1 Identification of porcine RUNX1 as an LPS-dependent gene expression regulator
2 in PBMCs by Super deepSAGE sequencing of multiple tissues

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22 Abstract

23 Genome-wide identification of gene expression regulators may facilitate our
 24 understanding of the transcriptome constructed by gene expression profiling
 25 experiment. These regulators may be selected as targets for genetic manipulations in
 26 farm animals. In this study, we developed a gene expression profile of 76,000+ unique
 27 transcripts for 224 porcine samples from 28 normal tissues collected from 32 animals
 28 using Super deepSAGE (serial analysis of gene expression by deep sequencing)
 29 technology. Excellent sequencing depth has been achieved for each multiplexed
 30 library, and principal component analysis showed that duplicated samples from the
 31 same tissues cluster together, demonstrating the high quality of the Super deepSAGE
 32 data. Comparison with previous research indicated that our results not only have
 33 excellent reproducibility but also have greatly extended the coverage of the sample
 34 types as well as the number of genes. Clustering analysis discovered ten groups of
 35 genes showing distinct expression patterns among those samples. Binding motif over
 36 representative analysis identified 41 regulators responsible for the regulation of these
 37 gene clusters. Finally, we demonstrate a potential application of this dataset to
 38 infectious and immune research by identifying an LPS-dependent transcription factor,
 39 runt-related transcription factor 1 (RUNX1), in peripheral blood mononuclear cells
 40 (PBMCs). The selected genes are specifically responsible for the transcription of
 41 toll-like receptor 2 (TLR2), lymphocyte-specific protein tyrosine kinase (LCK), vav1
 42 oncogene (VAV1), and other 32 genes. These genes belong to the T and B cell
 43 signaling pathways, making them potential novel targets for the diagnostic and
 44 therapy of bacterial infections and other immune disorders.

45 Introduction

46 The domestic pig (*Sus scrofa*) is an important farm animal for meat source
 47 worldwide and has been used as alternative models for studying genetics, nutrition,
 48 and disease as reviewed recently (Houpt et al. 1979; Verma et al. 2011; Bailey and
 49 Carlson 2019). The swine genome community has created a large amount of useful

50 data about the transcriptome of pigs (Schroyen and Tuggle 2015). The recently
 51 released pig genome sequence (Sscrofa 10.2) (Groenen et al. 2012) and associated
 52 annotation greatly enhance our knowledge of the pig biology (Dawson et al. 2013;
 53 Beiki et al. 2019). Currently, it is estimated that the porcine genome encodes for
 54 ~40,000 genes (Groenen et al. 2012). Transcriptome analysis indicated that the
 55 actively transcribed genes are only a fraction, perhaps 15,000 genes, in normal tissues
 56 (Hornshoj et al. 2007). Several research groups have created microarray transcriptome
 57 profiling data for normal human tissues (Haverty et al. 2002; Shmueli et al. 2003),
 58 normal mouse tissues (Su et al. 2002; Su et al. 2004), and normal rat tissues (Walker
 59 et al. 2004). In the pig, several Expressed Sequence Tag (EST) sequencing projects,
 60 microarray platforms, and deep sequencing projects have developed gene expression
 61 profiles across a range of tissues (Hornshoj et al. 2007; Freeman et al. 2012).
 62 Compared to the model organisms, the information of the pig transcriptome is still
 63 limited in terms of comprehensive tissue and gene coverage (Schroyen and Tuggle
 64 2015). Here we present Super deepSAGE (serial analysis of gene expression by deep
 65 sequencing) profiling data for the normal pig tissues with wide gene coverage and
 66 annotation. Using K-means clustering analysis and motif binding site enrichment
 67 analysis, we have identified regulators for co-expressed genes. A detailed analysis of
 68 one of the interesting transcription factors, runt-related transcription factor 1
 69 (RUNX1), illustrated the power of the data.

70 **Results and discussion**

71 **Development of the Super deepSAGE technology**

72 A flowchart of the Super deepSAGE experiment is summarized in Fig. 1.
 73 Dynabeads® M-270 Amine (Thermo Fisher Scientific, China) were coupled with
 74 -C6-SH labeled reverse transcription-primer with the sequence containing the
 75 5'-CAGCAG-3' recognition site of EcoP15I and an Oligo(dT) sequence at 3' end
 76 designed intentionally to complement the poly(A) sequence of mRNAs (Synthesized
 77 by Sangon Biotech, China). The coupling procedure was carried out following

