Transcription factor organic cation transporter 1 (OCT-1) affects the expression of porcine Klotho (*KL*) gene

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Klotho (KL), originally discovered as an aging suppressor, was a membrane protein that shared sequence similarity with the β-glucosidase enzymes. Recent reports showed Klotho might have a role in adipocyte maturation and systemic glucose metabolism. However, little is known about the transcription factors involved in regulating the expression of porcine *KL* gene. Deletion fragment analysis identified KL-D2 (-418 bp to -3 bp) as the porcine *KL* core promoter. MARC0022311 in *KL* intron 1 appeared a polymorphism (A or G) in Landrace × DIV pigs, and relative luciferase activity of *pGL3-D2-G* was significantly higher than *pGL3-D2-A*. This was possibly the result of a change in *KL* binding ability with transcription factor organic cation transporter 1 (OCT-1), which was confirmed using electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP). Moreover, OCT-1 regulated endogenous *KL* expression by RNA interference. Our study indicates SNP MARC0022311 affects porcine *KL* expression by regulating its promoter activity via OCT-1.

Transcription factor organic cation transporter 1 (OCT-1) affects the expression of porcine Klotho (*KL*) gene

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Short Title: OCT-1 affects porcine *KL* expression

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Abstract

Klotho (KL), originally discovered as an aging suppressor, was a membrane protein that shared sequence similarity with the β-glucosidase enzymes. Recent reports showed Klotho might have a role in adipocyte maturation and systemic glucose metabolism. However, little is known about the transcription factors involved in regulating the expression of porcine *KL* gene. Deletion fragment analysis identified KL-D2 (-418 bp to -3 bp) as the porcine *KL* core promoter. MARC0022311 in *KL* intron 1 appeared a polymorphism (A or G) in Landrace× DIV pigs, and relative luciferase activity of *pGL3-D2-G* was significantly higher than *pGL3-D2-A*. This was possibly the result of a change in *KL* binding ability with transcription factor organic cation transporter 1 (OCT-1), which was confirmed using electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP). Moreover, OCT-1 regulated endogenous *KL* expression by RNA interference. Our study indicates SNP MARC0022311 affects porcine *KL* expression by regulating its promoter activity via OCT-1.

Keywords

Pig; *KL* gene; OCT-1; MARC0022311

Introduction

Klotho (*KL*) gene encoded a membrane protein that shared sequence similarity with the β-glucosidase enzymes and its product might function as part of a signaling pathway that regulated aging *in vivo* and morbidity in age-related diseases (Ko et al., 2013). Mutant mice lacking the *KL* gene showed multiple aging disorders and a shortened life span (Kuro-o et al., 1997). *KL/KL* mice had the pattern of ectopic calcification certainly contributed by the elevated phosphate and calcium levels (Hu et al., 2011; Ohnishi et al., 2009). KL also acted as a deregulated factor of mineral metabolism in autosomal dominant polycystic kidney disease (Mekahli & Bacchetta., 2013). Mice that lacked Klotho activity were lean owing to reduced white adipose tissue accumulation, and were resistant to obesity induced by a high-fat diet (Ohnishi et al., 2011; Razzaque et al., 2012).

KL expression is regulated by thyroid hormone, oxidative stress, long-term hypertension and so on (Koh et al., 2001). Some transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR-γ) also could regulate *KL* expression (Zhang et al., 2008). A double-positive feedback loop between PPAR-γ and Klotho regulated adipocyte maturation (Chihara et al., 2006; Zhang et al., 2008). Briefly, chromatin immuno-precipitation (ChIP) and gel shift assays found a PPAR-responsive element within the 5'-flanking region of human *KL* gene. Additionally, PPAR-γ agonists increased *KL* expression in HEK293 cells and several renal epithelial cell lines, while the induction was blocked by PPAR-γ antagonists or small interfering RNAs (Zhang et al., 2008). Furthermore, Klotho could induce PPAR-γ synthesis during adipocyte maturation (Chihara et al., 2006). However, little is known about the transcription

48 factors involved in regulating the expression of porcine *KL* gene.

49 To investigate the transcriptional regulation of porcine *KL* gene, we identified the core promoter
50 of porcine *KL* gene, analyzed its upstream regulatory elements and revealed that transcription
51 factor OCT1 directly bound to the core promoter region of porcine *KL* gene and regulated its
52 expression.

53 **Materials and Methods**

54 **Ethics statements**

55 All animal procedures were performed according to protocols approved by the Biological Studies
56 Animal Care and Use Committee of Hubei Province, PR China. Sample collection was approved
57 by the ethics committee of Huazhong Agricultural University (No. 30700571 for this study).

58 **MARC0022311 polymorphism in pigs**

59 Nineteen Landrace × DIV crossbred pigs were genotyped with the Porcine SNP60 BeadChip
60 (Illumina) using the Infinium HD Assay Ultra protocol, which was conducted under the technical
61 assistance by Compass Biotechnology Corporation. DIV was a synthetic line derived by crossing
62 Landrace, Large White, Tongcheng or Meishan pigs. Raw data had high genotyping quality (call
63 rate > 0.99) and were analyzed with the GenomeStudio software.

