Acta Biochim Biophys Sin (2008): 85-90 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008.00373.x

# Expression patterns and subcellular localization of porcine (*Sus Scrofa*) lectin, galactose-binding, soluble 1 gene

Haifang Qiu, Shuhong Zhao, Mei Yu, Bin Fan, and Bang Liu*

Laboratory of Molecular Biology and Animal Breeding, Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan 430070, China

**Lectin, galactose-binding, soluble 1 (*LGALS1*) gene encodes galectin-1, an atypical secretory protein that plays an important role during myoblast proliferation and differentiation. In this study, the porcine *LGALS1* gene was cloned and characterized from pig muscle. The predicted protein sequence shared a high identity with its mammalian counterparts. Reverse transcription-polymerase chain reaction revealed that porcine *LGALS1* was expressed at 33 day post-coitus (dpc) and 65 dpc at a relatively high level, and then decreased to 90 dpc during fetal skeletal muscle development, suggesting that galectin-1 is a potent factor implicated in the formation of myofibers. *LGALS1* was found widely expressed in all tissues and transient transfection indicated that galectin-1 locates both in cytoplasm and nucleus. Genomic sequences and analysis predicted a promoter region at approximately 1.279–1.529 kb, but dual-luciferase reporter assay indicated that it has little promoter activity.**

*Keywords* expression pattern; subcellular localization; promoter activity; *LGALS1*

Galectins are structural proteins with at least one characteristic carbohydrate recognition domain with an affinity for β-galactosides [1–2]. To date, 15 different galectins have been characterized and they are numbered according to the chronology of discovery (galectin-1 to galectin-15). They are also widely distributed from lower to higher vertebrates [3]. Galectin-1 was the first discovered mammalian galectin [4] and it is secreted during differentiation and accumulates with laminin in the basement membrane surrounding each myofiber [5]. It is expressed in a wide range of vertebrate tissues, particularly in developing cardiac, smooth, and skeletal muscle. Studies showed that galectin-1 plays a role during skeletal muscle development [6] and that peak galectin-1 expression in muscle coincides with formation of myofibers [7]. The down-regulated expression of galectin-1 in migrating tumor cells could impair malignancy development in different ways [8–10].

Pig is an important meat animal, and meat production is determined by the number and size of myofibers. Meat quality is determined by the proportions of muscle fiber type [11]. In porcine muscle development, there are two major waves of fiber generation, a primary generation from 33 day post-coitus (dpc) to approximately 65 dpc, and a secondary generation from approximately 54 to 90 dpc [12]. Hence, 33 dpc, 65 dpc, and 90 dpc are key stages during prenatal skeletal muscle development. The study of lectin, galactose-binding, soluble 1 gene (*LGLAS1*) will contribute to the understanding of myofiber development.

Here, the sequences of porcine *LGALS1* were characterized, and its expression pattern, protein location, and activity of the predicted promoter region in PK15 cells were investigated.

## Materials and Methods

### Tissue sampling, RNA isolation, and cDNA preparation

Chinese indigenous Tongcheng pigs were used in this study. The fetal skeletal muscles from three ages, 33, 65, and 90 dpc, were harvested, frozen in liquid nitrogen, and stored at –80 ºC. Ten different tissues (heart, liver, spleen, lung, kidney, skeletal muscle, small intestine, lymph node, testis, and brain) were collected for spatial expression studies.

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, USA). RNA concentration was measured by a

Received: July 4, 2007 Accepted: September 27, 2007
This work was supported by the grants from the National Natural Science Foundation of China (Nos. 30371029 and 30571007), the Technologies Research and Development Project of Hubei Province (No. 2002AA201C27), and the Natural Science Foundation Creative Team Projects of Hubei Province (No. 2006ABC008)
*Corresponding author: Tel, 86-27-87282680; Fax, 86-27-87280408; E-mail, liubang@mail.hzau.edu.cn

Beckman DU 640 spectrophotometer (Beckman, Fullerton, USA). Then cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). Two micrograms of total RNA were combined with 5 mM oligo(dT)15 and 8 μl diethyl pyrocarbonate water, then incubated at 70 ºC for 5 min to denature secondary structures. After cooling the mixture rapidly to 0 ºC, 10 μl of 5×reverse transcriptase buffer, 250 mM dNTPs, 40 U RNase inhibitor (Promega), and 400 U Moloney murine leukemia virus reverse transcriptase were added to a total volume of 50 μl. The mixture was incubated at 42 ºC for 60 min, then at 95 ºC for 5 min to destroy the RNase, and then treated with RNase-free DNase (Fermentas, Vilnius, Lithuania).

