

ORIGINAL ARTICLE

## Molecular identification, characterization, and structure analysis of house musk shrew (*Suncus murinus*) leptin

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### ABSTRACT

**Objective:** House musk shrew (*Suncus murinus*), a small experimental animal with low body fat, may be a possible model for human lipodystrophy. Leptin is an adipocyte-derived hormone thought to have an important role in the pathophysiology of lipodystrophy. The objectives of this study were to clarify the structure and distribution of suncus leptin.

**Materials and methods:** To determine the primary structure of suncus leptin, we cloned the suncus *Lep* cDNA using the rapid amplification of cDNA ends method. The obtained amino acid (aa) sequence was compared with other mammals and the protein structure prediction was performed.

**Results:** The suncus *Lep* cDNA encodes 170 aa. The putative suncus leptin precursor has a predicted signal peptide of 21 aa, and the mature leptin comprises 149 aa. The mature leptin is 75%–82% homologous to that of other species. Insertion of the three aa, VPQ, not seen in other mammals was found. This VPQ insertion is thought to be due to a nucleotide insertion of nine bases by slippage-like microindels. The predicted 3D structure of suncus leptin exhibited a typical four  $\alpha$ -helix structure, however, the VPQ region protruded compared with human leptin. *Lep* mRNA expression was observed only in white and brown adipose tissues.

**Conclusion:** This study revealed the structure and distribution of suncus leptin. Because the addition of VPQ, which is not found in other mammals, was observed, suncus leptin attracts attention to its physiological action, and to the possibility of being a model of human lipodystrophy.

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### Introduction

Lipodystrophy syndromes in humans are genetic or acquired disorders in which full or partial deficiency of adipose tissues is seen. In many clinical cases, the adipose tissue deficiency in lipodystrophy is associated with diabetes, insulin resistance, and lipid metabolism disorders including ectopic fat deposition and non-alcoholic fatty liver disease [1]. Adipose tissue is well understood to be an important organ of energy metabolism and hormonal regulation, and leptin, the most important adipokine secreted from adipocytes, is related to the regulation of food intake and energy consumption in the whole body. In other words, adipose tissue deficiency causes leptin depletion in the

circulation, followed by the accompanying symptoms of leptin deficiency.

Shimomura et al. [2] have proved that the leptin deficiency caused insulin resistance in transgenic lipodystrophic mice and that leptin replacement remarkably improved insulin sensitivity. Metreleptin, a human recombinant methionyl leptin, has been reported to be effective for leptin replacement therapy for the complications associated with lipodystrophy, and is the only drug approved in the United States and Japan [1]. Metreleptin markedly improved fasting glucose and HbA1c, and decreased triglycerides and low-density lipoprotein cholesterol, but did not change high-density lipoprotein cholesterol [3,4,5,6,7]. Therefore, leptin replacement remarkably improved the accompanying symptoms seen

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in lipodystrophy, but details on how leptin deficiency affects the metabolic state of the whole body have not been elucidated.

House musk shrew (*Suncus murinus*), also called suncus, is an insectivore of the Soricidae family, which in the phylogenetic tree is closer to primates than to rodents. Suncus was established from wild animals as a laboratory animal in Japan. Because it has different characteristics from mice and rats, it has been widely used for physiological and morphological experiments. One of its features is the remarkable deficiency in whole body fat tissue, especially visceral fat [8]. Also, it has been reported that suncus easily develop fatty liver by fasting [9].

Despite the body fat deficiency, normal suncus have blood glucose levels (<7.0 mmol/l) comparable to humans and rodents, and normal glucose metabolism [10]. The question arises as to why normal suncus do not show insulin resistance and other symptoms seen in human lipodystrophy. Therefore, we explored the role of leptin, which is decreased in human lipodystrophy, and attempted to clarify leptin's function in suncus.

As the amount of body fat is scarce in suncus, the amount of circulating leptin and the function of leptin itself may be different from that of leptin in humans and other animals. Because there have been no reports on suncus leptin so far, it is difficult to estimate its function and to measure the blood leptin concentration in suncus. The objective of this study was to understand the structure of suncus leptin and its physiological role, especially its contribution to insulin sensitivity and glucose metabolism. To this end, we sequenced suncus *Lep* cDNA to clarify its protein structure and investigated the *Lep* mRNA expression in suncus tissues.

