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Molecular characterization of pig LEG1a protein

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Abstract *Liver-enriched gene 1* (*LEG1*) is a newly identified gene that plays an important role in the liver development of zebrafish and the innate immunity of platypus. However, little is known about *LEG1* in eutherians such as mice and humans. In this study, we explored the molecular characteristics of the LEG1 protein in a pig model (pLEG1a). A rabbit polyclonal antibody against pLEG1a was produced and validated. Then, we detected the pLEG1a protein in both the salivary gland and lung. Moreover, pLEG1a could be detected in the saliva, suggesting its secretory nature. Ectopic expression of *pLEG1a* in HEK293T cells further confirmed that pLEG1a was a secretory protein. In addition, glycosylation tests showed the existence of glycosylated bands which could be enzymatically removed, indicating that the secreted pLEG1a was *N*-glycosylated. In conclusion, the pLEG1a protein is similar to the LEG1 proteins of other species, especially eutherian LEG1s, in terms of expression profile, glycosylation, and secretion ability, suggesting that pLEG1a is a good model to further study the function of eutherian *LEG1s*.

Key words pig; LEG1a protein; secretory protein; glycosylation
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猪的肝富集基因1a蛋白的分子鉴定(英文)

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摘要 新发现的肝富集基因1(*live enriched-gene 1*, *LEG1*)在斑马鱼肝脏发育及鸭嘴兽的先天性免疫应答过程中发挥着重要的调控作用,但是*LEG1*在真兽亚纲物种(如小鼠和人)中的功能鲜有报道。本研究以猪为模型,对猪的肝富集基因1a蛋白(pLEG1a)进行了分子鉴定。实验首先制备并验证了pLEG1a蛋白的兔源多克隆抗体,并验证了pLEG1a在猪唾液腺和肺部的表达。同时,在唾液中检测到了pLEG1a蛋白的信号,表明pLEG1a蛋白可能是一种分泌蛋白。此外,当在HEK293T细胞中异位表达*pLEG1a*时,可在细胞培养液中检测到pLEG1a蛋白,进一步证明了pLEG1a是一种分泌型蛋白。另外,糖基化实验显示,唾液及HEK293T细胞中的pLEG1a存在糖基化条带,且这一条带可以通过糖基化酶去除,表明pLEG1a是一种*N*-糖基化蛋白。综上所述,本研究探究了pLEG1a蛋白与其他物种(尤其是真兽亚纲动物)LEG1蛋白在表达谱、糖基化和分泌能力方面的相似性,并且提出pLEG1a可能

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是研究真兽亚纲物种*LEG1*基因的良好模型。

关键词 猪；肝富集基因1a蛋白；分泌蛋白；糖基化

The *liver-enriched gene 1* (*LEG1* or *C6orf58*) is a newly identified, evolutionarily conserved gene with rare functional annotation. Zebrafish *LEG1s* (*zleg1s*) were first studied for their enrichment in the liver, suggesting their potential roles in liver development[1]. This hypothesis was later confirmed by knocking down *zleg1s* using a morpholino, resulting in a small liver phenotype under stress conditions[2]. Mammalian *LEG1* was first studied in the platypus, in which the monotreme lactation protein encoding gene (*MLP*) has anti-Gram-positive bacterial activity[3]. However, the function of *LEG1* in eutherian species remains poorly understood. Due to two tandem duplication events in mammals, *LEG1s* have three paralogs, *LEG1a*, *LEG1b*, and *LEG1c*, of which pigs have all three genes (*pLEG1a*, *pLEG1b*, and *pLEG1c*), while humans and mice only have *LEG1a* (*hLEG1a* and *mLeg1a*). An evolutionary study indicated that vertebrate *LEG1s* experienced purifying selection; however, different paralogs of *LEG1s* might retain distinct subfunctions of ancestral *LEG1*[4]. In a liver cell line, transcriptomic prediction confirms that the platypus *MLP* has antibacterial activity, and *pLEG1c* may play a dispensable role in the endoplasmic reticulum stress response and protein folding regulation, and *pLEG1a* and *pLEG1b* have little function in the liver[5]. However, further *in vivo* studies are required to elucidate the biological roles of the *LEG1* genes.