**cDNA isolation, sequencing, and analysis**

The full-length cDNA sequence of porcine *LGALS1* was obtained using the rapid amplification of cDNA ends (RACE). Gene-specific primers were designed using pig expressed sequence tag data from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (**Table 1**). RACE was carried out according to manufacturer's protocol of the SMART RACE cDNA kit (Clontech, Palo Alto, USA). The polymerase chain reaction (PCR) products were purified with a Gel Extraction Mini Kit (TaKaRa, Shiga, Japan) and cloned into plasmid pMD18-T (TaKaRa), then sequenced commercially. Bioinformatics analysis was carried out using PROSITE and TargetP (http://cn.expasy.org/tools/) [13].

**Genomic sequences and analysis**

Genomic DNA fragments were amplified by PCR in 20 μl of 1×PCR buffer (Fermentas) containing 50 ng porcine genomic DNA, 0.3 μM each primer, 75 μM each dNTP, 1.5 mM $MgCl_2$, and 2.0 U *Taq* DNA polymerase (Fermentas). The PCR parameters were 5 min at 95 ºC followed by 30 s at 94 ºC, 30 s at the annealing temperature (**Table 1**), and 30 s at 72 ºC for 35 cycles, followed by a final extension of 5 min at 72 ºC. The PCR products were purified, sequenced, and assembled.

The genomic DNA sequences were analyzed by CpG Island Searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx) and Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/) programs to find if there are some regulatory regions in this gene. Repetitive elements were identified using RepeatMasker (http://www.repeatmasker.org).

**Expression patterns of *LGALS1***

*LGALS1* expression patterns were determined by reverse transcription-PCR. One microliter of the resulting single-stranded cDNA was amplified 27 cycles with *LGALS1*-specific primers (**Table 1**). The housekeeping gene *β-actin* was used as an internal control. PCR products were separated by electrophoresis on 2.0% agarose gels and

**Table 1 Primers used in this paper**

| Gene name | Primer name | Primer sequence (5′→3′) | Binding region | $T_m$ (ºC) | Product size (bp) |
|---|---|---|---|---|---|
| *LGALS1* | 5' RACE | CCTTGCTGTTGCACACGATG | Exon 3 | 60 | 287 |
| | 3' RACE | ACCTGTGCCTGCACTTCAAC | Exon 3 | 60 | 390 |
| | Genomic1 PL | GGAACATCCTTGTCTCCTCAGTCA | Exon 1 | 60 | 1343 |
| | Genomic1 PR | GGTTTGAGATTCAGGTTGCTGG | Exon 2 | 60 | 1343 |
| | Genomic2 PL | GTCGCCAGCAACCTGAATCTC | Exon 2 | 62 | 1492 |
| | Genomic2 PR | ATGTCTCCGTGCATGTCGAAG | Exon 3 | 62 | 1492 |
| | Genomic3 PL | CTTCGACATGCACGGAGACAT | Exon 3 | 58 | 464 |
| | Genomic3 PR | ATCTGGCAGCTTGATGGTGAG | Exon 4 | 58 | 464 |
| | Semi-RT PL | GTCGCCAGCAACCTGAATCT | Exon 2 | 60 | 299 |
| | Semi-RT PR | TACCCATCTGGCAGCTTGA | Exon 3 | 60 | 299 |
| | pEGFP PL | GTCGACATGGCTTGTGGTCTGGTCG | Exon 1–Exon 2 | 60 | 423 |
| | pEGFP PR | GGATCCGCTTCACTCAAAGGCCACACAC | Exon 4 | 60 | 423 |
| | pGL3-Basic PL | GCTAGCTGGTTGAGAGTGGGGAGTTGC | Intron 1 | 60 | 1274 |
| | pGL3-Basic PR | AAGCTTGCTCAGCATGATGTGTGACACA | Intron 2 | 60 | 1274 |
| *β-Actin* | βSemi-RT PL | TCTGCCATCTCTACTACCCTAAGG | | 60 | 233 |
| | βSemi-RT PR | CTAAGAAGTGCTCCCTGAGCAG | | 60 | 233 |

βSemi-RT, semi-quantitative reverse transcription-polymerase chain reaction of *β-actin*; *LGALS1*, lectin, galactose-binding, soluble 1; pEGFP, enhanced green fluorescent protein plasmid; PL, left primer; PR, right primer; RACE, rapid amplification of cDNA ends.

visualized by ethidium bromide staining. The PCR fragments were purified and directly sequenced to confirm the correct amplification of the porcine *LGALS1* gene.

