups [18,19].

3. Results and discussion

3.1. Connectome of primo vessel in the lymphatic system of the rabbit

The primo vessels in previous research studies were observed in the lymph vessels near the vena cava lymph node [13,14]. The connectome of primo vessels in lymph vessels related to the five different lymph nodes of the rabbit as illustrated in Fig. 1. Fig. 1 showed visualization of the PVS in the internal lymph vessel between the inguinal lymph node and axillary lymph node. The primo vessels in the abdominal lymph vessels from two inguinal lymph nodes to two axillary lymph nodes with the middle part of the vena cava lymph node are shown in Fig. 1(A) with a schematic diagram of the vein vascular and lymphatic system in a rabbit. The inguinal lymph node, the abdominal lymph node, and the axillary lymph node were located near the subclavian, the cava vena, and the iliac veins, respectively. Fig. 1(B) showed the right and left axillary nodes, the abdominal node, and the right and left inguinal nodes into which AB solution was injected, which became blue as the AB solution flowed through the lymph node. Fig. 1(C) showed three magnification views of the blue-stained PVS in the lymph vessel. Fig. 1(C) showed the different images of the three primo vessels having a long-type length of 18 cm and a thickness (20—30 μm) much less than that of the lymph vessel that had a thickness of 100—150 μm.

3.2. RNA-Seq data analysis in the primo vessel and lymph vessel

The number of groups required for DEG analysis in the RNA-Seq experiment is three, which is better than two. In other words, the control group is a mixed group, and experimental groups are a pure primo vessel group and a pure lymph vessel group; the number of combined cases to be compared with each other can be increased. The findings from our study are to demonstrate an association with the primo vessel rather than with the lymphatic vessel. For many years, genes related to European oyster rabbits have been studied and explored by world-renowned researchers, and the characteristics and functions of these genes have been formally identified with *Oryctolagus cuniculus*. Thus, we used the "nucleotide" category from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) to select those candidate genes from European oyster rabbits, a category that contains 23,669 sequence entries. The advantage of the mixed group (the primo vessel + the lymph vessel) is that it is possible to supplement the DEG analysis of the pure primo vessel and the pure lymph vessel. In other words, the expression in the mixed group can be verified when the gene expressed in the primo vessel is not expressed in the lymphatic vessel.

Fig. 2 showed three scatter plots of gene expression as the RNA-Seq experimental results for combinations of three different experimental groups: Group 1 (primo vessel), Group 2 (primo vessel + lymph vessel), and Group 3 (lymph