64 ***In silico* sequence analysis**

65 *KL* gene sequence ENSSSCG00000009347 was available on the ENSEMBL online website
66 (http://asia.ensembl.org/index.html). We obtained the up-stream sequence for *KL* promoter
67 prediction. The potential promoter was analyzed using the online neural network promoter
68 prediction (NNPP) (http://www.fruitfly.org/seq tools/promoter.html) and Promoter 2.0 prediction

server (http://www.cbs.dtu.dk/services/Promoter/). Transcription factor binding sites were predicted using biological databases (BIOBASE) (http://www.gene-regulation.com/pub/programs.html) with a threshold of 0.90 and TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) with a threshold of 85.

Cell culture, transient transfection and luciferase assay

The porcine kidney (PK) cells and swine testis (ST) cells obtained from China Center for Type Culture Collection (CCTCC) were cultured at 37 ◦C in a humidified atmosphere of 5% CO2 using DMEM supplemented with 10% FBS (Gibco).

Four *KL* promoter deletion fragments were cloned into *pGL3-Basic* vector to determine the core promoter region. The plasmids contained pig KL-*D2* promoter and *KL* intron 1 fragments (g.1474 A and g.1474 G) were reconstructed, then transfected using lipofectamine 2000 (Invitrogen) into PK cells and ST cells. Plasmid DNA of each well used in the transfection containing 0.8 μg of *KL* promoter constructs and 0.04 μg of the internal control vector *pRL-TK* Renilla/luciferase plasmid. The enzymatic activity of luciferase was then measured with PerkinElmer 2030 Multilabel Reader (PerkinElmer).

RNA interference

Double-stranded small interfering RNAs (siRNAs) targeting *OCT-1* were obtained from GenePharma. Cells were co-transfected with 2 μl of siRNA, 0.2 μg of reconstructed plasmids using Lipofetamine 2000^{TM} reagent for 24 h. Transfection mixtures were removed, and fresh complete DMEM medium was added to each well. Finally, the enzymatic activity of luciferase was then measured with PerkinElmer 2030 Multilabel Reader (PerkinElmer).

Quantitative real time PCR (qPCR)

qPCR was performed on the LightCycler® 480 (Roche) using SYBR® Green Real-time PCR Master Mix (Toyobo). Primers used in the qPCR were shown in Table 1. qPCR conditions consisted of 1 cycle at 94 °C for 3 min, followed by 40 cycles at 94 °C for 40 sec, 61 °C for 40 sec, and 72 °C for 20 sec, with fluorescence acquisition at 74 °C. All PCRs were performed in triplicate and gene expression levels were quantified relatively to the expression of β-actin. Analysis of expression level was performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Student's t-test was used for statistical comparisons.

Western blotting

Western blotting was performed as described previously (Tao et al., 2014). Five μg proteins were boiled in 5 × SDS buffer for 5 min, separated by SDS-PAGE, and transferred to PVDF membranes (Millipore). Then, the membranes were blocked with skim milk and probed with anti-KL (ABclonal). β-actin (Santa Cruz) was used as a loading control. The results were visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and enhanced chemiluminescence.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein of PK and ST cells was extracted with Nucleoprotein Extraction Kit (Beyotime). The oligonucleotides (Sangon) corresponding to the OCT-1 binding sites of *KL* intron 1 (Table 1) were synthesized and annealed into double strands. The DNA binding activity of OCT-1 protein was detected by LightShift® Chemiluminescent EMSA Kit (Pierce). Ten μg nuclear extract was added to 20 fmol biotin-labeled double stranded oligonucleotides, 0.1 mM EDTA, 2.5% Glycerol,

1×binding buffer, 5 mM $MgCl_2$, 50 ng Poly (dI·dC) and 0.05% NP-40. In addition, control group added 2 pmol unlabeled competitor oligonucleotides, while the super-shift group added 10 μg OCT-1 antibodies (Santa Cruz). The mixtures were then incubated at 24 °C for 20 min. The reactions were analyzed by electrophoresis in 5.5% polyacrylamide gels at 180 V for 1 h, and then transferred to a nylon membrane. The dried nylon was scanned with GE ImageQuant LAS4000 mini (GE-Healthcare).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a commercially available ChIP Assay Kit (Beyotime) as previously described (Tao et al., 2015). Briefly, after crosslinking the chromatin with 1% formaldehyde at 37 °C for 10 min and neutralizing with glycine for 5 min at room temperature, PK and ST cells were washed with cold PBS, scraped and collected on ice. Then, cells were harvested, lysed and treated by sonication. Nuclear lysates were processed 20 times for 10 sec with 20 min intervals on ice water using a Scientz-IID (Scientz). An equal amount of chromatin was immune-precipitated at 4 °C overnight with at least 1.5 μg of OCT-1 antibody (Santa Cruz) and normal mouse IgG antibody (Millipore). Immune-precipitated products were collected after incubation with Protein A + G coated magnetic beads. The beads were washed, and the bound chromatin was eluted in ChIP elution buffer. Then the proteins were digested with Proteinase K for 4 h at 45 °C. The DNA was purified using the AxyPrep PCR Cleanup Kit (Axygen). The DNA fragment of OCT-1 binding sites in *KL* intron 1 was amplified with the specific primers (Table 1).