The LEG1 protein is characterized by the presence of the LEG1 domain (or domain of unknown function 781, DUF781), preceded by a signal peptide, indicating that it belongs to a secretory protein, as accumulating evidence has shown its existence in milk, saliva, and seminal plasma[3,6−7]. Studies of hLEG1a, zLEG1, and platypus MLP have suggested that glycosylation may be another hallmark of the protein family. As we proposed that *pLEG1s* are good models to study the gene family in eutherian species[4], the pLEG1 proteins need to be characterized at the protein level. In the current study, a pLEG1a−specific antibody was first validated and used to demonstrate that pLEG1a is similar to other LEG1 proteins. This information would facilitate the research of *LEG1* in eutherian species.

1 Materials and methods

1.1 Ethical statement

This work, involving the use of porcine tissue samples, has been approved by the Animal Welfare Committee of Zhejiang University (approval No. 11834).

1.2 Plasmid construction

Two types of expression plasmids for *pLEG1a* were generated as follows. For pCMV−pleg1a−C−FLAG, primers (forward: CTCAGTGGATCCGCCGCC ATGGCTTTCCTTCCTCCTTGG, and reverse: TCAG AAATGTTGGAATGCTGCAA), including *BamH*Ⅰ and *Xho*Ⅰ sites, were used to amplify *pLEG1a* from salivary gland cDNA. Then, the amplified *pLEG1a* fragment was digested with *Bam*HⅠ and *Xho*Ⅰ and ligated to pCMV−C−FLAG (Beyotime Biotechnology, Shanghai, China) to construct pCMV−pleg1a−C−FLAG. A similar strategy was used to construct pCAG−pleg1a−3×FLAG. For the generation of pCAG−pleg1b−3×HA, the *pLEG1b* sequence was directly synthesized and cloned into the pCAG−3× HA plasmid.

1.3 Cell culture and plasmid transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (HyClone) in a 6−well plate until reaching 80% confluency. Then, 3 μg of pMax (Lonza Group, Switzerland) and eukaryotic expression plasmids were used to transfect the cells with

lipofectamine 3000 (Thermo Fisher Scientific Inc., U. S.) according to the guidelines. Forty-eight hours later, the cells were harvested and subjected to protein extraction. The cell culture medium was concentrated by trichloroacetic acid (TCA) method[8]. Briefly, the cell culture medium was collected and centrifuged at 1 000 *g* for 5 min at 4 ℃. Then, the TCA solution (500 g TCA in 350 mL H_2O) was added to the sample at a volume ratio of 1∶4. After incubation for 10 min on ice, the mixture was centrifuged again at 1.3×10^4 *g* for 5 min. Then, the protein pellet formed after carefully removing the supernatant. Then, 200 μL of cold acetone was added to wash the pellet three times. Finally, the dried pellet could be directly dissolved in the protein loading buffer for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

1.4　Antibody preparation, protein extraction, and Western blotting analysis

The pCMV−pleg1a−C−FLAG plasmid was digested with *Bam*HⅠ and *Xho*Ⅰ. The *pLEG1a*−containing fragment was then purified and ligated to pET−32a to obtain the prokaryotic expression plasmid pET−32a−pleg1a. The subsequent antibody production was accomplished by Hangzhou HuaAn McAb Biotechnology Co. Ltd., which finally provided us with rabbit anti−pLEG1a polyclonal antibody and antiserum (No. 1869).

Whole protein extracts from the tissues or HEK293T cells were prepared using cell lysis buffer for Western and IP (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China) as a protease inhibitor. For tissue protein extraction, 200 μL of lysis buffer was added to 20 mg tissues, which were then homogenized using an automatic homogenizer (Shanghai Jingxin Company, China). After the adherent HEK293T cells were washed with phosphate buffered saline (PBS) twice, 200 μL of cold lysis buffer was added to the 6−well plate with gentle agitation for 5 min before collecting the lysed solution. After centrifugation of the lysates from tissues or cells, the supernatant was evaluated using the bicinchoninic acid protein assay kit (Beyotime Biotechnology, Shanghai, China). Approximately 30 μg of protein extracts was used for Western blotting analysis.