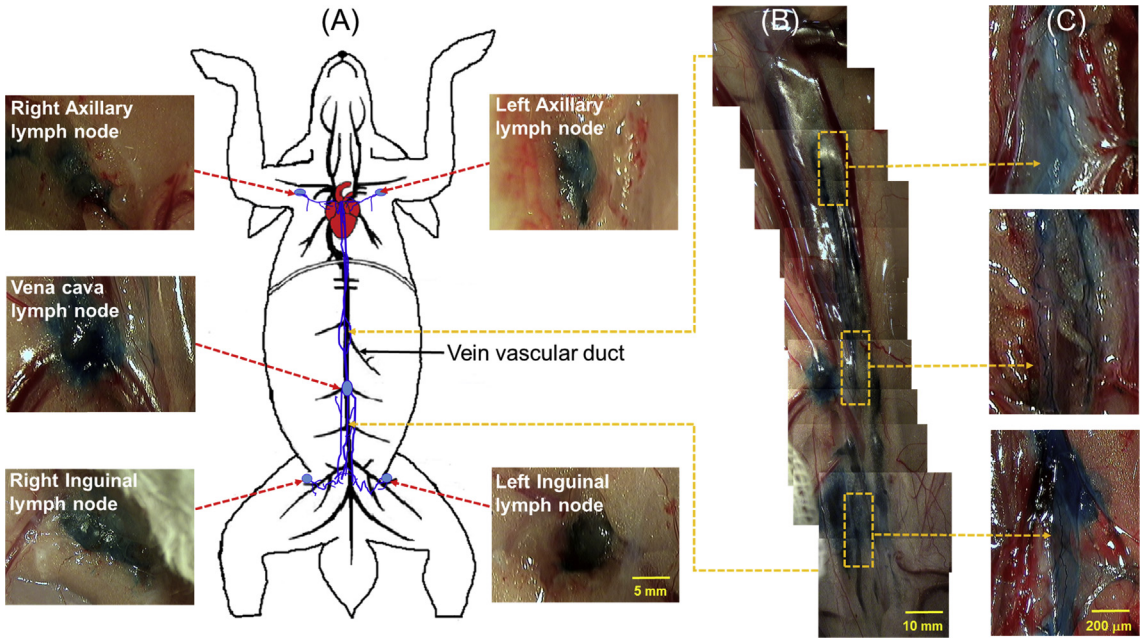


Figure 1 Visualization of the PVS in the internal lymph vessel between the inguinal lymph node and axillary lymph node. (A) A schematic diagram of the vein vascular duct and lymphatic vessel system in a rabbit. The inguinal lymph node, the abdominal lymph node, and the axillary lymph node were located near the subclavian, the cava vena, and the iliac veins, respectively. (B) The right and left axillary node, the abdominal node, and the right and left inguinal node into which AB solution was injected which became blue as the AB flowed through the lymph duct. (C) Three magnification views of the blue-stained PVS in the lymph vessel. Note that the primo vessel was much thinner than the lymph vessel. The thickness of the lymph vessel was 100—150 μm, whereas that of the primo vessel was 20—30 μm.

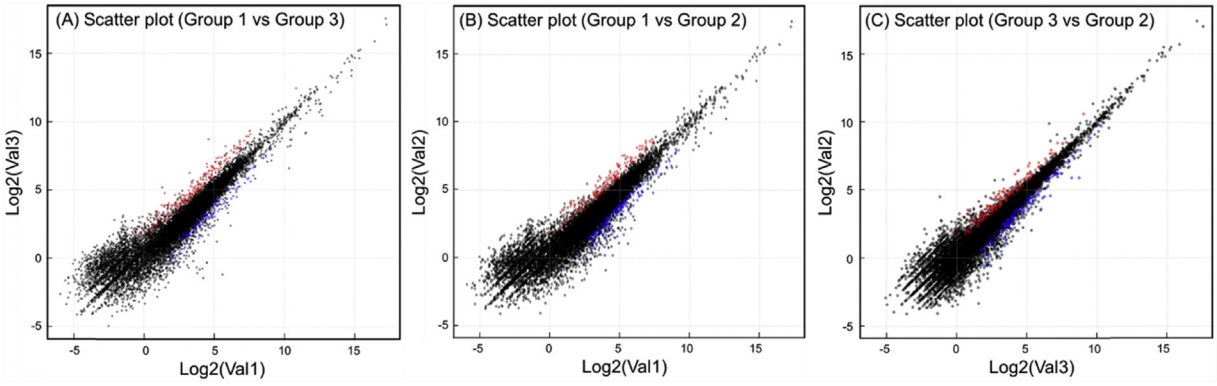


Figure 2 Scatter plots of gene expression for combinations of three different experimental groups: Group 1(primo vessel), Group 2(primo vessel + lymph vessel), and Group 3(lymph vessel) by RNA-Seq data analysis. (A) Group 1 vs Group 3, (B) Group 1 vs Group 2 and (C) Group 3 vs Group 2. The significant genes form the distribution of scattered genes noticed by the red and blue color dots.

vessel). Fig. 2(A) showed the scatter plots of the data obtained by RNA-Seq analysis of Group 1 vs Group 3, and Fig. 2(B) showed those of Group 1 vs Group 2. One combination of C-1 had a slightly different nonsymmetric distribution with each other. On the other hand, two different combinations (C-2 and C-3) of Group 3 vs Group 2 had a symmetric distribution of Log2(Val3) versus Log2(Val2), as shown in Fig. 2(C). The significant genes form the distribution of scattered genes noticed by the red and blue color dots in the three scatter plots as shown in Fig. 2.