For tissue-specific expression analysis, total RNAs were isolated from various tissues (heart, liver, spleen, lung, kidney, skeletal muscle, small intestines, lymph node, testis, and brain). For temporal expression analysis of *LGALS1*, total RNAs were isolated from skeletal muscle of various fetal developmental stages (33, 65, and 90 dpc), and the total RNAs of three individual fetuses were mixed in each stage in order to ensure authenticity [14].

### Subcellular localization in PK15 cells

The open reading frame of porcine *LGALS1* was amplified from its cDNA clone and subcloned into the *Sal*I-*Bam*HI site of the enhanced green fluorescent protein (EGFP) vector (pEGFP-N1; BD Biosciences Clontech, Palo Alto, USA) to yield a mammalian expression plasmid, pEGFP-*LGALS1*. The primers are listed in **Table 1** and the vector was sequenced for accuracy. PK15 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin under humidified air containing 5% $CO_2$ at 37 ºC and seeded onto cover slips. Transient transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

After 48 h, the cells in a 6-well plate were washed using phosphate-buffered saline, then fixed for 15 min with 4% para-formaldehyde. After the washing steps and incubation with 10 μM Hoechst 33342 for 10 min, the slides were mounted, sealed, and analyzed by confocal microscopy (TCS-SP2; Leica, Heideberg, Germany). Leica IM500 confocal software was used to generate images of individual fluorescent markers as well as overlay pictures that showed the relative distribution of the fusion protein.

### Transient transfection and dual-luciferase reporter assay

A 1.268 kb predicted regulatory region was amplified with the primers shown in **Table 1**. The amplified fragment was inserted into the *Nhe*I-*Hin*dIII site of pGL3-Basic (Promega) to construct the pGL3-1.268 kb vector. The pGL3-1.268 kb vector was co-transfected into PK15 cells in triplicate with an internal control pRL-TK (Promega). The cells were transfected with Lipofectamine 2000 in 24-well plates. Each well included $1.5\times10^5$ cells, 0.8 mg pGL3-1.268 kb, 0.08 mg pRL-TK, 2 μl Lipofectamine 2000, and 500 μl RPMI 1640 medium without serum or antibiotics. Empty pGL3-Basic (promoter-less) with pRL-TK was also transfected in triplicate in parallel as a control.

All cells were analyzed for dual-luciferase reporter gene expression 48 h after completion of the transfection procedure. The activities of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed with phosphate-buffered saline after harvest and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1×passive lysis buffer. Twenty milliliters of cell lysate was transferred into the luminometer tube containing 100 ml Luciferase Assay Reagent II (Promega). Firefly luciferase activity (M1) was measured, then Renilla luciferase activity (M2) was measured after adding 100 ml Stop & Glo reagent (Promega). The program of the luminometer was a 2 s pre-measurement delay followed by a 10 s measurement period for each assay.

## Results

### Molecular cloning and sequence analysis of porcine *LGALS1* gene

Analysis of the cDNA sequence of porcine *LGALS1* revealed the following results. The full-length cDNA of porcine *LGALS1* is 559 bp and contains an open reading frame of 408 bp encoding a protein of 135 residues with a calculated molecular mass of 14.72 kDa and an isoelectric point of 4.86. It contains a 5′-untranslated region of 71 bp and a 3′-untranslated region of 80 bp with a consensus AATAAA polyadenylation signal 21 bp before the poly(A) stretch. The sequence of porcine *LGALS1* had been submitted to GenBank (GenBank accession No. DQ367936). There are several phosphorylation, *N*-myristoylation and galaptin signature sites, but no protein-binding motifs, signal peptide, or transmembrane regions are common to any other known protein family predicted by ExPASy. A BLAST search (http://www.ncbi.nlm.nih.gov/blast) in the GenBank database indicated that the predicted protein shared high similarity with other mammals, 85% identity to human and rat, and 83% identity to mouse.