Pig saliva was collected from three pigs and subjected to Western blotting analysis to detect the presence of pLEG1a. Before direct loading for Western blotting analysis, approximately 5 mL of saliva was concentrated using the TCA precipitation protocol. Western blotting analysis was performed using a previously established protocol[1]. The antibodies used were rabbit anti-human LEG1 (antibody No. 897, a gift from Dr. PENG Jinrong), rabbit anti−pLEG1 (No. 1869), anti−FLAG (No. M2, Sigma Aldrich, Germany), and anti−HA (No. HA7, Sigma Aldrich, Germany) at a dilution of 1∶1 000. Ponceau staining (Beyotime Biotechnology, Shanghai, China) was employed to quantify the loaded protein according to the manufacturer's guidelines.

1.5　Glycosylation analysis

The bioinformatics tools NetNGlyc 1.0 and NetOGlyc 4.0[9] were applied for the prediction of potential glycosylated sites in pLEG1 proteins (GenBank accession Nos. XP_003121259.1, XP_020930551.1, XP_020940144.1) with default parameters.

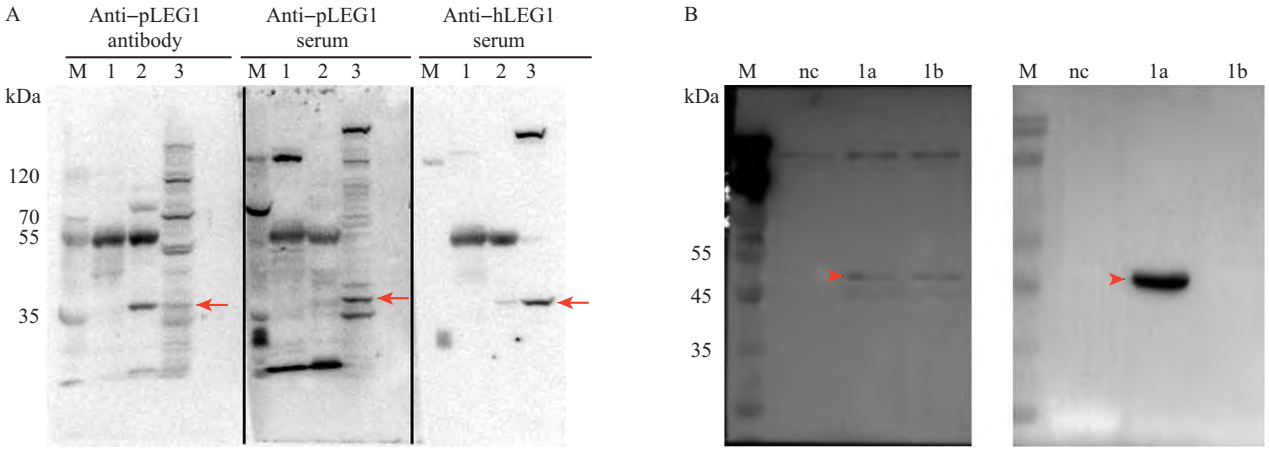
For experimental validation of *N*− or *O*−glycosylation of pLEG1, PNGase F and endo−*α*−*N*−acetylgalactosaminase plus neuraminidase (NEB), which cleave *N*−linked and *O*−linked glycans, respectively, were used for treating concentrated saliva and tissue protein extracts. The treated and untreated samples were analyzed by Western blotting for band shifts.