Fig. 3 shows the plot of DEG analysis for the remarkable 10 genes obtained from RNA-Seq data of Group 1(Primo vessel) vs Group 3(Lymph vessel) in the lymph vessel of a rabbit. Fragments Per Kilobase of exon per Million (FPKM) as an expression level is the average of duplicated experiments (at least 2 per experiment). The cutoff p-value for each gene is < 0.05. FPKMs of 10 genes such as IGHM, HLA-DRA, HIST1H4I, LPL, CD36, SRGN, DGAT2, SNCG, CD48, and GPD1 for the primo vessels were increased from double to triple times each other compared with those of lymph vessels. On the basis of RNA-Seq analysis, several gene markers will be selected to analyze quantitative real-time-PCR (qRT-PCR) for the expression of genes in the pure primo vessels and the lymph vessels. Table 2 shows DEG with the

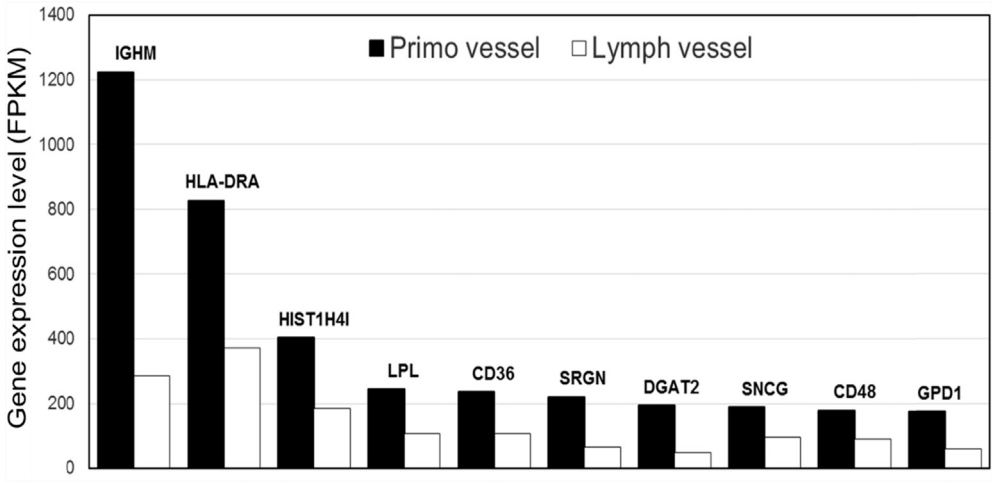


Figure 3 Expression level (FPKM) of 10 genes for Group 1 (primo vessel) vs Group 3 (lymph vessel). Samples were harvested and subjected to RNA-Seq. Each value is the average of duplicated experiments (at least 2 per experiment). The p-value for each gene is < 0.05. FPKM, Fragments Per Kilobase of exon per Million.

Table 2 Differentially expressed genes (DEG) of Group 1(primo vessel) vs Group 3 (lymph vessel) in the lymph vessel of a rabbit.

No	Gene name	Description	Value1	Value3	log2fc	p-value	q-value
1	IGHM	Immunoglobulin heavy constant mu	1224.2	286.36	−2.1	0.0087	0.621938
2	HLA-DRA	Major histocompatibility complex, class II, DR α	827.21	371.98	−1.15	0.038	0.997527
3	HIST1H4I	Histone cluster 1 H4 family member i	403.85	185.14	−1.13	0.0314	0.997527
4	LPL	Lipoprotein lipase	243.76	106.89	−1.19	0.03055	0.997527
5	CD36	CD36 molecule	236.32	105.18	−1.17	0.0241	0.997527
6	SRGN	Serglycin	220.61	65.81	−1.75	0.0018	0.307625
7	DGAT2	Diacylglycerol O-acyltransferase 2	193.33	48.93	−1.98	0.00005	0.041017
8	SNCG	Synuclein gamma	188.72	94.03	−1.01	0.03645	0.997527
9	CD48	CD48 molecule	178.66	89.17	−1	0.0382	0.997527
10	GPD1	Glycerol-3-phosphate dehydrogenase 1	174.9	58.36	−1.58	0.0013	0.271127
11	CYP2F1	Cytochrome P450 family 2 subfamily F member 1	165.55	55.37	−1.58	0.0009	0.229211
12	C1QC	Complement C1q C chain	144.88	45.3	−1.68	0.00095	0.229211
13	GMFG	Glia maturation factor gamma	142.16	63.08	−1.17	0.03745	0.997527
14	S100A8	S100 calcium-binding protein A8	114.01	37.83	−1.59	0.04055	0.997527
15	MPEG1	Macrophage-expressed gene 1	106.59	37.28	−1.52	0.00215	0.339176
16	MS4A1	Membrane-spanning 4-domains A1	100.12	34.63	−1.53	0.00155	0.293427
17	BIRC3	Baculoviral IAP repeat containing 3	96.53	31.75	−1.6	0.00095	0.229211
18	EZR	Ezrin	96.27	28.67	−1.75	0.00085	0.229211
19	GRN	Granulin precursor	87.61	42.83	−1.03	0.0291	0.997527
20	CTSS	Cathepsin S	87.18	39.8	−1.13	0.0211	0.985798
21	KRT19	Keratin 19	82.74	23.48	−1.82	0.00045	0.184575
22	VCAM1	Vascular cell adhesion molecule 1	75.18	20.8	−1.85	0.00055	0.205083
23	CTSH	Cathepsin H	68.58	33.41	−1.04	0.0386	0.997527
24	CD53	CD53 molecule	60.97	30.37	−1.01	0.03765	0.997527
25	CIDEA	Cell death—inducing DFFA-like effector a	57.8	21.16	−1.45	0.00305	0.41242
26	RHOH	Ras homolog family member H	57.28	16.88	−1.76	0.0206	0.974935
27	UPK3B	Uroplakin 3B	56.3	13.87	−2.02	0.0098	0.634679
28	CLEC12A	C-type lectin domain family 12 member A	51.63	16	−1.69	0.0047	0.530583
29	TSC22D4	TSC22 domain family member 4	42.97	19.53	−1.14	0.04325	0.997527
30	PLAC8	Placenta-specific gene 8	42.54	15.65	−1.44	0.00345	0.446866