## Materials and Methods

### Animals

The BK suncus strain, a hybrid between the BAN strain, established from wild populations in Bangladesh [11], and the KAT strain, founded from wild populations in Kathmandu, Nepal [12], was used in this study. The animals were kept individually in aluminum cages provided with an empty beverage can for a retreat. Animals were bred and maintained in standard laboratory conditions: room temperature of 23°C–26°C, 12-h light/12-h dark cycle, and *ad libitum* access to water and commercial pelleted food for aquaculture rainbow trout (FEED ONE, Yokohama, Kanagawa, Japan).

The animal experiments in this study were approved by the Institutional Animal Care and Use Committee (approval number: 30S-15) and carried out according

to Nippon Veterinary and Life Science University Animal Experimentation Regulation.

### RNA extraction

Adult (>1 year) male suncus were euthanized with an intraperitoneal injection of 300 mg/kg pentobarbital sodium (Somnopentyl®; Kyoritsu Seiyaku, Tokyo). Then, tissues were collected within 30 min, frozen in liquid N<sub>2</sub> and stored at –80°C until RNA extraction. Total RNAs were extracted from various tissues including brain, liver, quadriceps muscles, esophagus, stomach, small intestine, large intestine, pancreas, gallbladder, heart, kidney, lung, spleen, bladder, testis, inguinal-subcutaneous white adipose tissue (WAT), epididymal WAT, and interscapular brown adipose tissue (BAT), using NucleoSpin® RNA (Takara, Otsu, Shiga, Japan). The total RNA was cleaned by on-column DNase I digestion. The amounts of RNA were measured using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Tokyo, Japan).

### cDNA cloning of suncus *Lep* mRNA

The cDNA cloning for suncus *Lep* gene was performed by the conventional Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods. One pair of PCR primers for the *Lep* gene (SunLepF, 5'-gaa gcc caa ccc agg aag aa-3', and SunLepR, 5'-gct gct tct cct gga gac tc-3') was designed based on an unpublished suncus genome resource derived from the KAT strain (courtesy of the Suncus Genome Project). Partial cDNA fragments encoding the *Lep* gene were amplified using subcutaneous adipose tissue cDNA. The PCR products were ligated into pMD20 T vector (Takara) and sequenced using BigDye Terminator v3.1 and Applied Biosystems 3130xl/3730xl DNA Analyzers (Applied Biosystems, Foster City, CA). After identifying the sequence encoding the partial *Lep* cDNA of suncus, 3'-RACE was performed using the SMARTer 3' Kit (Takara) to obtain 3'-terminal cDNA sequences. The 3'-RACE-PCR was performed with a universal primer mix provided by the manufacturer and a gene-specific SunLepF primer (5'-gat tac gcc aag ctt aag cct cac tct tct cca cag agg t-3') in accordance with the manufacturer's instructions. The obtained PCR fragments were cloned and sequenced by the walking-primer method. A partial 5'-untranslated region (UTR) was cloned by conventional RT-PCR with the SunLepR1 primer (5'-atg tct gca gtt cct gag cc-3') and the SunLep5UTR primer (5'-ccc cgg agg act tca gca gc-3'), which was designed based on the suncus genome resource. For cDNA cloning, PCR was performed using a high-fidelity enzyme, PrimeSTAR® *HS DNA Polymerase* (Takara). Obtained PCR fragments were cloned and sequenced as depicted above.

### Sequence analyses and phylogeny

A search for a signal peptide sequence was carried out using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). The theoretical isoelectric point (pI) and the molecular weight (MW) of the polypeptide were calculated using the compute pI/MW tool on the ExPASy Server ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)) [13]. Alignment of multiple sequences was performed using the ClustalW program, and the phylogenetic tree was created by the Neighbor-Joining (NJ) method in MEGA X [14]. The protein sequence reference numbers used in the alignment analysis of leptin protein are as follows: horse (NP\_001157452; 167 aa), pig (NP\_999005; 167 aa), cow (NP\_776353; 167 aa), human (NP\_000221; 167 aa), mouse (NP\_032519; 167 aa), rat (NP\_037208; 167 aa), common shrew *Sorex araneus* (XP\_004608342; 165 aa), chimpanzee (NP\_001180601; 167 aa), Rhesus monkey (NP\_001036220; 167 aa), dog (AB020986; 167 aa ; [15]), cat (AB041360; 167 aa; [16]), and gray short-tailed opossum (XP\_001366398; 167 aa).