2　Results

2.1　Establishment of a polyclonal antibody for pLEG1a

In a previous report, we demonstrated that *pLEG1a* was specifically expressed in salivary

glands, while no expression signals were found in any tissues for *pLEG1b* and *pLEG1c* by reverse transcription−polymerase chain reaction (RT−PCR)[4]. However, due to the lack of suitable antibodies recognizing the newly identified protein, the expression of pLEG1a protein has yet to be determined. To solve this problem and characterize pLEG1a, we produced a rabbit anti−pLEG1a polyclonal antibody (No. 1869) based on the pLEG1a sequence. According to the RT−PCR results, we tested the specificity of the antibody using proteins extracted from the heart, salivary glands, and HEK293T cells overexpressing *pLEG1a*. The results of Western blotting analysis showed that the anti−pLEG1a purified antibody and antiserum both readily recognized a band of approximately 38 kDa (corresponding to the molecular weight of pLEG1a) in the proteins from the salivary glands and the cells rather than the heart, in accordance with the result obtained using anti−hLEG1a serum (No. 897) (Fig. 1A). This experiment indicated that the 38 kDa band is pLEG1a because it can be detected using different anti−pLEG1a antibodies. We also noted that the cells transfected with the pCMV−pleg1a−C−FLAG plasmid had a low level of pLEG1a expression, resulting in the nonspecific bands in Fig. 1A. Thus, we reconstructed the expression plasmids using the CAG promoter and 3×FLAG or 3×HA tags in our subsequent experiments. The newly constructed plasmids for *pLEG1a* and *pLEG1b* were used to transfect HEK293T cells. Then, the extracted cells were blotted with anti−pLEG1a (No. 1869) antibody, which could detect both pLEG1a and pLEG1b proteins (Fig. 1B). To confirm the result of the anti−pLEG1a antibody, we employed the anti−FLAG antibody to detect the blot again, in which a protein band with a similar size was detected in the *pLEG1a*−transfected cells (Fig. 1B). This result confirmed that the raised antibody could be used to identify the pLEG1a and pLEG1b proteins.



A. Detection of pLEG1a by anti−pLEG1a (No. 1869) and anti−hLEG1a (No. 897) antibodies and antiserums (M: Marker; 1: Heart; 2: Salivary gland; 3: pCMV−pleg1a−C−FLAG transiently transfected HEK293T cells. Red arrow corresponds to an ≈38 kDa band); B. Detection of pLEG1a and pLEG1b by anti−pLEG1a (No. 1869) antibody [M: Marker; nc: Non-specific control; 1a: pCAG−pleg1a−3×FLAG−transfected cell extract; 1b: pCAG−pleg1b−3×HA−transfected cell extract. Left panel: Extracts from cells transfected with pCAG−pleg1a−3×FLAG and pCAG−pleg1b−3×HA were blotted with anti−pLEG1a (No. 1869) antibody, and the red arrowhead indicates the detected band. Right panel: The same samples were blotted with an anti−FLAG antibody, resulting in only one band detected from the pCAG−pleg1a−3×FLAG cell extract (red arrowhead)].

Fig. 1 Western blotting analysis of the pLEG1 protein

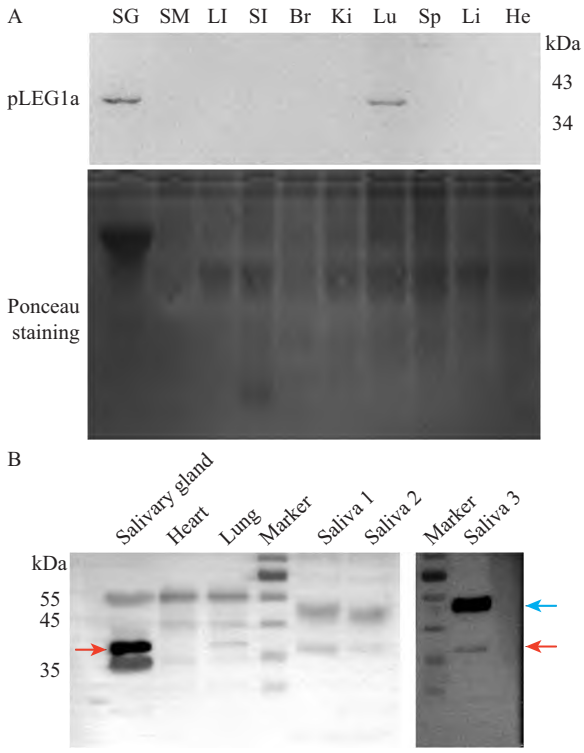
2.2 Expression profile of the pLEG1a protein