gene name, description of gene, Value1 [FPKM of Group 1 (Primo vessel)], Value3 [FPKM of Group 3 (Lymph vessel)], log2fc = log2(Value1/Value3), p-value, and q-value for 30 genes according to the order of expression level. Especially the selected HLA-DRA gene having the description of major histocompatibility complex, class II, and DR α is 2.22 times higher in primo vessels than in lymph vessels. Also, the subcellular locations of HLA-DRA gene from information of

GeneCards have the highest confidence in the compartment of plasma membrane, extracelluar membrane, and endoplasmic reticulum.

Fig. 4 shows the plot of DEG analysis for the remarkable 10 genes obtained from RNA-Seq data of Group 1 (primo vessel) vs Group 2 (primo + lymph vessel) in the lymph vessel of a rabbit. FPKM as an expression level is the average of duplicated experiments (at least 2 per experiment). The cutoff p-value for each gene is < 0.05. FPKMs of 10 genes such as IGHM, HIST1H1E, RPL18A, CD74, HIST1H4I, HIST1H1D, CORO1A, DGAT2, HIST1H2BG, and HIST1H3E for the primo vessels were increased from double to triple times each other compared with those of lymph vessels. On the basis of RNA-Seq analysis, several gene markers will be selected to analyze qRT-PCR for the expression of genes in the pure primo vessels and the primo and lymph vessels. Table 3 shows DEG with the gene name, description of gene, Value1 [FPKM of Group 1 (primo vessel)], Value2 [FPKM of Group 2 (primo + lymph vessel)], log2fc = log2(Value1/Value2), p-value, and q-value for 30 genes according to the order of expression level. The selected HIST1H1E gene having the description of histone cluster 1 H1 family member e is 2.41 times higher in primo vessels than in the mixed Group 2 (primo + lymph vessel). Also, the subcellular locations of HIST1H1E gene from information of GeneCards have the highest confidence in the compartment of extracelluar membrane, nucleus, and cytosol.

Fig. 5 shows the plot of DEG analysis for the remarkable 10 genes obtained from RNA-Seq data of Group 3 (lymph vessel) vs Group 2 (primo + lymph vessel) in the lymph vessel of a rabbit. FPKM as an expression level is the average of duplicated experiments (at least 2 per experiment). The cutoff p-value for each gene is < 0.05. FPKMs of 10 genes such as FABP4, ALAS2, CD36, BNIP3L, LPL, MMRN1, ADIPOQ, CXCL8, COL4A1, and THRSP for the primo vessels and the lymph vessels were increased from double to triple times each other compared with those of lymph vessels. On the basis of RNA-Seq analysis, several gene markers will be selected to analyze qRT-PCR for the expression of genes in the lymph vessels and the primo and lymph vessels. Therefore, the remarkable genes, as shown in Figs. 3–5, can be selected to FABP4, DGAT2, CYP2F1, CIDEA, DNASE1L3, F8, STAB2, ACACB, CAMP, MMP12, IGHM, HLA-DRA, HIST1H1E, and HIST1H4I. Table 4 shows DEG with the gene name, description of gene, Value3 [FPKM of Group 3 (lymph vessel)], Value2 [FPKM of Group 2 (primo + lymph vessel)], log2fc = log2(Value3/Value2), p-value, and q-value for 30 genes according to the order of expression level. The selected FABP4 gene having the description of fatty acid–binding protein 4 is 2.95 times higher in primo + lymph vessels than in lymph vessels. Also, the subcellular locations of FABP4 gene from information of GeneCards have the highest confidence in the compartment of extracelluar membrane, nucleus, and cytosol.