### 3D structure analysis

The protein tertiary structure prediction was subsequently conducted by homology modeling using the SWISS-MODEL server (<http://swissmodel.expasy.org/>) [17]. Templates were searched with BLAST against the SWISS-MODEL template library (SMTL, last update: 2018-11-14, last included PDB release: 2018-11-09). The quality of the candidate template was predicted from the target-template alignment, and the highest quality template was selected for model building. Models were constructed based on the target-template alignment using ProMod3. Coordinates that were preserved between the target and the template were copied from the template to the model. Insertion was remodeled using a fragment library. Based on the human obesity protein PDB data (1AX8) and predicted suncus leptin PDB data, the alignment of human and suncus leptin 3D structures was drawn using PyMOL (<http://www.pymol.org>).

### Tissue distribution determination by RT-PCR

Determination of the distribution and the expression of *Lep* mRNA in suncus tissues was performed by RT-PCR using DNase I-digested total RNA from the various tissues described above. First strand cDNAs were synthesized using the PrimeScript™ RT reagent Kit (Takara) according to the manufacturer's protocol. The *Lep* gene was amplified by conventional RT-PCR using the *Lep*-specific primer pair, SunLepF and SunLepR same as described in the cloning section, which provided a 607 bp fragment. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal standard, and a 452 bp

fragment was obtained using the primer pair, *GapdhF* (5'-acc aca gtc cat gcc atc ac-3') and *GapdhR* (5'-tcc acc acc ctg tgg ctg ta-3'). PCR was carried out using KAPA2G Fast HS ReadyMix with dye (Kapa Biosystems, Woburn, MA) according to the manufacturer's protocol. The PCR products were analyzed by electrophoresis on 2.0% (w/v) agarose gels visualized with Midori Green Advance (Nippon Genetics, Tokyo, Japan).

### Results and Discussion

Lipodystrophy is a disorder that causes metabolic disorders including insulin resistance and hepatic steatosis by decreasing circulating leptin due to loss of body fat. Even though the visceral fat is remarkably scarce in suncus, this metabolic abnormality has not been identified in normal suncus. Because leptin replacement therapy ameliorates insulin resistance in lipodystrophy [1], the pathophysiological role of leptin associated with insulin resistance development is very interesting. Therefore, in this study, to elucidate why suncus do not show insulin resistance, we cloned the suncus *Lep* gene, analyzed the structure of its protein product, leptin, and confirmed the tissue distribution of *Lep* mRNA expression.

### cDNA cloning of suncus *Lep* cDNA

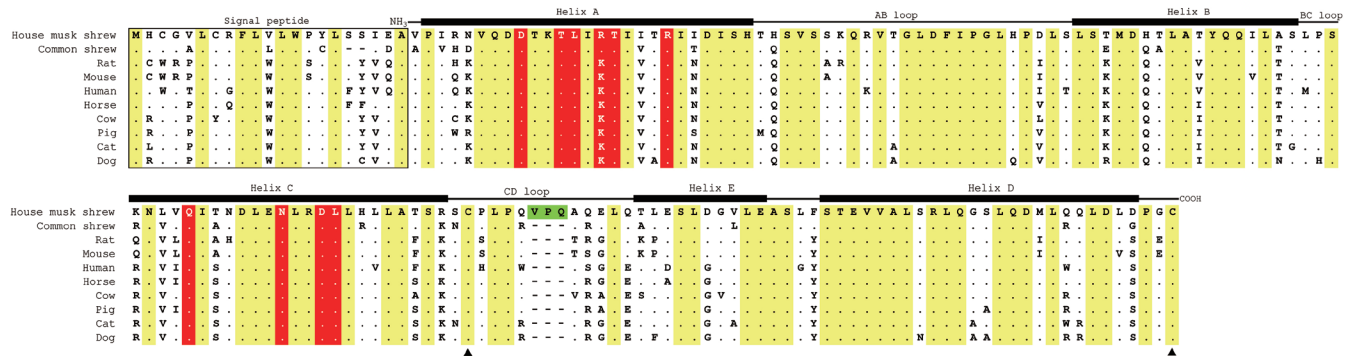
Cloning of the suncus *Lep* cDNA was carried out by combining the 3' RACE method with RT-PCR of partial 5'-UTR. By assembling the obtained sequences and determining the consensus sequence, the nucleotide sequence of suncus *Lep* cDNA containing the complete coding region was determined.