Results

MARC0022311 status in pigs

MARC0022311 in *KL* intron 1 appeared a polymorphism (A or G) in 19 Landrace× DIV pigs, with 12 AA pigs and AG pigs genotyped using the Illumina PorcineSNP60 chip (Supplementary dataset). The SNP (MARC0022311) in pig *KL* intron 1 was renamed as *KL* g.1474 A>G according to the standard mutation nomenclature (den Dunnen & Antonarakis, 2000).

Identification of promoter region of the porcine *KL* gene

An 833 bp contig in 5' flanking region of pig *KL* gene was obtained by PCR. To determine the promoter region, four promoter deletions (KL-D1: -178 bp to -3 bp, KL-D2: -418 bp to -3 bp, KL-D3: -599 bp to -3 bp and KL-D4: -835 bp to -3 bp) were cloned into fluorescent vector based on the prediction of NNPP online software and Promoter 2.0 (Fig. 1A). Luciferase activity analysis in both PK and ST cells revealed that KL-D2 (-418 bp to -3 bp) was essential for its transcriptional activity and was defined as the *KL* promoter region (Fig. 1B).

MARC0022311 SNP affects the *KL* expression

Intron SNPs could not change the amino acid sequence, but might alter gene promoter activity by affecting the binding of transcription factors (Van Laere et al., 2003). The plasmids contained KL-D2 and the wild-type A (g.1474 A) or mutant G (g.1474 G) sequence were named as *pGL3-D2-A* and *pGL3-D2-G,* respectively. Results showed that luciferase activity of *pGL3-D2-G* was significantly higher than *pGL3-D2-A* in both PK cells ($P < 0.05$) and ST cells ($P < 0.01$) (Fig. 2A), indicating the binding of certain regulatory elements affected *KL* promoter activity.

The SNP (MARC0022311) located in the first intron of *KL* gene (+1474 bp) was predicted to change the binding of OCT-1 by BIOBASE and TFsearch (Fig. S1). siRNAs were used to knock

down *OCT-1* in PK and ST cells. After silencing *OCT-1*, luciferase activity of *pGL3-D2-G* was significantly lower than *pGL3-D2-A* in both PK cells and ST cells ($P < 0.05$) (Figs. 2B and 2C). Furthermore, compared with the negative control, the luciferase activity of *pGL3-D2-A* was significantly decreased ($P < 0.05$) (Figs. 2B and 2C). Thus, MARC0022311 regulated the promoter activity via OCT-1.

However, inhibition of *OCT-1* expression significantly suppressed *KL* expression in PK and ST cells ($P < 0.05$) (Fig. 3), possibly because OCT-1 could stimulate *KL* expression by binding *KL* gene at other sites.

Transcription factor OCT-1 binds to the *KL* intron 1 both *in vitro* and *in vivo*

To address whether *KL* intron 1 contained OCT-1 binding sites *in vitro*, we used two oligonucleotides (A allele and G allele oligonucleotides) with differing only at SNP MARC0022311 position, as porcine OCT-1 probes in EMSA. EMSA revealed a highly specific interaction with allele A oligonucleotide, and a 100 fold excess of mutant allele G oligonucleotide could not outcompete the interaction (Fig. 4A). A super-shift was obtained when nuclear extracts from PK and ST cells were incubated with OCT-1 antibodies, providing further biochemical evidence for the presence of OCT-1 *in vitro* (Fig. 4A). We found the *KL* genotype at g.1474 A>G locus was AA in PK and ST cells by PCR-sequencing, indicating the endogenous binding of OCT-1 to *KL* in above two cell lines (Fig. S2). The chromatin was immune-precipitated using an OCT-1 antibody and DNA fragments of the expected size were used as a template to perform PCR amplification. ChIP analysis showed that OCT-1 interacted with *KL* intron 1 (Fig. 4B). These results showed that transcription factor OCT-1 bound to *KL* intron 1

both *in vitro* and *in vivo*.

Discussion

KL gene encodes a type-I membrane protein that is related to beta-glucosidases (Ko et al., 2013). *KL* might function as part of a signaling pathway that regulated morbidity in age-related diseases such as atherosclerosis and cardiovascular disease, and mineral metabolism diseases such as ectopic calcification (Ko et al., 2013; Kuro-o et al., 1997; Hu et al., 2011; Ohnishi et al., 2009). Overexpression of *KL* in the preadipocyte 3T3-L1 cell line can induce expression of several adipogenic markers, including *PPARγ*, CCAAT/enhancer binding protein alpha (*C/EBPα*) and CCAAT/enhancer binding protein delta (*C/EBPδ*), and facilitate the differentiation of preadipocytes into mature adipocytes (Chihara et al., 2006). Eliminating *KL* function from mice resulted in the generation of lean mice with almost no detectable fat tissue, and induced a resistance to high-fat-diet-stimulated obesity (Razzaque et al., 2012; Ohnishi et al., 2011).