To determine the expression profile of pLEG1a, we used anti−pLEG1a to detect various tissue extracts. The results showed that pLEG1a was detectable in the salivary glands (Fig. 2A), consistent with the previous RT−PCR results[4]. Unexpectedly, another bright band

was found in the lung extract, where *pLEG1* genes were not expressed according to RT-PCR. Both bands (≈38 kDa) detected corresponded to the predicted molecular weight of pLEG1a. These results indicated that pLEG1a existed in both the salivary gland and lung but was absent in the heart, liver, spleen, kidney, brain, intestine, and skeletal muscle. As LEG1s from other species are regarded as secretory proteins, we wanted to determine whether pLEG1a is also present in pig saliva. Western blotting was employed using concentrated saliva from three pigs, in which two bands in each saliva sample were detected (Fig. 2B). The lower band displayed a similar size to those identified in the lungs and salivary glands. In contrast, the upper band (between 45 kDa and 55 kDa) with the same size as those in Fig. 1B was distinct from the nonspecific 55 kDa band found in other tissues (Fig. 2B). This result indicated that pLEG1a could be secreted into the saliva in two forms.

2.3　*N*-glycosylated salivary pLEG1a

As two bands could be recovered in the pig saliva, we speculate that the upper band might be due to a glycosylation event, which is a common phenomenon in other species[3,6-7,10-11]. Thus, bioinformatics tools were first utilized to predict the potential glycosylation sites of pLEG1 proteins, and the results showed that pLEG1s exhibit several potential *N*-glycosylation sites and *O*-glycosylation sites. For example, pLEG1a was predicted to show no *O*-glycosylation but contain several potential *N*-glycosylation sites. In contrast, pLEG1b and pLEG1c were both predicted to be *N*- and *O*-glycosylated (Fig. 3A). Subsequently, the proteins from the heart and salivary gland were treated with either PNGase F or endo-α-*N*-acetylgalactosaminidase plus neuraminidase (*O*-glycosidase/neuraminidase). However, no band shift was observed (Fig. 3B), demonstrating that pLEG1a is not glycosylated in tissues. Next, the concentrated saliva was treated with PNGase F or *O*-glycosidase/neuraminidase. The results showed that a band shift occurred following treatment with PNGase F instead of *O*-glycosidase/neuraminidase, which eliminated



A. Detection of pLEG1a in various tissues using a rabbit anti-pLEG1a antibody (No. 1869) (SG: Salivary gland; SM: Skeletal muscle; LI: Large intestine; SI: Small intestine; Br: Brain; Ki: Kidney; Lu: Lung; Sp: Spleen; Li: Liver; He: Heart); B. Detection of pLEG1a in concentrated saliva by Western blotting [Saliva 1, 2, and 3 indicate the saliva collected from three different pigs (Western blotting analysis for saliva 3 was performed in a separate blot). The red arrows show the nonglycosylated pLEG1a, while the blue arrow denotes the glycosylated pLEG1a, which is slightly lower than the nonspecific 55 kDa band in the tissues].

Fig. 2　Expression of the pLEG1a protein

the upper band. Moreover, the shifted band showed a similar molecular weight to those in the salivary gland and saliva (Fig. 3C). Thus, salivary pLEG1a is a *N*-glycosylated protein exhibiting no *O*-glycosylation. In addition, the presence of the 38 kDa band in the saliva suggested that pLEG1a is not completely glycosylated, resulting in a low level of nonglycosylated protein.