The cloned suncus *Lep* cDNA was 3026 bp, including a 142 bp partial 5'-UTR and a 2371 bp 3'-UTR. A putative polyadenylation signal (aataaa) existed 15–20 bp upstream from the poly (A) tail. The 513 bp putative open reading frame encoded a 170 aa polypeptide. The calculated MW of the leptin precursor is 18.9 kDa, and the theoretical pI is 5.17. The putative suncus leptin precursor possesses a predicted signal peptide of 21 hydrophobic residues at its amino-terminal, suggesting that the mature leptin comprises 149 aa with a calculated MW of 16.4 kDa and a theoretical pI of 4.98. The suncus *Lep* cDNA sequence was deposited in the DNA Data Bank of Japan (DDBJ) under the accession number LC432494.

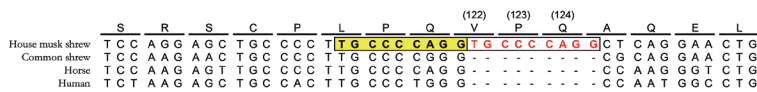
### Sequence analyses

Subsequently, we compared the peptide sequence of the suncus leptin precursor with its counterparts in several representative mammalian species, including human, mouse, rat, horse, cow, pig, cat, and dog. The leptin sequence of the common shrew (*Sorex araneus*), also known as a Eurasian

**A**



**B**



**Figure 1.** Nucleotide and amino acid multiple sequence alignment of suncus leptin with other representative domestic mammals. (A) Protein sequence alignment was performed using ClustalW. Identical residues across all sequences are represented by dots (.) and absent amino acids are indicated by dashes (-). The predicted signal peptide is boxed. Conserved residues are indicated by yellow shading. The amino acid residues important for binding to the leptin receptor are indicated by red shading. The two conserved cysteine residues are indicated by arrowheads. The three amino acid residues, VPQ, seen only in suncus are indicated by green shading. The secondary structure of the human mature leptin is indicated above the sequence alignment. (B) Partial nucleotide sequences of leptin were aligned. Nine nucleotide sequences conserved in most aligned animals are indicated by yellow shading. The same nine nucleotides appearing immediately after are surrounded by a red box.

shrew, which belongs to the same insectivore group (family *Soricidae*) as the suncus (house musk shrew), was also used for alignment comparison.

As shown in Fig. 1A, the results of protein sequence alignment indicated that the leptin precursor is highly conserved within these species and that suncus leptin is highly homologous with rat (77%), mouse (77%), human (75%), horse (82%), cow (80%), pig (80%), cat (78%), dog (76%), and common suncus (*Sorex*) (81%). These results suggest that the suncus leptin is also highly conserved among other mammals. Interestingly, the highest homology against the suncus leptin precursor was observed in horse and not in humans, rodents or even the common shrew.

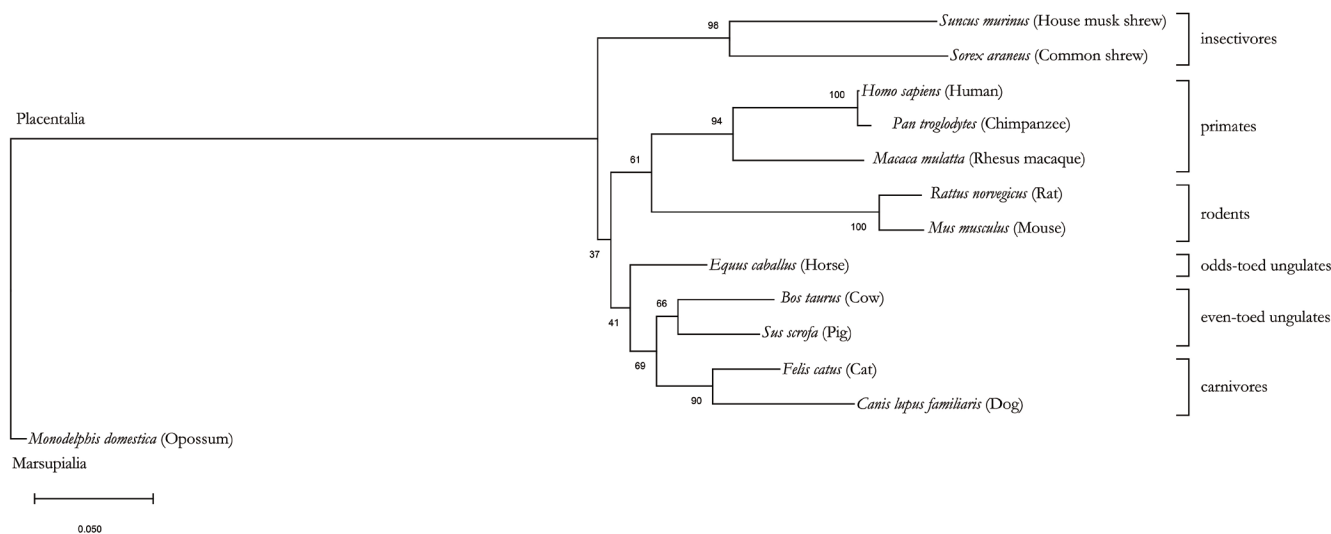
The sequence alignment revealed an insertion of 3 aa in suncus leptin, which was not observed in the other examined mammalian proteins. This insertion sequence can also be considered as PQV or PQA. Thus, we compared the nucleotide sequence alignment to confirm which insertion sequence is more reasonable.