Here we found the SNP MARC0022311 located in *KL* intron 1 showed a polymorphism in the tested pigs (Supplementary dataset). A number of SNPs were proved to have major effects on the phenotypic variations (Markljung et al., 2009; Milan et al., 2000; Ren et al., 2011; Van Laere et al., 2003). Previous research reported that a G to A transition in intron 3 of porcine insulin-like growth factor 2 (*IGF2*) affected the binding of ZBED6 and significantly up-regulated *IGF2* expression in skeletal muscle (Markljung et al., 2009; Van Laere et al., 2003). We predicted the SNP MARC0022311 located in *KL* intron 1 could change the binding of transcription factors including OCT1 by BIOBASE and TFsearch online software (Fig. S1).

The Octamer-binding proteins (OCTs) are a group of highly conserved transcription factors that

195 specifically bind to the octamer motif (ATGCAAAT) and closely related sequences that are
196 found in promoters and enhancers (Zhao, 2013). OCT1 regulates the expression of a variety of
197 genes, including immunoglobulin genes (Dreyfus, Doyen & Rougeon, 1987), β-casein gene
198 (Zhao, Adachi & Oka, 2002), miR-451/ AMPK signaling (Ansari et al., 2015), sex-determining
199 region Y gene (Margarit et al., 1998), synbindin – related ERK signaling (Qian et al., 2015).
200 In the present study, luciferase activity of *pGL3-D2-G* was significantly higher than *pGL3-D2-A*
201 in PK cells and ST cells and the following *OCT-1* RNAi results showed that luciferase activity of
202 *pGL3-D2-G* significantly decreased, confirming OCT-1 was the repressor. Therefore, we
203 supposed that OCT-1 could bind to the first intron of *KL* when the SNP was allele A, and then
204 depressed activity of *KL* promoter.
205 However, the expression of *KL* was significantly inhibited after silencing *OCT-1*. There were
206 several OCT-1 binding sites in porcine *KL* gene. One hundred and sixty six OCT-1 binding sites
207 were predicted in intron 1 (36324 bp in length) by BIOBASE and TFsearch online software (Fig.
208 S3A). ChIP analysis showed that OCT-1 interacted with all of three tested regions (1395 bp to
209 1525 bp, 14322 bp to 14436 bp, 30970 bp to 31141 bp) in PK cells (Fig. S3B). In consequence,
210 we hypothesized that OCT-1 could bind *KL* gene at multiple sites, and the positive regulation of
211 *KL* gene might be dominant.
212 Klotho physiologically regulate mineral and energy metabolism by influencing the activities of
213 fibroblast growth factors (FGFs) including FGF-2, FGF-19, FGF-23 and their receptors (FGFRs)
214 (Guan et al., 2014; Razzaque et al., 2009; Wu et al., 2008). Taken together, KL exerts is function
215 via OCT-1 - KL- FGF- FGFR pathway.

Conclusions

In summary, SNP MARC0022311 affected OCT-1 binding ability with the *KL* promoter. And the *KL* promoter activity was significantly decreased with allele A of MARC0022311 compared with allele G. Our study indicates SNP MARC0022311 affects porcine *KL* expression by regulating its promoter activity via OCT-1.

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315 **Figure Legends**

316 Fig. 1: Deletion analysis of pig *KL* promoter. (A) Schematic diagram of *KL* promoter ,
317 MARC0022311 (*K*L g.1474 A>G) and OCT-1 binding site in intron 1. (B) Promoter activities of
318 a series of deleted constructs determined by luciferase assay. Left panel, relative location of four
319 deletion fragments. The nucleotides were numbered from the potential transcriptional start site
320 assigned as +1. Right panel, the relative luciferase activities of four reconstructed vector
321 contained sequence from KL-D1 to KL-D4. *** $P<0.001$.

322 Fig. 2: MARC0022311 in pig *KL* intron 1 affected promoter activity in PK and ST cells. (A)
323 Luciferase assays of reporter constructs using pig KL-D2 promoter and intron 1 fragments
324 (g.1474 A and g.1474 G). (B) Luciferase detection after co-transfection of *OCT-1* siRNA with
325 *pGL3-D2-A* and *pGL3-D2-G* in PK cells. (C) Luciferase detection after co-transfection of *OCT-1*
326 siRNA with *pGL3-D2-A* and *pGL3-D2-G* in ST cells. * $P<0.05$. ** $P<0.01$.

327 Fig. 3: OCT-1 up-regulated *KL* expression by RNAi. (A) PK cells were treated with 2 μl *OCT-1*
328 siRNA and 2 μl NC for 24 h. Knockdown of *OCT-1* was confirmed by qPCR. *KL* mRNA and
329 protein expressions were analyzed by qPCR and Western blotting. (B) ST cells were treated with
330 2 μl *OCT-1* siRNA and 2 μl NC for 24 h. Knockdown of *OCT-1* was confirmed by qPCR
331 analysis. *KL* mRNA and protein expressions were analyzed by qPCR and Western blotting. *
332 $P<0.05$. ** $P<0.01$.