By using the reverse transcriptase-polymerase chain reaction (RT-PCR), we already found that lymphatic endothelial cell markers such as FLT4, LYVE-1, PROX-1, and PDPN were highly expressed in the primo vessel compared with those in the lymphatic endothelium, suggesting pivotal roles of the primo vessel in the lymph vessel during inflammation caused by lipopolysaccharide treatment. Furthermore, lymph-related genes including MTF2, HIF1a, AGTR1, and AGTR2 were also overall increased in the primo vessel, and these genes except PPARG were remarkably increased after acupuncture electric stimulation in two acupoints, implying central role of the primo vessel by gene activation. By using the differential gene expression of RNA-Seq experiments for the pure primo vessel without lipopolysaccharide treatment, the selected and remarkable three genes are HLA-DRA, HIST1H1E, and FABP4. From these results, the transcriptome profiling of the primo tube by next-generation sequencing analysis as a result of gene expression analysis of primo vessel and the epitope monoclonal antibody of primo-specific expression gene based on this profiling will be used.

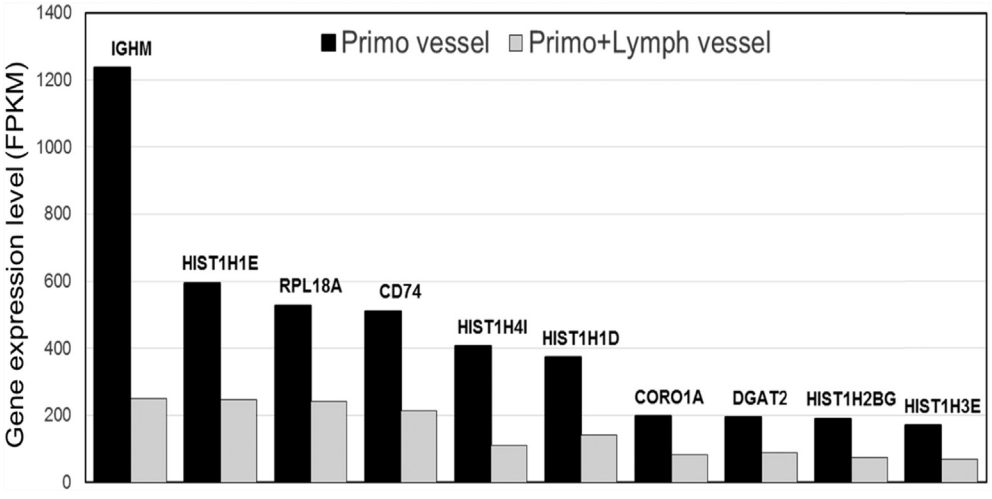


Figure 4 Expression level (FPKM) of 10 genes for Group 1 (primo vessel) vs Group 2 (primo + lymph vessel). Samples were harvested and subjected to RNA-Seq. Each value is the average of duplicated experiments (at least 2 per experiment). The p-value for each gene is < 0.05. FPKM, Fragments Per Kilobase of exon per Million.

Table 3 Differentially expressed genes (DEG) of Group 1 (primo vessel) vs Group 2 (primo + lymph vessel) in the lymph vessel of a rabbit.