78 protocol reported by Hill and Mirkin (Hill and Mirkin 2006) using the succinimidyl
79 4-(p-maleimidophenyl)butyrate (SMPB) crosslink reagent (Thermo scientific,
80 Shanghai, China). Ten micrograms of mRNA were reverse-transcribed (cDNA
81 synthesis system, Invitrogen) with the Oligo(dT) magnetic beads to generate
82 single-stranded cDNA using protocol recommended by the manufacturer. The product
83 was converted to double-stranded cDNA using random primer and then digested with
84 NlaIII (NEB, Beijing, China). The biotin-labeled linkers (linker-5EA) with
85 phosphorylated 5' termini and 3' end overhang (5'-CATG-3'), containing the EcoP15I
86 recognition site were prepared by annealing commercially synthesized
87 oligonucleotides. The magnetic beads-bound cDNA was washed and linked to
88 linker-5EA by T4 DNA ligase (NEB, Beijing, China). As a result, each cDNA
89 fragment bounded to the magnetic beads is flanked by two inverted repeats of
90 EcoP15I recognizing sites. The type III restriction enzyme EcoP15I cleaves the DNA
91 downstream of the recognizing site (25 nt in one strand and 27 nt in the other strand)
92 leaving a 5' end overhang of two bases (Meisel et al. 1992; Moncke-Buchner et al.
93 2009). Linker-ligated cDNAs on the magnetic beads were digested with ten units of
94 EcoP15I under conditions described previously (Matsumura et al. 2003). The
95 supernatant containing released biotin-labeled fragments were added to streptavidin
96 magnetic beads (Promega, Beijing, China), and the biotin-labeled fragments of the
97 cDNAs were captured. Finally, barcoded linkers (linker-3EA) with two random base
98 overhangs at 5' end and phosphorylated termini were prepared and ligated to the
99 cDNA ends by T4 DNA ligase (NEB, Beijing, China). The resulting products were
100 amplified by polymerase chain reaction (PCR), and the 119 bp product was separated
101 by polyacrylamide gel electrophoresis (PAGE) and recovered from the gel. The
102 barcoded libraries prepared from different samples were combined into a single
103 multiplex sequencing reaction at the end of library construction and submitted for
104 deep sequencing. The sequence information of synthetic oligos, linkers, and primers
105 are available in Supplemental document 1.

106 The serial analysis of gene expression (SAGE) was first developed by
107 Velculescu et al. (Velculescu et al. 1995) and improved by Saha et al. (Saha et al.
108 2002), Matsumura et al. (Matsumura et al. 2003), and Nielsen et al. (Nielsen et al.

2006). The traditional SAGE library construction protocol includes multiple steps, and the separation of the linker-tag fragment is challenging to perform, and the PAGE purification often produces low yield. The library construction protocol in this study was improved by introducing two magnet beads: 1) Dynabeads® M-270 Amine coupled with –C6-SH labeled Oligo(dT) reverse transcription primer; 2) The streptavidin magnetic beads which can capture biotin-labeled linkers (linker-5EA). The magnetic beads used in this protocol can capture and purify the DNA fragments and is technically less demanding than PAGE separation. This modification increased the yield of linker-tag fragments and resulted in the robustness of the technique. Also, the primers and linkers were designed compatible with multiplexed deep sequencing technology, saving the sequencing cost.

Fig. 1. Flowchart of Super deepSAGE library construction. There are three major steps included in the protocol: A) reverse transcription with oligo(dT) coupled magnetic beads, synthesis of the secondary chain, and digestion with NlaIII; B) add 5' end linker and digest with EcoP15I, and C) add 3' end linker and PCR amplification. For details see materials and methods section.

126 Animals, samples collection, and deep sequencing

127 A total of 224 tissue samples across 28 different tissues were collected from a
 128 slaughtering farm located in Hubei province in China. The samples were collected
 129 from 32 animals from a Duroc $\times$ Landrace $\times$ Yorkshire (DLY) commercial crossbreed
 130 pig populations consisting of 16 males and 16 females with a median age of 21 days.
 131 The endometrium, placenta, and conceptus were collected from Landrace $\times$ Yorkshire
 132 (LY) sows of 65 days of gestation. The detailed sample information is available in
 133 Table 1. In the computational extraction of tags from sequence data, the in-house
 134 designed program removes the two bases at the 5' end. This 'digital removal' is
 135 performed to minimize the less accurate effect of two random bases, at the 5' end of
 136 linker-3EA, and could potentially reduce the length of tags, and affect the
 137 representative ability of the data. However, direct link with a linker that has two
 138 random bases at the 5' end forming stick ends will 1) enhance the efficiency of the
 139 link assay, and 2) no additional blunt ending process was needed. The inaccuracy
 140 caused by this linkage process was removed by the 'digital removal' procedure,
 141 thereby lowering the systematic bias in the data.

142 Table 1. Detailed information of the collected samples

ID	Code	Dupli cates	Age (day)	Male/ femal e	Breeds	Tissue
1	AC	8	21	4/4	DLY	Adrenal cortex
2	AM	8	21	4/4	DLY	Adrenal medulla
3	CPT.SPH	8	21	4/4	DLY	Conceptus spherical
4	CPT.TUB	8	21	4/4	DLY	Conceptus tubular
5	FT.AB	8	21	4/4	DLY	Abdominal fat tissue
6	MS.DI	8	21	4/4	DLY	Diaphragm muscle
7	STOM	8	21	4/4	DLY	Stomach
8	CPT.FIL	8	21	4/4	DLY	Conceptus filamentous
9	MS.LD	8	21	4/4	DLY	Longissimus dorsi
10	LNG.TRA	8	21	4/4	DLY	Lung porcine trachea