333 Fig. 4: Binding of OCT-1 with *KL* intron 1 was analyzed by EMSA and ChIP. (A) The probe
334 was incubated with nuclear extract in the absence or presence of 100-fold excess of various
335 competitor probes (mutant or non-labeled probe) or anti-OCT-1. The specific super-shift (DNA-

protein-antibody complex) bands were both observed in PK and ST cells. The sequences of various probes were demonstrated under the panel. (B) ChIP assay of OCT-1 binding to the *KL* intron 1 in PK cells and ST cells. The interaction of OCT-1 *in vivo* with *KL* intron region was determined by chromatin immunoprecipitation analysis. DNA isolated from immune-precipitated material was amplified by PCR to amplify *KL* fragement. Total chromatin was used as the input. Normal mouse IgG was used as a negative control.

343 Table 1. Primers and DNA oligos used in this study.

Supplementary files

Fig. S1: Transcription factor binding site prediction of the procine *KL* intron 1 containing MARC0022311 (KL g.1474 A>G). Quadrilateral frame indicated the substitutions and extra binding site of OCT-1. (A) Predicted by BIOBASE online software. (B) Predicted by TFserach online software.

Fig. S2: Genotyping results of MARC0022311. (A) PK cells. (B) ST cells. MARC0022311 was marked in gray backgound.

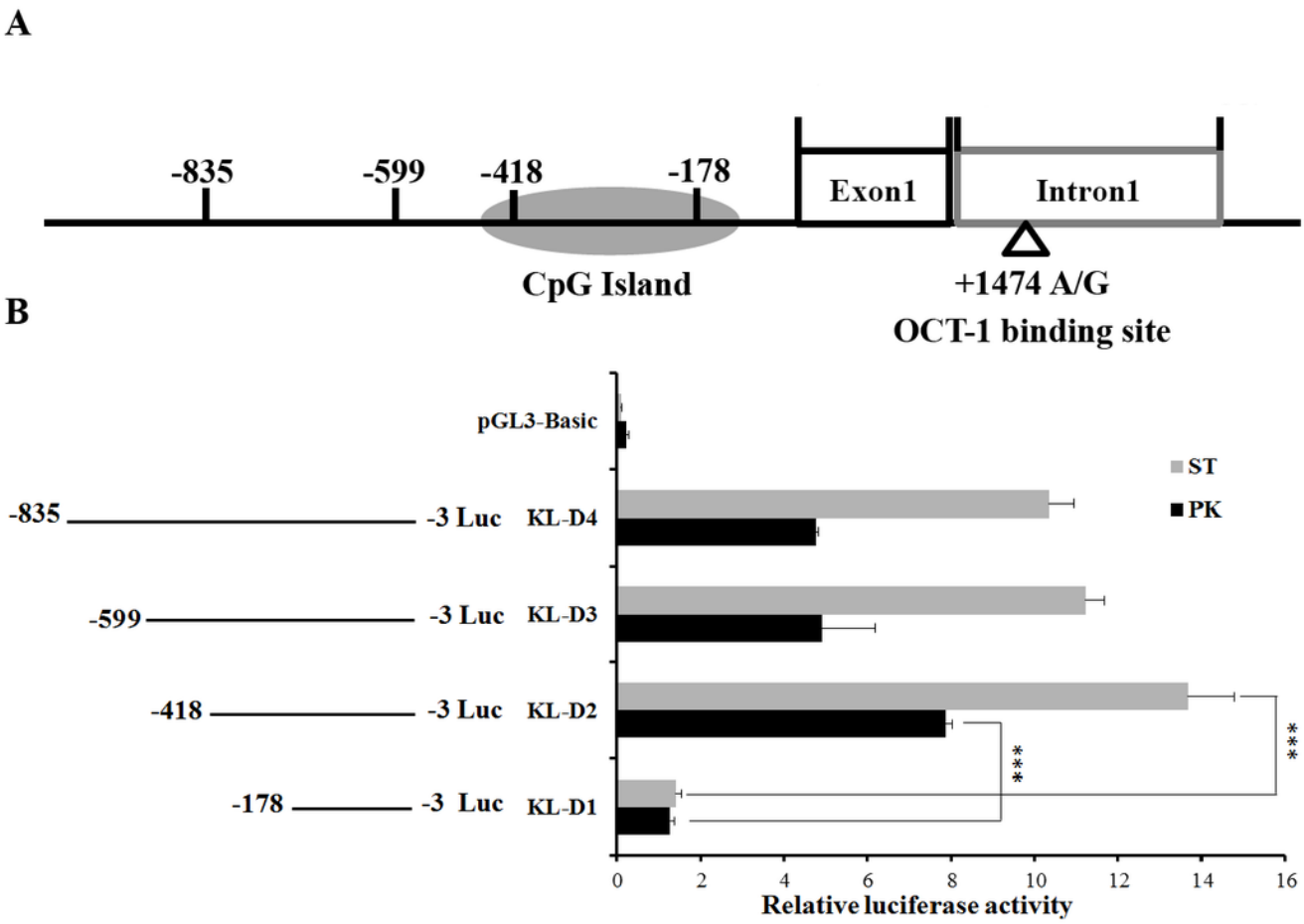
Fig. S3. OCT-1 binding sites in the porcine *KL* intron 1. (A) Frequency distribution of the predicted OCT-1 binding sites. X-axis indicated the length of the porcine *KL* intron 1 in bp. Y-axis was the frequency of the predicted OCT-1 binding sites. (B) ChIP analysis of three candidate OCT-1 binding sites (1395 bp to 1525 bp, 14322 bp to 14436 bp, 30970 bp to 31141 bp) in *KL* intron 1 in PK cells. Primers used for ChIP-PCR was shown in Table 1. Input and R were positive control, while IgG was the negative control.