The genomic DNA sequences of porcine *LGALS1* (GenBank accession No. DQ367937) were obtained by PCR amplification. It is interesting that there is a CpG island predicted by CpG Island Searcher, from 1.168 kb to 2.281 kb, the GC level is 61.4%, and ObsCpG/ExpCpG is 0.705. A promoter is also found in this region, from 1.279 to 1.529 kb, the score is 78.02%, and there are some important transcription factors such as Sp1, myosin-specific factor, and UCE-2. In human and mouse, there

are also similar structures analyzed by the same method. Three short interspersed sequence nucleotide elements (124–296, 729–835, and 2555–2671), one long interspersed sequence nucleotide elements (2391–2495), and one simple repeat (2279–2309) were also discovered in this gene.

### Spatial and temporal expression patterns of *LGALS1*

Porcine *LGALS1* was expressed at the highest level in the skeletal muscle with prominent expressions detected in the lung, lymph node, and testis, and lower levels detected in heart, liver, spleen, kidney, small intestine, and brain (**Fig. 1**). These data are generally in agreement with the expression pattern of *LGALS1* in both human and mouse [15].

As shown in **Fig. 2**, porcine *LGALS1* was expressed at 33 and 65 dpc at a relatively high level, then decreased at 90 dpc.

### Cellular localization of porcine *LGALS1* in PK15 cells

The cellular location of *LGALS1* was studied by fluorescence and confocal analysis of PK15 cells transiently transfected with pEGFP-*LGALS1*. Hoechst 33342 was used to label nuclei. LGALS1 fusion protein was found to localize both in cytoplasm and nuclei (**Fig. 3**). Green fluorescence was detected through control cells, transfected with GFP vector alone.

### Promoter activity of the cloned 1.268 kb fragment

The firefly luciferase expression driven by the 1.268 kb predicted promoter of *LGALS1* was examined to evaluate the promoter activity. The relative luciferase activity of the experimental sample is presented by the ratio of the activities of firefly luciferase and Renilla luciferase (M1/M2). The result showed that the relative activity (M1/M2) was 0.240, 48 h after pGL3-1.268 kb was co-transfected into PK15 cells with pRL-TK. This was only 12-fold higher than that of pGL3-Basic co-transfection

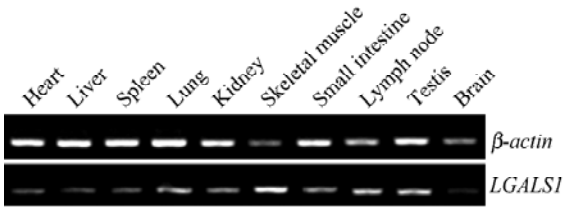


**Fig. 1 Porcine lectin, galactose-binding, soluble 1 gene (*LGALS1*) expression pattern in heart, liver, spleen, lung, kidney, skeletal muscle, small intestine, lymph node, testis, and brain**

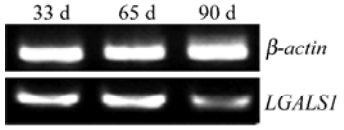


**Fig. 2 Temporal distribution of porcine lectin, galactose-binding, soluble 1 gene (*LGALS1*) in fetal skeletal muscle from three developmental stages (33, 65, and 90 day post-coitus)** Each lane contains a pool of RNA from three pigs.

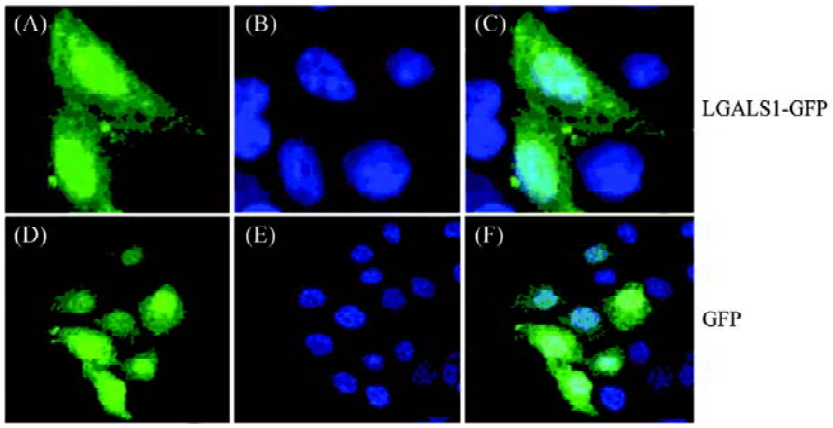


**Fig. 3 Cellular localization of porcine lectin, galactose-binding, soluble 1-green fluorescent protein (LGALS1-GFP) fusion protein in PK15 cells** The recombinant plasmid was transiently transfected into PK15 cells using Lipofectamine reagent. (A, D) The LGALS1-GFP fusion protein was distributed both in cytoplasm and nuclei (excited at 488 nm). (B, E) Nuclei were stained with Hoechst 33342 (excited at 360 nm). The fluorescent signals were analyzed by confocal microscopy. (C, F) The overlay images were produced by merging two images together. Magnification, 400×.