For this alignment, we used part of the nucleotide sequence of the common shrew (*Sorex*), human and equine *Lep* gene (Fig. 2B). Taylor et al. [18] have reported that the majority of insertions (52%) seen in the human,

mouse and rat genomes were found to have a sequence identical to the inserted sequence, directly adjacent to the insertion or deletion event. An indel is an insertion or deletion of a nucleotide sequence in the genomic DNA [19], and a microindel is defined as indels producing a net change of 1–50 nucleotides [20]. In contrast to microindels occurring in non-coding regions, microindels within protein-coding regions can be serious and sometimes lethal. The inserted nucleotide sequence was compared with the immediately adjacent 5' and 3' sequences. In our study, by examining the flanking sequences including the insertion site, an identical sequence was found 5' upstream.

Focusing on the suncus nucleotide sequence, we found that an upstream region with the same nine nucleotides was present, adjacent to the nucleotide sequence region corresponding to the inserted amino acids. Sometimes indels are recognized as slippage, if the adjacent sequences are identical to the inserted sequence [18].

As shown in Fig. 1B, the nine nucleotide sequence, TGCCCCAGC, from the second nucleotide (T) of the L119 codon to the first nucleotide (G) of the V122 codon appears again immediately after.



**Figure 2.** Phylogenetic tree based on the Neighbor-Joining (NJ) method was constructed based on deduced amino acid sequences of leptin using MEGA X. The evolutionary history of leptin was inferred using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved 13 amino acid sequences. The leptin sequence of the gray short-tailed opossum (*Monodelphis domestica*) was used as the outgroup. Evolutionary analysis was conducted with MEGA X [14].

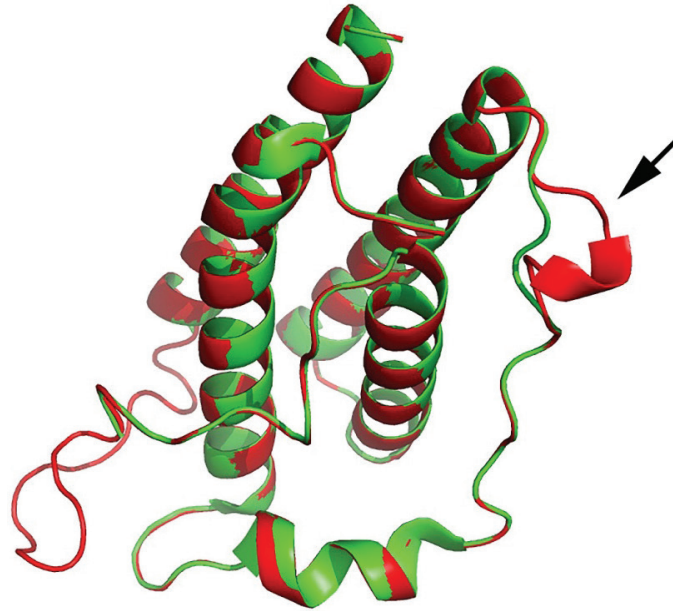
In the coding region, a frameshift mutation is generated downstream of the insertion site unless the indel length is a multiple of three. Due to the nature of the genetic triplet code, however, microindels with multiples of three nucleotides are unlikely to be deleterious. Because the insert seen in the suncus *Lep* gene is 9 bp, it resulted in the insertion of 3 aa without causing a frameshift mutation. Also, it did not cause a nonsense mutation, as the insertion sequence did not produce a stop codon. In the case of insertion of multiples of three, even if a frameshift occurs locally by the insertion, because the reading frame returns to the original position after the insertion fragment, it does not affect the downstream amino acids. The original nucleotide sequence is thought to be TTG CCC CAG GCT, encoding L119-P120-Q121-A122, but as nine nucleotides (TGCCCCAGC) were inserted at the second position of the Ala codon, a partial frame shift occurred and the reading frame changed to TTG CCC CAG G [TG CCC CAG G]CT, resulting in L119-P120-Q121-[V122-P123-Q124]-A125.