Supplementary dataset: SNP genotyping results in 19 Landrace× DIV pigs using the Porcine SNP60 BeadChip (Illumina). DIV was a synthetic line derived by crossing Landrace, Large White, Tongcheng or Meishan pigs.

1

Deletion analysis of pig *KL* promoter.

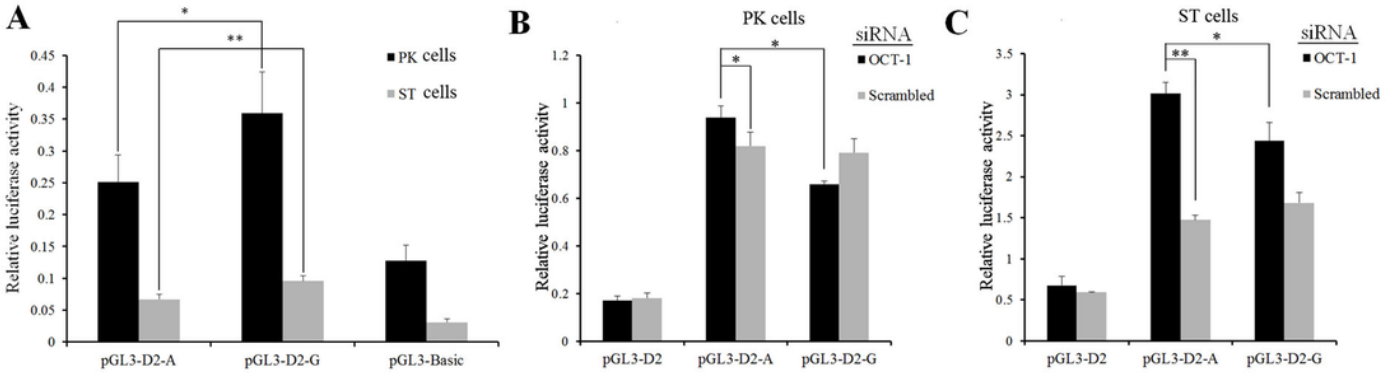
(A) Schematic diagram of *KL* promoter , MARC0022311 (*K*L g.1474 A>G) and OCT-1 binding site in intron 1. (B) Promoter activities of a series of deleted constructs determined by luciferase assay. Left panel, relative location of four deletion fragments. The nucleotides were numbered from the potential transcriptional start site assigned as +1. Right panel, the relative luciferase activities of four reconstructed vector contained sequence from KL-D1 to KL-D4. *** $P<0.001$.



2

MARC0022311 in pig *KL* intron 1 affected promoter activity in PK and ST cells.