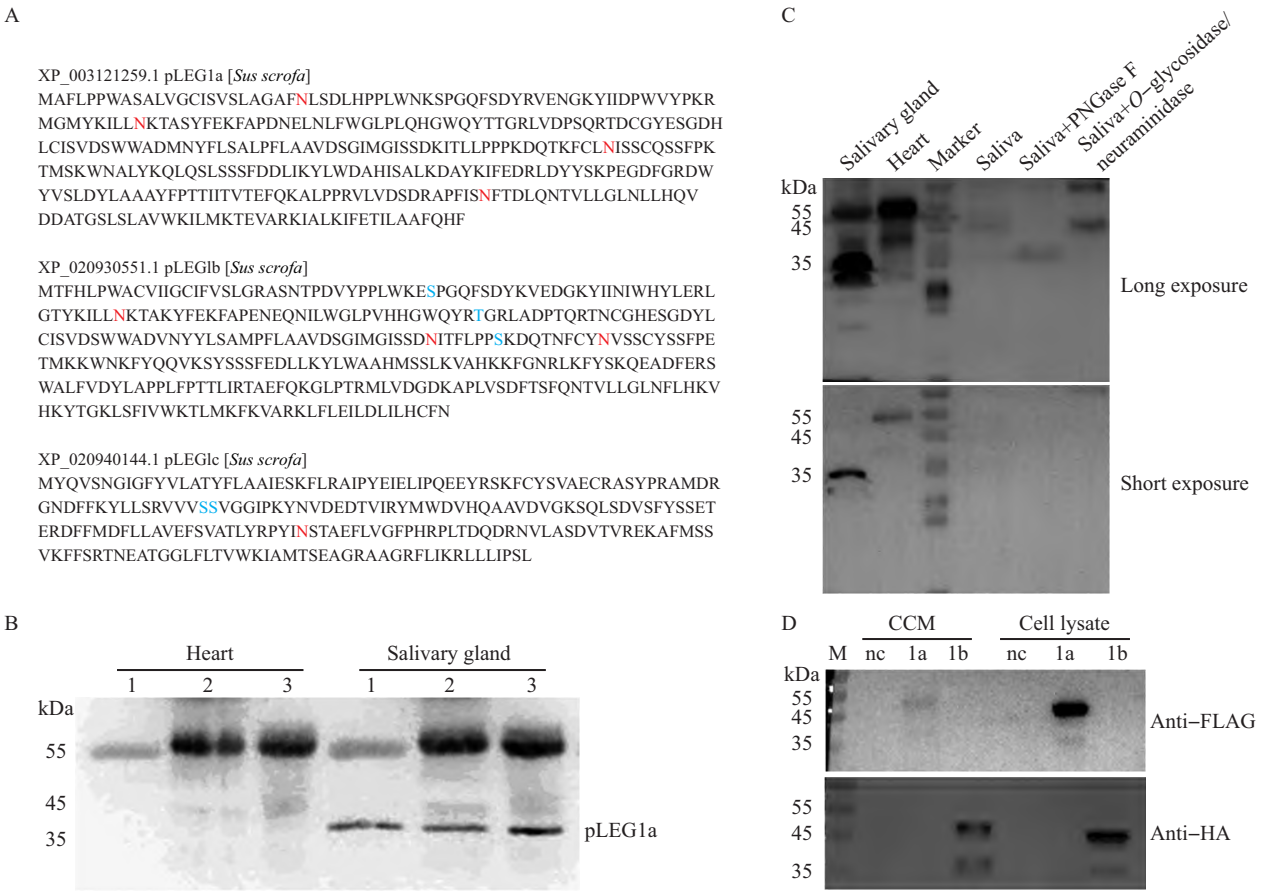
2.4　Secretory protein pLEG1a

To further confirm that pLEG1a is a secretory protein, we used pCAG-pleg1a-3×FLAG and pCAG-pleg1b-3×HA to transiently transfect HEK293T cells. Extended exposure showed that anti-FLAG and anti-HA antibodies also recognized two

bands both in cell lysates and cell culture media, including a lower band with similar size to those detected in the lungs and salivary glands and an upper band corresponding to the size observed in the saliva (Fig. 3D). These results suggested that pLEG1a is a secretory protein.