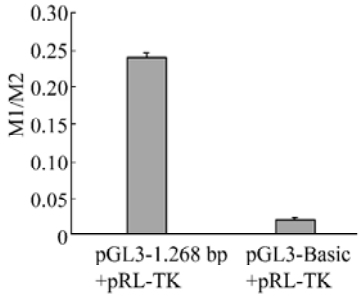


**Fig. 4 Dual-luciferase reporter assay for porcine lectin, galactose-binding, soluble 1 gene (*LGALS1*)** The 1.268 kb fragment was obtained by polymerase chain reaction and cloned into pGL3-Basic. Dual-luciferase assay was carried out 48 h after PK15 cells were transfected. The relative activities (M1/M2) were 0.240±0.007 for pGL3-1.268 kb with internal control pRL-TK, and 0.020±0.003 for pGL3-Basic with pRL-TK. Data are presented as mean±SD ($n$=3).

with pRL-TK (**Fig. 4**).

## Discussion

*LGALS1* is secreted during differentiation and binds to lamin [16], and it inhibits cell-matrix interaction. The inhibition of cell-matrix adhesion has been proposed to play an important role in muscle formation in mouse [5, 16]. Muscle mass is largely determined by the number of muscle fibers and the size of those fibers. In this study, the full-length cDNA of porcine *LGALS1* gene was obtained. The porcine *LGALS1* gene was mapped to SSC5p11-p15 [17]. In the latest released Pig QTL Database (http://www.animalgenome.org/QTLdb/pig.html) [18], several QTLs for the proportion of muscle fiber types and their size, which affects muscularity as well as functional properties of the musculature and meat quality [19], were mapped to this small chromosomal region, indicating that this gene might be a positional candidate gene for these traits.

The temporal expression data indicate that porcine *LGALS1* was expressed at 33 and 65 dpc at a relatively high level, then decreased at 90 dpc, results similar to those of Tang *et al* [20]. In mouse, myoblasts release galectin-1 while undergoing differentiation, but not while proliferating. It is expressed at a high level when maximum cell fusion is occurring during muscle development [6,7,21]. It was reported that the numbers of pig skeletal muscle fiber stopped increasing at approximately 90 dpc [12], and then the fiber began to hypertrophy; our results are in agreement with these reports. Therefore, it could be inferred that *LGALS1* acts as an enhancing factor of myofiber formation during muscle development and it is more important for increasing muscle fiber numbers than for fiber hypertrophy.

We further examined *LGALS1* expression in porcine tissues by reverse transcription-PCR. Porcine *LGALS1* showed a wide distribution in tissues. Then we detected its protein, galectin-1, distribution in PK15 cells, and we found it localized both in cytoplasm and nuclei. This might be relative to its various biological functions. There is no predicted signal peptide in this protein, so we deduce that galectin-1 might be secreted by non-classical mechanisms, not through classical vesicle-mediated exocytosis. And there are reports suggesting that galectin-1 is secreted by non-classical mechanisms in rat and mouse [7,22]. However, the real mechanisms are not clear and need further study.

Introns are non-coding DNA sequences that widely reside in the genome of eukaryote. The regulatory elements of introns often affect the efficiency of gene expression [23], so we tried to test if there are regulatory elements within *LGALS1*. Through prediction, we found a CpG island and a possible promoter region. But the luciferase activity of the predicted promoter region is rather weak, only 12-fold higher than that of pGL3-Basic. In general, an over 50-fold increase in luciferase activity characterizes a typical promoter region [24,25], so the result indicated that the predicted promoter region has little promoter activity. There should be other regions that regulate the gene expression of *LGALS1*. The result also indicated that not all regulatory regions are within or nearby CpG islands.

In summary, we have isolated and characterized the porcine *LGALS1* gene. Data presented here provides biochemical and structural bases for future studies of porcine *LGALS1* function. It will potentially lead to a better understanding of the mechanism of *LGALS1* function in muscle fiber formation, thereby influencing muscle development.

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