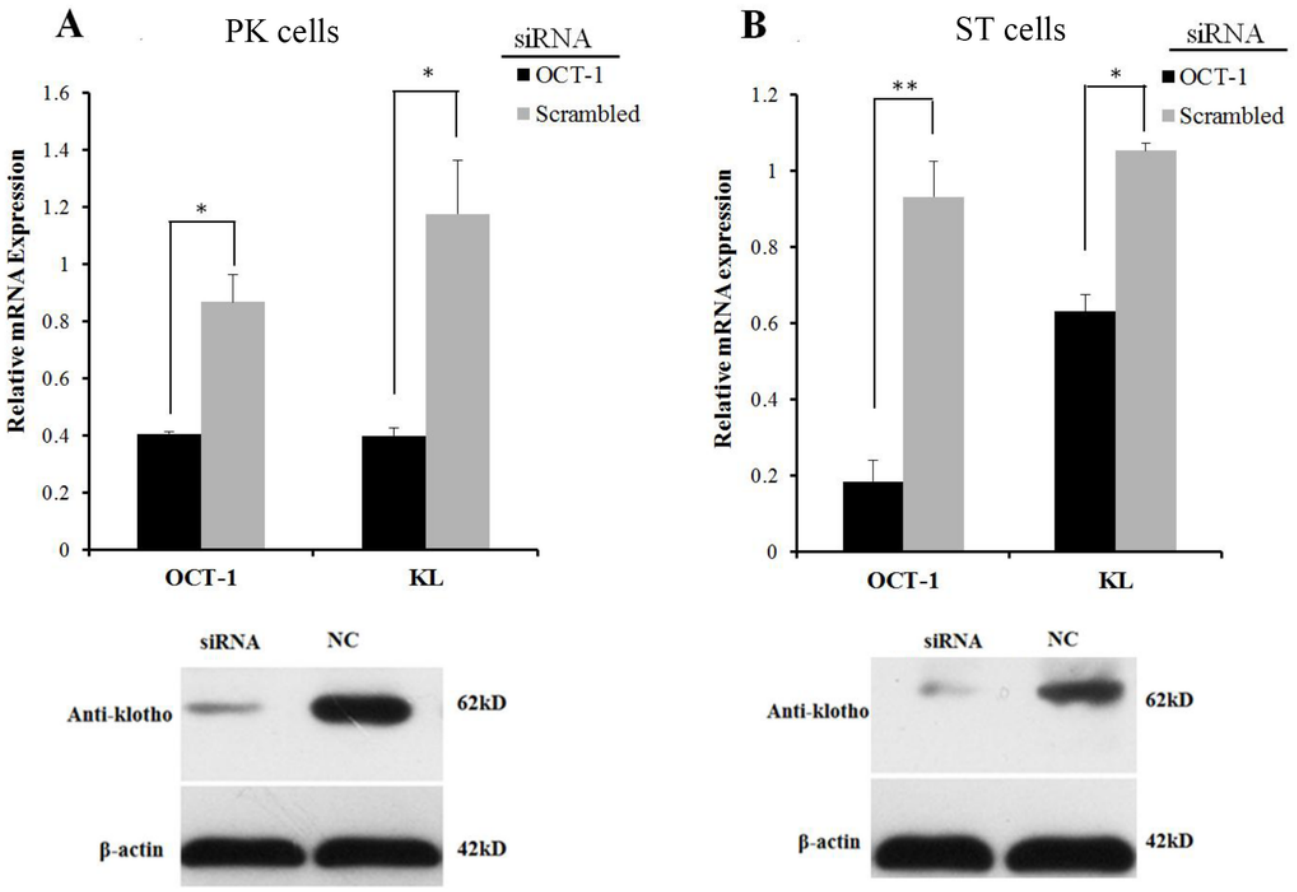
(A) Luciferase assays of reporter constructs using pig KL-D2 promoter and intron 1 fragments (g.1474 A and g.1474 G). (B) Luciferase detection after co-transfection of *OCT-1* siRNA with *pGL3-D2-A* and *pGL3-D2-G* in PK cells. (C) Luciferase detection after co-transfection of *OCT-1* siRNA with *pGL3-D2-A* and *pGL3-D2-G* in ST cells. * $P<0.05$. ** $P<0.01$.



3

OCT-1 up-regulated *KL* expression by RNAi.

(A) PK cells were treated with 2 μl *OCT-1* siRNA and 2 μl NC for 24 h. Knockdown of *OCT-1* was confirmed by qPCR. *KL* mRNA and protein expressions were analyzed by qPCR and Western blotting. (B) ST cells were treated with 2 μl *OCT-1* siRNA and 2 μl NC for 24 h. Knockdown of *OCT-1* was confirmed by qPCR analysis. *KL* mRNA and protein expressions were analyzed by qPCR and Western blotting. * $P<0.05$. ** $P<0.01$.



4

Binding of OCT-1 with *KL* intron 1 was analyzed by EMSA and ChIP.

(A) The probe was incubated with nuclear extract in the absence or presence of 100-fold excess of various competitor probes (mutant or non-labeled probe) or anti-OCT-1. The specific super-shift (DNA-protein-antibody complex) bands were both observed in PK and ST cells. The sequences of various probes were demonstrated under the panel. (B) ChIP assay of OCT-1 binding to the *KL* intron 1 in PK cells and ST cells. The interaction of OCT-1 *in vivo* with *KL* intron region was determined by chromatin immunoprecipitation analysis. DNA isolated from immune-precipitated material was amplified by PCR to amplify *KL* fragement. Total chromatin was used as the input. Normal mouse IgG was used as a negative control.