11	PLACT	8	21	4/4	DLY	Placenta
12	LNG.BRO	8	21	4/4	DLY	Lung porcine bronchus
13	LNG.DIS	8	21	4/4	DLY	Lung porcine distal
14	SPL	8	21	4/4	DLY	Spleen
15	FT.BF	8	21	4/4	DLY	Back fat tissue
16	KID	8	21	4/4	DLY	Kidney
17	ADE	8	21	4/4	DLY	Adenohypophysis
18	MP.BMD	8	21	4/4	DLY	Bone-marrow derived macrophage
19	MS.BF	8	21	4/4	DLY	Biceps femoris
20	EDMT	8	21	4/4	DLY	Endometrium
21	BLD	8	21	4/4	DLY	Blood
22	PBMC	8	21	4/4	DLY	Peripheral blood mononuclear cell
23	HT	8	21	4/4	DLY	Heart
24	CC	8	21	4/4	DLY	Cerebral cortex
25	MP.MD	8	21	4/4	DLY	Monocyte derived macrophage
26	MP.ALV	8	21	4/4	DLY	Porcine alveolar macrophages
27	MLN	8	21	4/4	DLY	Mesenteric lymph nodes
28	LIV	8	21	4/4	DLY	Liver

143 Analysis of the complexity and diversity of Super deepSAGE data across tissues

144 Rarefaction analysis of size-fractionated library for each sample was performed
145 to determine the complexity and diversity of the tissues in pig (Wang et al. 2018). The
146 sequencing depth achieved using eight samples-multiplexed deep sequencing technic
147 reached near-saturation of transcript discovery within all size ranges. Saturation was
148 reached very early in Super deepSAGE sequencing data due to the lower complexity
149 of the tags (number of tags) in libraries (Fig. 2A-F showed the first six deep
150 sequencing runs). Samples from the same sequencing run were compared using reads

151 from different size-fractionated libraries to further investigate the diversity of the
152 relationship between sequencing depth and transcript discovery. In all deep
153 sequencing runs, tissues exhibited transcriptome diversity in terms of both total
154 numbers of reads and the number of transcripts discovered. For example, the muscle
155 tissue (MS.DI_2) saturated much sooner than the conceptus (CPT.SPH_8) and have
156 less number of transcripts discovered in the first deep sequencing run (Fig. 2A).
157 Similar sequencing depth and diversity were obtained using size-fractionated data
158 from each of the sequencing run and transcript as outcome measures (Supplemental
159 Fig. SA-D).

160

161 Fig. 2. Rarefaction analysis of covered genes/transcripts in porcine tissues and
162 cells Super deepSAGE library. Plot A to F shows the covered Kilo transcripts per Kilo

reads in the first six Super deepSAGE sequencing runs. The samples in each sequencing run were randomized and detailed information is given in Table 1.

Data quality and internal consistency control using principal component analysis (PCA)

Principal component analysis (PCA) was used to check if the samples clustered together according to their tissue source (Son et al. 2005). Even though the samples were collected from 32 individual animals from different families, genders, and ages (Table 1), the PCA plot showed that the samples from the same tissues clustered together and were distinct from other samples (Fig. 3). The transcripts in conceptus, blood, and macrophages had relatively distinct expression profile and segregation apart from the rest of the samples when plotted using the first two components of the PCA analysis (Fig. 3A). The adenohypophysis, cerebral cortex, heart, and muscle were aggregate and separated from other samples when plotted using the third and fourth component (Fig. 3B). The adrenal, liver, mesenteric lymph nodes, peripheral blood mononuclear cell, and spleen were slightly away from other samples when plotted using the fifth and sixth component (Fig. 3C). When removing those samples from the datasets and re-calculating the PCAs, the remaining samples; fat, placenta, endometrium, kidney, lung, and stomach grouped differently according to the tissue/cell types (Fig. 3D to F). Tissues having similar cellular composition and biological function, like alveolar macrophages and monocyte-derived macrophages or heart and skeletal muscles, clustered closely together but were separated from each other.

185

186 Fig. 3. Principal components analysis of the Super deepSAGE sequencing data. A)
 187 to D) shows the top eight principal components of all 224 samples from the 28 tissues
 188 (two principal components per each plot). Samples separated in plot A to D were
 189 removed, and PCA was re-calculated with the remaining samples (fat, placenta,
 190 endometrium, kidney, lung, and stomach grouped). E) and F) shows the top four
 191 principal components of all the remaining samples (two principal components per
 192 each plot).

193 Comparison of the Super deepSAGE data with previously published microarray 194 research

195 The expression profiles were compared with microarray data published
 196 previously (Hornshøj et al. 2007). There is a total of 18,306 common genes for seven
 197 tissues, while high correlations ($r=0.85-0.93$ and p -values less than 1.0×10^{-30}) were

calculated between the gene expression profiles generated by the two platforms (Fig. 4). Similar dynamic range was observed in both platforms for transcripts with relative expression level between 0.55 and 0.95. Differences in expression profiles were apparent between the two platforms with several genes exhibiting relatively higher or lower expression values in either platform deviated from the diagonal line (Fig. 4). All transcripts had an expression value in the microarray, due to background hybridization or noise, regardless of whether it was truly expressed or not. The overall dynamics of the fitted curve showed that the Super deepSAGE is more sensitive than that microarray for the low expressed genes showing a concaved trend at the lower ends (with relative expression level less than 0.55 in Fig. 4). For those genes with high expression levels, variability is high in both Super deepSAGE and microarray platforms.