### Phylogenetic analysis

*Suncus* belonging to the Insectivore order are phylogenetically very different from rats and rabbits and are believed to have conserved features with the common ancestor of primates and rodents [21].

A phylogenetic tree from the mammalian leptin aa sequence was created and compared among selected mammalian species, especially Placentalia. The reference leptin sequence consists of human, chimpanzee, and macaque monkey as primates, rat and mouse as rodents, horse as odd-toed ungulates, cow and pig as even-toed ungulates, cat and dog as carnivores, house musk shrew (*S. murinus*) and common shrew (*Sorex arenius*) as insectivores. As an outgroup, the leptin sequence of opossum, which is a marsupial, was used. The result of the phylogenetic tree analysis was almost the same as the previous report [22] (Fig. 2).

Primates and rodents are thought to have evolved from the common ancestor, insectivores. Hence, the Soricidae family of the insectivores is thought to preserve characteristics of common ancestry of primates and rodents. Searching for the predicted *Lep* gene registered in the unpublished suncus genomic resource confirmed that the KAT strain also had a VPQ insertion. Therefore, we revealed that this microindel exists not only in the BK strain but also in the KAT strain. However, we were unable to examine the *Lep* gene of the BAN strain, so it remains unclear whether the BAN strain has this microindel. In other words, it is unknown whether the microindel in the BK strain is derived from the KAT or the BAN strain, or both. Considering the phylogenetic tree analysis and the VPQ



**Figure 3.** Predicted tertiary structure of suncus leptin. The alignment of human and suncus leptin 3D structures was done using PyMOL. Prediction of the 3D structure of suncus leptin was carried out using a SWISS-Model based on the human leptin structure (PDB ID: 1AX8). Ribbon models based on human (green) and suncus (red) leptin were overlaid. The arrow indicates the additional amino acid residues, VPQ, seen in suncus leptin.

insertion, we think that microindels occurred in the house musk shrew group, KAT strain and/or BAN strain, after a divergence in the Soricidae family. This will be clarified by analyzing the BAN strain in the future. Furthermore, it is zoologically interesting to consider the evolution and distribution of suncus.

### 3D structure analysis

A 3D model of the suncus leptin structure was created based on a known structure of the human obesity protein (leptin; PDB code: 1AX8), displaying a typical four  $\alpha$ -helix structure (Fig. 3). From these results, we confirmed that our cloned *Lep* cDNA encodes a suncus leptin precursor protein with a structural resemblance to leptin proteins from other mammals.

Two characteristic cysteine residues of suncus leptin predict the formation of a disulfide bond in the leptin molecule, which is essential for the 3D configuration and biological activity of human leptin [23].

As shown in Fig. 1A, the predicted secondary structure of leptin has four  $\alpha$ -helix structures, and the two cysteine residues necessary for maintaining the 3D structure are conserved. As in previous reports [23,24], important sites for binding to the receptor are also preserved.