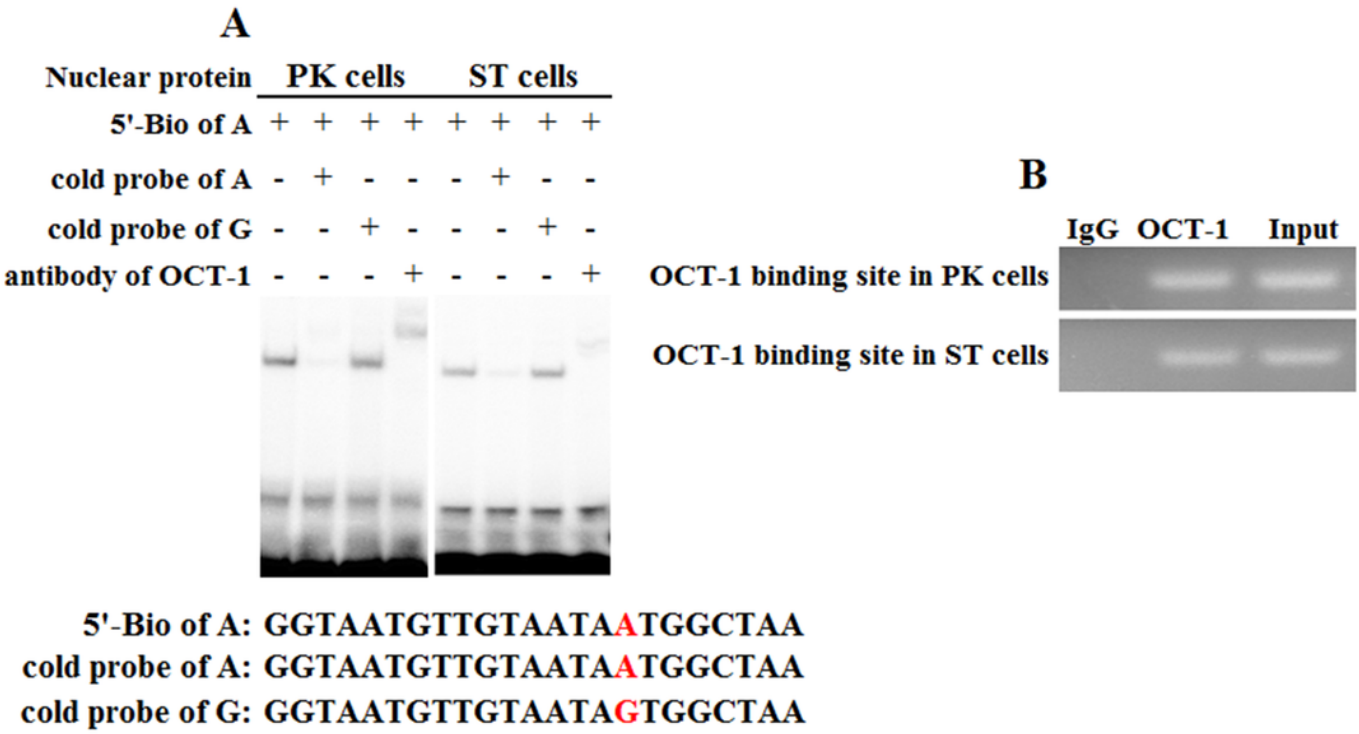


Table 1(on next page)

Primers and DNA oligos used in this study.

1 **Table 1. Primers and DNA oligos used in this study.**

Primer	Primer sequence (5'-3')	Amplicon Length (bp)	Tm (℃)
5'-Bio of A (+)	GGTAATGTTGTAATAATGGCTAA		60
5'-Bio of A (-)	TTAGCCATTATTACAACATTACC		
cold probe of A (+)	GGTAATGTTGTAATAATGGCTAA		60
cold probe of A (-)	TTAGCCATTATTACAACATTACC		
cold probe of G (+)	GGTAATGTTGTAATAGTGGCTAA		60
cold probe of G (-)	TTAGCCACTATTACAACATTACC		
*KL*_ChIP_PF	TGAAGACCACTGCTACACACTT		59
*KL*_ChIP_PR	AGCAAACAGGTTTTGTGGAGC		
*KL*_D1_PF	*CGG***GGTACC**TTGTTGGATGTTTTGTT TGTCTAGCTAGC	193	58
*KL*_D_PR	*CG***ACGCGT**CCCTGTGAAGGCTTGTTT		
*KL*_D2_PF	*CGG***GGTACC**TATGAGGAGGTGGGTT GGCTAGCTAGC	433	59
*KL*_D_PR	*CG***ACGCGT**CCCTGTGAAGGCTTGTTT		
*KL*_D3_PF	*CGG***GGTACC**CACTTAACCTCTTATTC TTGAGTTACTAGCTAGC	614	59
*KL*_D_PR	*CG***ACGCGT**CCCTGTGAAGGCTTGTTT		
*KL*_D4_PF	*CGG***GGTACC**CACATAAAAGTTAGAAA ATCAGAGAACTAGCTAGC	850	59
*KL*_D_PR	*CG***ACGCGT**CCCTGTGAAGGCTTGTTT		
OCT1_qPCR_PF	TGAACAATCCGTCAGAAACC	196	58
OCT1_qPCR_PR	TGAGCAGCAGCCTGTAAACT		
KL_qPCR_PF	ACCCGTATTTATTGATGGAGAC	173	57
KL_qPCR_PR	GGAACTTCATCTGAGGGTCTAA		
KL_intron1_ChIP_PF	GCCGTAGATAATTGAAGC	130	50
KL_intron1_ChIP_PR	TCTGTGGTAGCAAACAGG		
KL_intron2_ChIP_PF	GCCAGTGTAAGGTGTTACC	114	51
KL_intron2_ChIP_PR	ATTCTCCAAAGAAGACATACA		
KL_intron3_ChIP_PF	CAAGATTGTACCGTGGAG	171	50
KL_intron3_ChIP_PR	GGTCATTTGACATCATTCT		

2 Protective bases and induced enzyme sites were in italic and bold respectively.

3