3 Discussion

In our previous study, *pLEG1a* was proven to be similar to human and mouse homologs evolutionarily and molecularly[4]. Additionally, at the RNA level, *hLEG1a*, *mLeg1a*, and *pLEG1a* all exhibit salivary gland-specific expression patterns in contrast to those in zebrafish and platypus[1,3−4,12]. These results indicate that human, mouse, and pig orthologs might preserve similar functions due to subfunctionalization, and *pLEG1a* might be a good model to study eutherian *LEG1a* functions. In the current work, we aimed to characterize pLEG1a proteins and determine whether pLEG1a is similar to other LEG1s at the protein level. First, to determine the expression profile of the pLEG1a protein, we prepared an anti−pLEG1a (No. 1869) antibody based on the pLEG1a sequence. Then, we identified the antibody validity by detecting pLEG1a in tissues and cells. Using the antibody, we



A. Glycosylation prediction of pLEG1a, pLEG1b, and pLEG1c (*N*−glycosylation sites are highlighted in red, while *O*−glycosylation sites are highlighted in blue); B. Deglycosylation test of pLEG1a in the heart and salivary gland extracts (1: No treatment; 2: PNGase F treatment; 3: *O*−glycosidase/neuraminidase treatment); C. Deglycosylation test of saliva pLEG1a using PNGase F or *O*−glycosidase/neuraminidase [The same polyvinylidene difluoride membrane was exposed for a short time (lower panel) and a long time (upper panel)]; D. Western blotting analysis of transiently transfected HEK293T cells expressing either pCAG−pleg1a−3×FLAG or pCAG−pleg1b−3×HA using anti-epitope antibodies (CCM: Cell culture medium; M: Marker; nc: Non-specific control; 1a: pCAG−pleg1a−3×FLAG−transfected cell extract; 1b: pCAG−pleg1b−3×HA−transfected cell extract).

Fig. 3 Glycosylation test of pLEG1a

found that pLEG1a was observed in both the salivary gland and lung tissues. However, there was no *pLEG1* transcription in the lung tissues according to our previous work[4]. As several proteomic studies have shown that LEG1 proteins are secretory proteins that are detected in milk, saliva, and seminal plasma[3,6,10−11,13], we assumed that pLEG1a might be enriched in the respiratory mucus and show a low level of expression in the mucosal epithelial cells. Therefore, future studies using more tissues, such as milk, mucus, and seminal plasma, will be needed.

Glycosylation is an important biological process that plays a critical role in protein stability, trafficking, cell growth, and host−pathogen interactions[14]. It has been reported that glycosylation of zLEG1 is critical in the liver development of zebrafish and that glycosylated MLP can confer antibacterial activity to pups[2−3]. In addition, *N*−glycosylated hLEG1a was detected in human saliva[7,11]. Thus, glycosylation might be another hallmark of LEG1 proteins. In our experiment, *N*−glycosylated pLEG1a was observed in concentrated saliva but not in tissue-derived proteins. Furthermore, both *N*−glycosylated and nonglycosylated bands could be detected in the saliva of pigs, with glycosylated pLEG1 as the predominant form. *N*− and *O*−glycosylated sites were bioinformatically predicted in pLEG1 proteins; however, only *N*−glycosylation events could be experimentally validated, in accordance with previous experiments conducted on human, mouse, zebrafish, and platypus LEG1s[2−3,7,11−12]. Therefore, the glycosylation pattern might indicate important biological functions of the pLEG1a protein.

Ectopic overexpression of the *pLEG1a* and *pLEG1b* genes in HEK293T cells also confirmed that pLEG1a and pLEG1b are both secretory proteins. Interestingly, two bands with molecular weights corresponding to the glycosylated and nonglycosylated pLEG1s were detected in the experiment, although the possible nonglycosylated bands had lower intensities, consistent with the results from saliva. Nonglycosylated secretory proteins, such as transthyretin and growth hormone[15−18], have been reported before, indicating an alternative protein secretion pathway. Additionally, a study that mutated all potential *N*−glycosylated sites of carnosinase suggested that a trace amount of the nonglycosylated protein was still secreted. A previous report on zLEG1 protein also observed that when mutating N70A in zLEG1, the nonglycosylated protein could be secreted. These results indicated that either an alternative secretory pathway exists or that these proteins have altered enzymatic activity[19]. Based on these studies, the pLEG1a protein is similar at the protein level to other LEG1 proteins, especially eutherian LEG1s, in terms of the expression pattern, glycosylation, and secretion ability. Therefore, pLEG1a might be a good model to study the function of the newly identified gene.

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