As compared by microarray, reliable gene expression profiles can be generated by Super deepSAGE in seven known tissues. Of the 50 highest expressed Super deepSAGE tags, 38 (76%) found corresponding probesets in the 50 highest expressed genes, and only three tags showed a statistically significant difference between Super deepSAGE and microarray data. Two possibilities could cause such discrepancies between Super deepSAGE and microarray data: 1) the SAGE tag was derived from two or more different transcripts, which were differentially expressed in the samples tested, and 2) the microarray probeset can target two or more transcripts due to sequence similarity of transcripts. For example, the transcripts from the same gene family will always produce the same SAGE tag (attributable to the lower resolution power of Super deepSAGE) and preferred to hybrid to the same microarray probeset (can be minimized by design probesets in the none-conserved region). Regardless of some discrepancy, we conclude that Super deepSAGE data are overall compatible with the microarray data and provide faithful gene expression profiles.

224

225 Figure 4. Comparison of the expression profiles of the 18,306 common transcripts
 226 between Super deepSAGE and microarray platforms. Scatter plots show the averages
 227 (between biological duplicates) of $\log_2$ transformed expression values of transcripts
 228 between two platforms. The relationship between the expression profiles generated in
 229 the two platforms is depicted as a smoothing spline (red).

230 Identification of tissue-specific expression of transcripts

231 A total of 4,165 transcripts showed significant up or down-regulation at least in

one tissues in comparison to the average tag count for all other 27 tissues. K-means clustering was then performed by trying a different number of centers (K from 5 to 28) and several random sets (S from 10 to 1000). Finally, we selected K = 10 and S = 400 to produce clustering result with clean and clear expression pattern (by visualization), highly reproducible for each duplicated run (Fig. 5). The result indicated that Cluster 1 has the largest number of transcripts, and most of these transcripts were expressed low in tissues, except macrophages, PBMCs, blood, and conceptus which were moderately expressed. The conceptus specifically expressed transcripts were in Cluster 2, while the conceptus, macrophages, PBMCs, and blood de-expressed transcripts were in Cluster 4. The macrophages, PBMCs, blood, mesenteric lymph nodes, and spleen specific expressed transcripts were in Cluster 5. The genes specifically expressed in heart and skeletal muscle were in cluster 10. The cerebral cortex specifically expressed genes were in Cluster 6, and liver specifically expressed transcripts were in Cluster 7. The adrenal cortex, adrenal medulla, cerebral cortex, and adenohypophysis specifically expressed transcripts were in Cluster 8. Transcript in Cluster 3 and Cluster 9 were ubiquitously expressed or expressed in multiple tissues.

Gene expression data obtained from transcriptional profiling experiments have inspired several applications, such as the identification of differentially expressed genes (Huang et al. 2011; Huang et al. 2018) and the creation of gene classifiers for improved diagnoses of diseases such as cancer (Wesolowski and Ramaswamy 2011; Tonella et al. 2017). The gene expression profile of 224 samples created in this study is complicated that traditional models were difficult to apply to this data to find differentially expressed genes. An *ad hoc* method comparing each tissues to the average tag count for all other 27 tissues was performed, and a very stringent threshold was set (fold change >5.0, p-value <1.0×10⁻⁶) to filter the tissues specifically expressed transcripts. The K-means clustering algorithms which group similar transcripts and separate dissimilar transcripts by assigning them to different clusters have proven to be useful for identifying biologically relevant gene clusters for different biological status (Yao et al. 2016). Even though very useful, the K-means clustering algorithm is particularly sensitive to initial starting conditions and converges to the point that is the local minimum (Selim and Ismail 1984).

263 Furthermore, the number of clusters (parameter K) is difficult to be determined. In
 264 this study, global-seeding procedures of BF98 (Bradley and Fayyad 1998) have been
 265 introduced into the algorithm to improve the consistency and quantity of clustering
 266 results. The BF98 method employed a bootstrap-type procedure to determine the
 267 initial seeds for the centers. Several subsamples (recommended $n = 10$) of the data set
 268 were clustered using K-means. Each clustering operation produced a different
 269 candidate set of centroids from which a new data set was constructed. This data set
 270 was clustered using K-means, and the centroids were chosen as the initial seeds. The
 271 optimal BF98 clustering result on the Super deepSAGE data was obtained by
 272 “visualization” of the result performed by using $K=10$ and number of subsamples
 273 $S=1000$ after trying K from 5 to 28 and S from 20 to 1,000. The “visualization”
 274 method is straightforward for that deterring the best parameter for the K-means
 275 clustering procedure, but when the K reached 10, definite, compact and representative
 276 gene clustering was formulated, and when the S is higher than 200, consistent
 277 clustering result was produced for each duplicated clustering run.

278

279 Fig. 5. K-means clustering analysis of differentially expressed genes across
 280 tissues. Data adjustment (median center and normalization) was performed before the
 281 clustering analysis. The color codes of red, white, black, and dark green represent
 282 high, average, low, and absence of expression, respectively. A detailed view of
 283 expression pattern and internal structure of each gene cluster were constructed by

284 hierarchical clustering and is shown in plot areas from 1–10.