No	Gene name	Description	Value1	Value2	log2fc	p-value	q-value
1	IGHM	Immunoglobulin heavy constant mu	1236.7	251.11	−2.3	0.0016	0.466185
2	HIST1H1E	Histone cluster 1 H1 family member e	596.05	246.86	−1.27	0.014	0.996488
3	RPL18A	Ribosomal protein L18a	529.52	241.36	−1.13	0.02845	0.996488
4	CD74	CD74 molecule	512.35	214.03	−1.26	0.01755	0.996488
5	HIST1H4I	Histone cluster 1 H4	407.98	111.07	−1.88	0.003	0.568857
6	HIST1H1D	Histone cluster 1 H1 family member d	374.51	142.43	−1.39	0.00855	0.727696
7	CORO1A	Coronin 1A	197.97	82.25	−1.27	0.0158	0.996488
8	DGAT2	Diacylglycerol O-acyltransferase 2	195.31	89.65	−1.12	0.03315	0.996488
9	HIST1H2BG	Histone cluster 1 H2B family member g	191.75	75.18	−1.35	0.03155	0.996488
10	HIST1H3E	Histone cluster 1 H3 family member e	170.07	69.64	−1.29	0.0445	0.996488
11	CYP2F1	Cytochrome P450 family 2 subfamily F member 1	167.25	27.71	−2.59	0.00005	0.0746625
12	C1QC	Complement C1q C chain	146.36	69.48	−1.07	0.0368	0.996488
13	ITGB2	Integrin subunit beta 2	127.1	52.59	−1.27	0.01325	0.977065
14	MS4A1	Membrane-spanning 4-domains A1	101.14	43.74	−1.21	0.01755	0.996488
15	EZR	Ezrin	97.25	29.22	−1.73	0.0012	0.421624
16	CYP4B1	Cytochrome P450 4B1	92.54	24.69	−1.91	0.0003	0.188621
17	CD79B	CD79b molecule	86.05	40.09	−1.1	0.03405	0.996488
18	KRT19	Keratin 19	83.59	24.57	−1.77	0.00145	0.462907
19	RAC2	Ras-related C3 botulinum toxin substrate 2	79.12	27.49	−1.53	0.01255	0.954919
20	SLC44A2	Solute carrier family 44 member 2	74.62	35.88	−1.06	0.0425	0.996488
21	SASH3	SAM and SH3 domain containing 3	71.93	35.71	−1.01	0.0426	0.996488
22	HLA-DOB	Major histocompatibility complex, class II, DO β	65.77	29.93	−1.14	0.03235	0.996488
23	PTPN6	Protein tyrosine phosphatase, nonreceptor type 6	59.45	28.15	−1.08	0.0398	0.996488
24	UPK3B	Uroplakin 3B	56.88	4.54	−3.65	0.0215	0.996488
25	RASSF2	Ras association domain family member 2	55.05	22.21	−1.31	0.02045	0.996488
26	PPP6R1	Protein phosphatase 6 regulatory subunit 1	53.68	26.33	−1.03	0.04895	0.996488
27	SYK	Spleen-associated tyrosine kinase	51.96	23.68	−1.13	0.0242	0.996488
28	PSME1	Proteasome activator subunit 1	51.37	22.35	−1.2	0.0493	0.996488
29	DBNL	Drebrin-like protein	50.16	23.38	−1.1	0.0426	0.996488
30	CXCL13	C-X-C motif chemokine ligand 13	49.12	22.88	−1.1	0.04975	0.996488

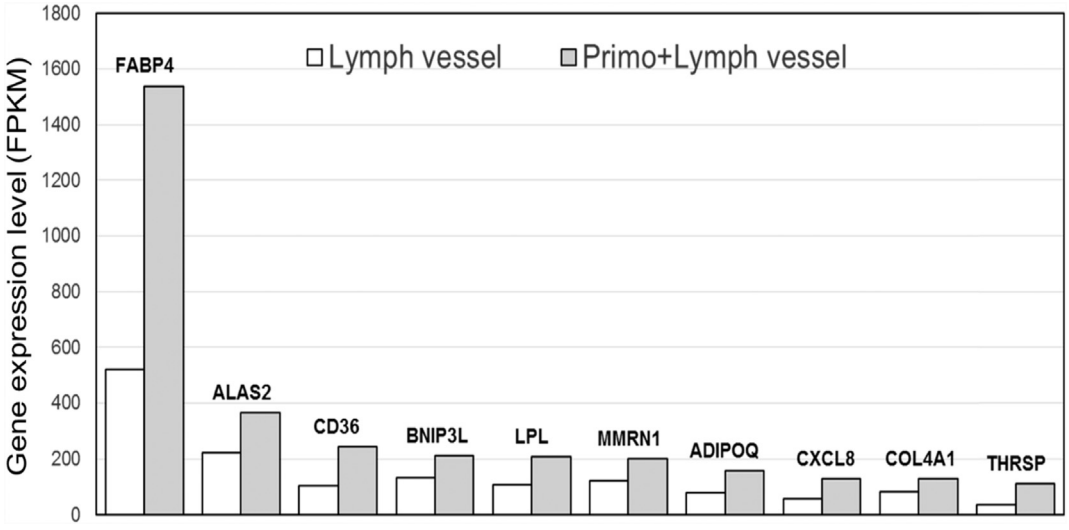


Figure 5 Expression level (FPKM) of 10 genes for Group 3 (lymph vessel) vs Group 2 (primo + lymph vessel). Samples were harvested and subjected to RNA-Seq. Each value is the average of duplicated experiments (at least 2 per experiment). The p-value for each gene is < 0.05. FPKM, Fragments Per Kilobase of exon per Million.

Table 4 Differentially expressed genes (DEG) of Group 3 (lymph vessel) vs Group 2 (lymph vessel) in the lymph vessel of a rabbit.

No	Gene name	Description	Value3	Value2	log2fc	p-value	q-value
1	FABP4	Fatty acid—binding protein 4	520.29	1538.6	1.56	0.00005	0.033588
2	ALAS2	5′-aminolevulinate synthase 2	220.1	366.18	0.734	0.0097	0.938763
3	CD36	CD36 molecule	104.85	243.86	1.22	0.0001	0.060105
4	BNIP3L	BCL2-interacting protein 3—like protein	131.25	209.92	0.678	0.02945	0.995425
5	LPL	Lipoprotein lipase	106.55	206.9	0.957	0.0013	0.324686
6	MMRN1	Multimerin 1	121.34	198.54	0.71	0.01405	0.995425
7	ADIPOQ	Adiponectin, C1Q and collagen domain containing	79.16	156.56	0.984	0.00075	0.225395
8	CXCL8	C-X-C motif chemokine ligand 8	57.19	129.01	1.17	0.00025	0.1142
9	COL4A1	Collagen type IV alpha 1 chain	82.08	128.12	0.642	0.0268	0.995425
10	THRSP	Thyroid hormone—responsive protein	34.25	109.45	1.68	0.00235	0.442065
11	FABP5	Fatty acid—binding protein 5	66.25	108.57	0.713	0.0214	0.995425
12	GPD1	Glycerol-3-phosphate dehydrogenase 1	58.18	107.62	0.887	0.00995	0.948521
13	CCL21	C—C motif chemokine ligand 21	56.43	101.66	0.849	0.0071	0.818792
14	DGAT2	Diacylglycerol O-acyltransferase 2	48.78	89.06	0.868	0.0108	0.97115
15	CIDEA	Cell death—inducing DFFA-like effector a	21.09	87.44	2.05	0.00005	0.033588
16	MPEG1	Macrophage-expressed gene 1	37.16	75.25	1.02	0.0014	0.324686
17	YPEL3	Yippee-like 3	40.72	72.98	0.842	0.03655	0.995425
18	POSTN	Periostin	17.67	72.56	2.04	0.00005	0.033588
19	MGST1	Microsomal glutathione S-transferase 1	32.41	71.65	1.14	0.00175	0.367071
20	CDO1	Cysteine dioxygenase type 1	30.79	60.94	0.985	0.01025	0.954712
21	STX12	Syntaxin 12	35.89	60.94	0.764	0.0312	0.995425
22	CHRDL1	Chordin-like 1	38.56	59.64	0.629	0.0374	0.995425
23	TNFAIP6	TNF alpha—induced protein 6	34.67	58.23	0.748	0.04015	0.995425
24	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3	29.79	52.31	0.812	0.0124	0.995425
25	BIRC3	Baculoviral IAP repeat—containing 3	31.65	50.45	0.673	0.0371	0.995425
26	VCAM1	Vascular cell adhesion molecule 1	20.73	42.86	1.05	0.00055	0.190333
27	MARCKSL1	MARCKS-like 1	20.78	42.81	1.04	0.01135	0.993963
28	FOLH1	Folate hydrolase 1	25.43	41.53	0.708	0.0272	0.995425
29	RGS5	Regulator of G-protein signaling 5	18.9	40.67	1.11	0.0115	0.993963
30	C4BPA	Complement component 4—binding protein alpha	24.79	40.25	0.699	0.02675	0.995425