285 **Identification of over-represented motif for tissues specifically expressed** 286 **transcripts**

287 The CLOVER software (Frith et al. 2004) with JASPAR PWM database (Khan et
288 al. 2018) was used to identify over-represented transcription factor binding motifs for
289 each cluster of genes. The promoter regions for each cluster of transcript (1,000 bp
290 upstream) were obtained using the Ensemble Biomart tool (Kasprzyk 2011). The
291 promoter regions for the whole transcript detected in this project, which possesses
292 similar GC content, were used as background. Motifs having a p-value of ≤ 0.05 were
293 selected as significant (Table 2, top 5 motifs). The most significantly enriched motif in
294 Class 1 is MZF1. TFAP2A and TFAP2C were also significantly enriched with a raw
295 score higher than 30. In Class 2, there was only one significantly enriched motif,
296 RHOXF1. In Class 3 and 4, there were five and four motifs with p-value < 0.05
297 respectively, but the raw score was lower than ten. In Class 5, there were at least five
298 motifs with p-value < 0.05 , and three of them, RUNX1, ASCL1, and Myod1 had a
299 raw score higher than 30. In Class 6, the significantly enriched motifs with the highest
300 score were SNAI2 and FIGLA, whereas, in Class 7, the significantly enriched motifs
301 with the highest score was NR4A2. In Class 8, there was only one motif ZEB1
302 enriched in the promoter region of these transcripts. In Class 9, all the enriched motifs
303 had a raw score of less than ten. In Class 10, the top three motifs were Ascl2, Myog,
304 and Tcf12.

305 The transcription factors interact with the DNA recognition motifs, regulates
306 transcription of a large number of genes, and play important roles in fundamental
307 biological processes, including growth, development, and disease (Latchman 1997).
308 To understand gene expression regulation in the Super deepSAGE data obtained in
309 this study, identifying the over-represented or under-represented motifs in the
310 sequence showing similar expression pattern and which factors bind to them, is
311 necessary. Over-representation indicated the motif candidates playing a regulatory
312 role in the sequences, while under-representation indicated that the motif would have

313 a harmful dis-regulatory effect. In each gene clusters showing a similar expression
314 pattern, Clover successfully detects motifs known to function in the sequences and
315 generate interesting and testable hypotheses.

316 Table 2. Significantly over-represented binding motifs in the promoter region of
317 transcripts showing a similar expression pattern

Gene cluster	Jaspar ID	TF name	Occurrence	Gene count	Raw score	FDR
Class 1	MA0056.1	MZF1	291	291	91.6	0
Class 1	MA0810.1	TFAP2A(var.2)	165	165	33.1	0.002
Class 1	MA0524.2	TFAP2C	149	149	32.3	0.003
Class 1	MA0811.1	TFAP2B	143	143	29.1	0.004
Class 1	MA0507.1	POU2F2	53	53	8.35	0.006
Class 2	MA0719.1	RHOXF1	142	142	6.96	0.008
Class 3	MA1105.1	GRHL2	28	28	6.99	0.003
Class 3	MA0842.1	NRL	60	60	3.67	0.006
Class 3	MA0164.1	Nr2e3	52	52	2.42	0.003
Class 3	MA0029.1	Mecom	23	23	2.25	0.009
Class 3	MA0117.2	Mafb	49	49	1.69	0.004
Class 4	MA0691.1	TFAP4	20	20	4.93	0.004
Class 4	MA0091.1	TAL1::T CF3	16	16	2.43	0.006
Class 4	MA0616.1	Hes2	19	19	0.8	0.003
Class 4	MA0089.1	MAFG:: NFE2L1	21	21	-1.09	0.005
Class 5	MA0002.2	RUNX1	135	135	55.5	0
Class 5	MA1100.1	ASCL1	79	79	46.8	0.008
Class 5	MA0499.1	Myod1	59	59	31.6	0.003
Class 5	MA1124.1	ZNF24	30	30	18.6	0.002
Class 5	MA1109.1	NEURO	57	57	9.76	0.004

D1						
Class 6	MA0745.1	SNAI2	75	75	18.7	0.001
Class 6	MA0820.1	FIGLA	77	77	17.6	0.003
Class 6	MA0138.2	REST	6	6	7.13	0.002
Class 6	MA0665.1	MSC	36	36	6.63	0.002
Class 6	MA0691.1	TFAP4	30	30	5.79	0.003
Class 7	MA0160.1	NR4A2	59	59	15.5	0
Class 7	MA0693.2	VDR	31	31	4.29	0.004
Class 7	MA0017.2	NR2F1	27	27	4.12	0.009
Class 7	MA1142.1	FOSL1::J UND	38	38	3.25	0.001
Class 7	MA0059.1	MAX::M YC	16	16	1.48	0.008
Class 8	MA0103.3	ZEB1	21	21	10.9	0.002
Class 9	MA0084.1	SRY	22	22	9.59	0.01
Class 9	MA0130.1	ZNF354 C	35	35	9.47	0.007
Class 9	MA0463.1	Bcl6	21	21	7.12	0.007
Class 9	MA0799.1	RFX4	7	7	5.64	0.007
Class 9	MA0798.1	RFX3	7	7	5.38	0.009
Class 10	MA0816.1	Ascl2	66	66	28.6	0.009
Class 10	MA0500.1	Myog	58	58	28.5	0.01
Class 10	MA0521.1	Tcf12	57	57	27.2	0.008
Class 10	MA0665.1	MSC	36	36	9.17	0.002
Class 10	MA0108.2	TBP	57	57	4.39	0.007

318 **Case report: confirmation of the regulatory roles of RUNX1 in PBMCs in pig**

319 **Confirmation of the RUNX1 binding site in the promoter region of TLR-2, LCK,** 320 **and VAV1**

321 The toll-like receptor 2 (TLR-2), lymphocyte-specific protein tyrosine kinase
322 (LCK), and vav1 oncogene (VAV1) plasmid containing the 1Kb promoter sequence
323 were used in *in vivo* studies (wild type). To show the regulation effect of RUNX1, the
324 binding site of RUNX1 in TLR-2, LCK, and VAV1 was mutated or deleted. Reporter
325 vectors constructed by the wild type, mutated, or deleted promoter sequences were
326 transfected into the peripheral blood mononuclear cells (PBMCs), and luciferase
327 activity was monitored. Binding site deletion significantly attenuated the expression
328 of the downstream reporter luciferase activity ($p<0.05$), indicating that the RUNX1
329 could interact with the target site and regulate the expression of the downstream
330 reporter gene (Fig. 6A-C). The mutated vectors showed significant attenuation of the
331 activity of downstream luciferase at 40, 44, and 48 hours post-transfection ($p<0.05$)
332 indicating a regulatory relationship between RUNX1 and the targets. Another
333 experiment was performed using mouse macrophage cells (RAW 264.7) to validate
334 the hypothesis further. Consistent with the previous results, deletion/mutations to the
335 RUNX1 binding sites in TLR-2, LCK, and VAV1 promoter sequence significantly
336 attenuated the activity of downstream luciferase at 40, 44, and 48 hours
337 post-transfection (Fig. 6D-F). The luciferase reporter activity after transfection with
338 the wild-type vector was significantly higher in macrophage cells than in the PBMC
339 assays, suggesting that the endogenous RUNX1 expression in mouse macrophage cells
340 was higher than in PBMCs.

341

342 Fig. 6. Luciferase reporter assay of the RUNX1 targets. One wild-type promoter
343 construct (containing the predicted RUNX1 binding site), two mutant constructs
344 (mutated or not containing the binding site) were investigated. The mutant construct
345 (black) was identical to the wild-type, except that the RUNX1 binding site was
346 deleted or mutated. The line graphs show the luciferase activity after the reporter
347 plasmids were transfected into PBMCs (A-C) or macrophages (D-F). Three RUNX1
348 target gene have been investigated (A and D: TLR-2, B and E: LCK, C and F: VAV1).
349 The error bars represent the mean $\pm$ standard deviation of three duplicate samples.

350 RNA flow cytometry analysis of RUNX1 targets in LPS and RUNX1 inhibitor 351 treated PBMCs

352 To show the effect of RUNX1 on three targets; TLR2, LCK, and VAV1, pig
353 PBMCs were stimulated with LPS and/or RUNX1 inhibitor, for 6 hours, during which
354 their TLR2, LCK, VAV1, CD14 protein levels were monitored. Two subsets of cells
355 readily emerged from CD14/TLR2 analysis in PBMCs: a CD14^{hi}/TLR2^{lo}
356 (CD14^{high}/TLR2^{low}) and a CD14^{lo}/TLR2^{lo} population (Fig. 7D). The percentage of
357 CD14^{hi}/TLR2^{lo} cells increased in LPS plus RUNX1 inhibitor treated samples, but the
358 proportion of CD14^{lo}/TLR2^{lo} cells remained unchanged. The percentages of TLR2^{hi}
359 (for both CD14^{hi} and CD14^{lo}) cells increased seven-fold in LPS alone treated samples
360 compared with the non-treated controls. Four subsets of cells readily emerged from
361 CD14/LCK analysis in PBMCs treated with LPS or RUNX1 inhibitor: a
362 CD14^{hi}/LCK^{lo}, CD14^{hi}/LCK^{hi}, CD14^{lo}/LCK^{hi}, and CD14^{lo}/LCK^{lo} population (Fig. 7E).
363 The percentage of CD14^{hi}/LCK^{hi}, and CD14^{lo}/LCK^{hi} cells increased in LPS plus
364 RUNX1 inhibitor treated samples, and the proportion of CD14^{hi}/LCK^{lo} cells was
365 decreased. The percentages of CD14^{hi}/LCK^{hi} cells increased by 40% in LPS alone
366 treated samples compared with the non-treated controls. Two subsets of cells readily
367 emerged from CD14/VAV1 analysis in PBMCs: a CD14^{hi}/VAV1^{lo} and a
368 CD14^{lo}/VAV1^{lo} population (Fig. 7F). The percentage of VAV1^{hi} (for both CD14^{hi} and
369 CD14^{lo}) cells increased four-fold in LPS plus RUNX1 inhibitor treated samples. The
370 percentages of VAV1^{hi} (for both CD14^{hi} and CD14^{lo}) cells increased seven-fold in
371 LPS alone treated samples compared with the non-treated controls and is two-fold
372 higher than in LPS plus RUNX1 inhibitor treated samples.