4. Conclusions

The connectome of the PVS dyed with AB from an inguinal lymph node to an axial lymph node was visualized in a long-type primo vessel passed over around the vena cava of a rabbit. The AB injection into two inguinal nodes, one abdominal node, and two axially nodes revealed the desired primo vessel in the same lymph vessel. The length of a long-type primo vessel was more than an average length of 18 cm. The average diameters of the primo vessels and the lymph vessels were about 20—30 μm and 100—150 μm, respectively. Three different samples as the isolated primo vessel, the mixed primo and lymph vessel, and the separated lymph vessel were separated and stored in TRIzol liquid for the RNA-Seq data analysis to select the remarkable genes of the pure primo vessel. From the RNA-Seq data of primo and lymph vessels of the rabbit, we analyzed the gene expression levels for three different combinations which were the average of duplicated experiments (at least 2 per experiment). The differentially expression levels of 30 genes were compared in combinations of three experimental groups. Specially, FPKMs of the 10 genes such as IGHM, HLA-DRA, HIST1H41, LPL, CD36, SRGN, DGAT2, SNCG, CD48, and GPD1 for primo vessels were increased double or triple times each other, compared with those of lymph vessels. On the basis of RNA-Seq analysis, several specific gene markers will be selected to develop the remarkable monoclonal antibodies for the connectome of the primo vessel.

Disclosure statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) funded by the Republic of Korea government (Ministry of Science and ICT) under Grant No. 2016R1E1A2A01953467.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jams.2018.10.008.

References

[1] Campbell NA, Reece JB, Urry LA, et al. Biology. 10th ed. Pearson Education; 2015.
[2] Kierszenbaum AL. History and Cell Biology: An Introduction to Pathology. New York: Mosby; 2002.
[3] Kim BH. Study on the reality of acupuncture meridians. J Jo Sun Med 1962;9:5—13. In Korean.
[4] Kim BH. On the acupuncture meridian system. J Jo Sun Med 1963;90:6—35. In Korean.
[5] Kim BH. Sanal theory. J Jo Sun Med 1965;108:39—62. In Korean.
[6] Kim BH. Sanal and hematopoiesis. J Jo Sun Med 1965;108:1—6. In Korean.
[7] Kim BH. On the Kyungrak system. J Acad Med Sci DPR Korea 1963;90:1—41.
[8] Soh KS. Bonghan circulatory system as an extension of acupuncture meridians. J Acupunct Meridian Stud 2009;2: 93—106.
[9] Yoo JS, Ayati MH, Kim HB, et al. Characterization of the primo-vascular system in the abdominal cavity of lung cancer mouse model and its differences from the lymphatic system. PLoS One 2010;5:e9940.
[10] Noh YI, Rho M, Yoo YM, et al. Isolation and morphological features of primo vessels in rabbit lymph vessels. J Acupunct Meridian Stud 2012;5:201—5.
[11] Lee HR, Rho M, Hong YJ, et al. Primo vessel stressed by lipopolysaccharide in rabbits. J Acupunct Meridian Stud 2015; 8:301—6.
[12] Shin JY, Ji JO, Choi DW, et al. Expression of genes in primo vasculature floating in lymphatic endothelium under lipopolysaccharide and acupuncture electric stimulation. J Acupunct Meridian Stud 2018;11. Online Published.
[13] Noh YI, Yoo YM, Kim RH, et al. Observation of a long primo vessel in a lymph vessel from the inguinal node of a rabbit. Evid Based Complement Alternat Med 2012;2012: 429106.
[14] Lee SH, Bae KH, Kim GO, et al. Primo Vascular System in the Lymph Vessel from the Inguinal to the Axillary Nodes. Evid Based Complement Alternat Med 2013;2013:472704.
[15] Wang L, Wang S, Li W. RSeQC: quality control of RNA-seq experiments. Bioinformatics 2012;28:2184—5.
[16] Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 2012;7:562—78.
[17] Wang L, Feng Z, Wang X, et al. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 2010;26:136—8.
[18] Young MD, Wakefield MJ, Smyth GK, et al. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 2010;11:1—12.
[19] Chung O, Jin S, Cho YS, et al. The first whole genome and transcriptome of the cinereous vulture reveals adaptation in the gastric and immune defense systems and possible convergent evolution between the Old and New World vultures. Genome Biology 2015;16:215—26.