373 RUNX1 is a master regulator of hematopoiesis and plays a vital role in T and B
374 cells development. RUNX1 is critical in inducing the production of genes in immune
375 cells, such as interleukin-2 (IL-2, (Wong et al. 2011), IL-3 (Uchida et al. 1997),
376 colony-stimulating factor 1 receptor (CSF1R, (Zhang et al. 1996), CSF2 (Frank et al.
377 1995), and cluster of differentiation 4 (CD4, (Taniuchi et al. 2002). However, its roles
378 in LPS-mediated inflammation in PBMCs remains unclear. In this study, regulations
379 of TLR-2, LCK, and VAV1 have been confirmed by flow cytometry. TLR2 is an

essential receptor for the recognition of a variety of pathogen-associated molecular
 pattern (PAMPs) from Gram-positive bacteria, including bacterial lipoproteins,
 lipomannan, and lipoteichoic acids (Medzhitov 2001). LCK encoded protein is a key
 signaling molecule in the selection and maturation of developing T-cells (Davis and
 van der Merwe 2011). The VAV1 encoded protein is important in hematopoiesis,
 playing a role in both T-cell and B-cell development and activation (DeFranco 2001;
 Helou et al. 2015). These results suggested that RUNX1 might be a new potential
 target for resolving inadequate or uncontrolled inflammation in PBMCs.

388

Fig. 7. Simultaneous staining of the target gene and CD14 protein in rested and
 stimulated PBMCs. Plots of PBMCs that were left untreated (A) or were stimulated
 with LPS plus RUNX1 inhibitor for 6 hours (B) or were stimulated with LPS only (C),
 and labeled with antibodies that bind to CD14 (PE-A) and target protein (FITC-A,
 D-F).

394 **Real-time PCR analysis of RUNX1 targets in LPS and RUNX1 inhibitor treated** 395 **PBMCs**

396 To investigate if the expression patterns of the 23 RUNX1 target genes could be
397 modeled by LPS and RUNX1 inhibitor treatment *in vivo*, we performed real-time
398 PCR assay after treating PBMCs with two different doses of LPS (1 ng/mL, 10
399 ng/mL), and RUNX1 inhibitor (1 ng/mL, 10 ng/mL). Samples were collected six
400 hours post-stimulation. A total of 21 genes were induced in response to at least one
401 dose of LPS stimulation, as expression levels for these genes were different when
402 compared to non-stimulated control. A total of 10 genes were down-regulated in
403 response to the RUNX1 inhibitor treatment. Hierarchical clustering analysis was used
404 to determine whether the response of LPS stimulation response was similar to the
405 patterns detected in RUNX1 inhibitor treatment, and if any differences were observed
406 depending on the dosage of LPS and RUNX1 inhibitor used. As shown in Fig. 8, the
407 expression patterns of samples with RUNX1 inhibitor treatment, RUNX1 inhibitor
408 plus LPS treatment, and non-simulated controls clustered together. Different dose of
409 the RUNX1 inhibitor did not affect the samples, as observed by the mixing up of
410 respective samples in the heatmap. The LPS treated samples were unique and were
411 separated from the RUNX1 inhibitor-treated groups and control groups. Similar to the
412 RUNX1 inhibitor, different doses of LPS dose did not affect the samples as well. The
413 expression patterns of RUNX1 inhibitor plus LPS treatment samples were similar
414 with controls and samples treated with RUNX1 alone because they were mixed in the
415 heatmap.

Fig. 8. RUNX1 target gene expression in PBMCs treated with LPS or RUNX1 inhibitor. Cells were treated in vitro with two different doses of LPS (1 ng/ml, 10 ng/ml) and RUNX1 inhibitor (1 ng/ml, 10 ng/ml). Color codes of yellow, black, and blue represent expression levels of high, average, and low, respectively, across the treatments shown.

Super deepSAGE is a useful data resource in pig study

Gene expression analysis is extensively applied in the understanding of the molecular mechanisms underlying a wide range of biological process such as host-pathogen interactions. Our dataset of transcript levels in normal tissues was developed as a reference datasets that can be compared to attained information of biological event specifically related aberrations in transcript levels. Therefore, one major focus of this manuscript was to demonstrate the biological importance of these

429 profiles. We report that >40% of the measured transcripts were differentially
430 expressed between the different tissues. We show that statically the transcripts were
431 co-regulated by a few important transcription factors. We describe one of the many
432 transcription factors that regulated gene expression in PBMCs. To our knowledge, this
433 data set is the largest to date for the analysis of transcriptional profiles within normal
434 tissues from pigs and is complementary to previously published data sets. These data
435 will improve the annotation of the pig genome, support versatile biological research,
436 and increase the utility of the pig as a meat source animal and model in medical
437 research.

438 **Materials and methods**

439 **Sample collection and RNA extraction**

440 Soon after anesthesia by electric shock, specimens were excised, snap-frozen in
441 liquid nitrogen, and kept in a deep freezer (-80°C) until RNA extraction. RNA
442 extraction from the tissue samples and cells was conducted using the RNeasy Mini
443 Kit (Qiagen, Shanghai, China) following the manufacturer's protocols. The
444 BioAnalyzer 2100 (Agilent) was used to assess the integrity of total RNAs, and RIN
445 number of less than 0.7 was removed from the study.

446 **Super deepSAGE sequencing and data procession**

447 The sequencing data were filtered by removing sequences that had poor quality
448 (score <0.5) for more than 20% of all the bases. All the data discussed in this study
449 have been deposited to the NCBI GEO database (Edgar et al. 2002) under accession
450 number GSE134461. Tag sequence was extracted, counted, and assigned for each
451 transcript, and then normalized for each sample by quantile normalization method
452 (Pan and Zhang 2018). For the tags assigned to multiple transcripts, the average copy
453 numbers of those tags were used. The principal component analysis (PCA) was
454 performed using the log₂ tag counts of all the transcripts across all the samples using
455 R statistical software version 3.5. The tissue specific transcripts expressed were

identified by comparing samples from each tissue to the overall tag count across all samples (average), and a threshold was set to fold change >5.0 , p-value $<1.0 \times 10^{-6}$ according to a method implemented in limma package (Ritchie et al. 2015). Clustering analysis was performed by first using K-means clustering method to separate the transcripts to several big groups, and then using Hierarchical clustering to build the internal structure of the transcripts within the groups according to the method reported by Gu et al. (Gu et al. 2016).

Luciferase reporter assay

The three predicted target genes, TLR-2, LCK, and VAV1, were also conserved in human and mouse. For these three genes, a 1Kb nucleotide promoter segment that included RUNX1 target sites was inserted upstream of a firefly luciferase ORF (pGL3, Promega, Beijing, China), and luciferase activity was compared to that of an analogous reporter with point substitutions disrupting the target sites, or analogous reporter that the binding site deleted completely (see detailed sequence information in Supplemental document 2). The logic behind the luciferase reporter assay is that deletion/mutation of a RUNX1 binding site should allow the down-regulation of its target genes, and hence the target gene should be expressed differently between the wild type and mutated constructs. The pGL3-Control activity was used for the normalization of firefly luciferase activity. For the assay, the cells were plated in a 96-well plate at 3,000 cells per well. After overnight incubation, the cells were treated with a transfection mixture consisting of 35 μL of serum-free medium, 0.3 μL of TransFastTM Transfection Reagent (Cat. E2431), and 0.02 μg of pGL3 and pGL3-Control vector per well. After one hour incubation, 100 μL of the serum-containing medium was added to the wells. At 24 to 48 hours of post-transfection, EnduRenTM Live Cell Substrate (Cat. E6481) was added to a final concentration of 60 μM , and luciferase activity was monitored.

482 **PBMC Isolation and Stimulation**

483 Peripheral blood mononuclear cells (PBMCs) were isolated from whole normal
484 blood collected from five animals aged 21 days using BD Vacutainer^{VR} Cell
485 Preparation Tubes (Becton Dickinson, Shanghai, China). The samples were processed
486 according to the manufacturer's instructions within two hours of blood collection.
487 PBMCs were harvested from the tube, washed with phosphate-buffered saline (Life
488 Technologies), and centrifuged for 10 min at 300g prior to use. To induce gene
489 expression, PBMCs were resuspended in RPMI-1640 medium (Life Technologies)
490 supplemented with 10% fetal bovine serum (Life Technologies) at 1.5×10^6 cells/mL
491 in a 96-well V-bottom polypropylene plate (Corning Incorporated). LPS
492 (Sigma-Aldrich, Shanghai, China) and RUNX1 inhibitor (Ro 5-3335, R&D Systems,
493 Shanghai, China) were added at 5 ng/mL and 10 ng/mL, respectively, according to the
494 manufacturer's instructions. Untreated PBMCs were used as control samples.

495 **Surface staining and cytometry acquisition**

496 Phenotypic surface staining was performed in BD PharmingenTM stain buffer
497 (BSA, BD Biosciences, Shanghai, China) for 30 min at room temperature in the dark,
498 using anti-CD14 PE (BD Biosciences, Shanghai, China). Cells were washed and
499 suspended in BD Pharmingen stain buffer (BSA, BD Biosciences, Shanghai, China),
500 anti-TLR-2 FITC, anti-LCK FITC, anti-VAV1 FITC (BD Biosciences, Shanghai,
501 China), was then added separately, and the mixture was incubated for 20 min at room
502 temperature. Finally, cells were washed and acquired on a BD LSRFortessaTM cell
503 analyzer (BD Biosciences, Shanghai, China). The flow cytometry data were deposited
504 in Flow Repository database (ref) under accession FR-FCM-Z268.

505 **Data access**

506 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134461>

507 <http://flowrepository.org/id/RvFrphtLijqf34kFNTA1gdB6BdXEskSDTdhZ4VwfM1q>
 508 [bgTIPfmqbL8o5eVTIhiUH](http://flowrepository.org/id/RvFrphtLijqf34kFNTA1gdB6BdXEskSDTdhZ4VwfM1q)

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516 **Ethics approval and consent to participate**

517 All procedures involving animals were ethical and were approved by the Animal
 518 Care and Use Committee of Hubei Province (China, YZU-2018-0031).

519 **Disclosure declaration**

520 The authors declare that the research was conducted in the absence of any
 521 commercial or financial relationships that could be construed as a potential conflict of
 522 interest.

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