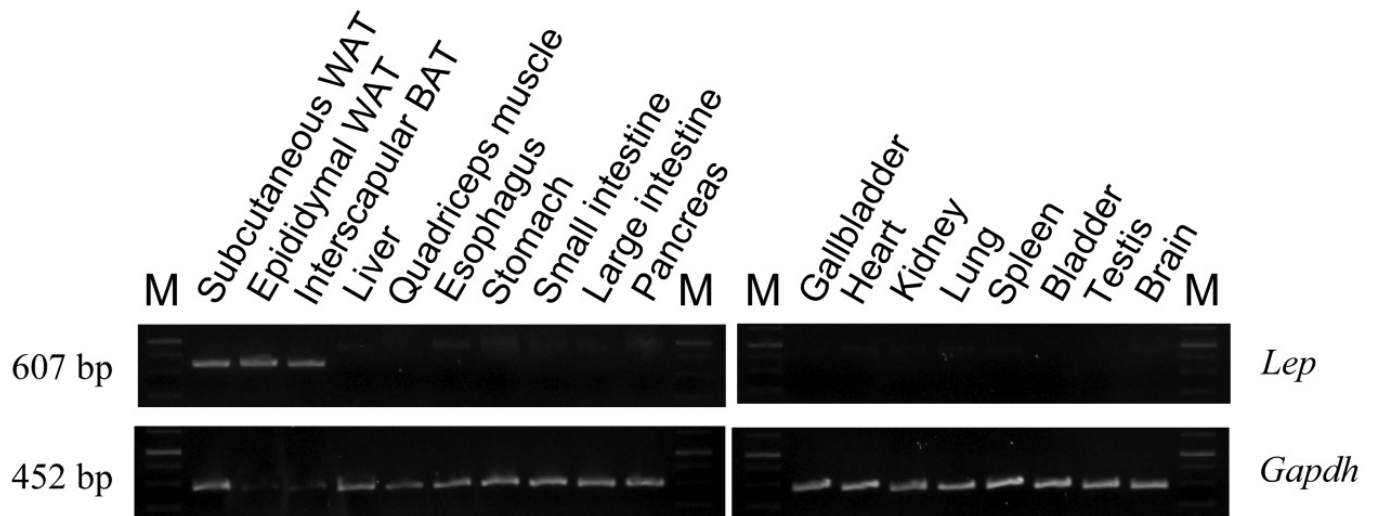
One template was found when the 3D model of suncus leptin was drawn using the human obesity protein, leptin

(1AX8), as a model, which confirmed that suncus leptin has a tertiary structure similar to that of human leptin. Next, the 3D structures of human leptin and suncus leptin were merged and visualized, and a particularly different structure was searched between them (Fig. 3).

As the VPQ sequence is inserted into the CD loop, the structure is protruding (indicated by the arrow in the figure), however, this part is not predicted to affect the binding to the leptin receptor. Therefore, we speculated that the suncus leptin has the same physiological activity as leptin of other mammals, but for detailed analysis, binding experiments of a recombinant protein with the leptin receptor are necessary. We plan to produce a recombinant suncus leptin protein to conduct a more detailed analysis.

### Determination of tissue distribution

In mammals, leptin is a protein that is mainly produced and secreted by adipose tissue. Leptin regulates the energy state of the whole body and adjusts the food intake. In suncus, subcutaneous WAT is a major fat site, and epididymal WAT is in the peritoneal cavity. In mice and rats, fat accumulates with age in adipose tissues such as omental, mesenteric, retroperitoneal, perirenal, and pericardial WAT, but fat accumulation in these areas has not been seen in suncus.



**Figure 4.** Tissue distribution of *Lep* mRNA in suncus. RT-PCR analysis was performed using total RNA from inguinal-subcutaneous WAT, epididymal WAT, interscapular BAT, liver, quadriceps muscle, esophagus, stomach, small intestine, large intestine, pancreas, gallbladder, heart, kidney, lung, spleen, bladder, testis, and brain. Amplification products were analyzed on a 2% agarose gel and stained with Midori Green Advance. *Gapdh* (452 bp) was used as an internal control, and amplified products were observed in all tissues examined. The sizes of the PCR products are indicated on the left. M: 100 bp DNA ladder marker.

Because suncus have less body fat than other mammals, the tissue distribution of *Lep* gene expression was investigated to confirm whether leptin is expressed in tissues other than adipose tissue. *Lep* gene expression was observed only in WAT (subcutaneous and epididymal) and BAT, similar to the distribution seen in other mammals (Fig. 4).

Suncus is not a hibernating animal, but like rodents, it has interscapular BAT. It has been reported that BAT in suncus expresses uncoupling protein 1 (UCP-1) and dissipates excess energy as heat (non-shivering thermogenesis) [25]. Expression of the *Lep* gene was confirmed in BAT as well as other adipose tissues (Fig. 4), however, it was not observed in non-adipose tissues.

## Conclusion

In this study, we explored the possibility of suncus as a model animal of human lipodystrophy. To elucidate why suncus, which has less body fat than other mammals, does not show insulin resistance under normal conditions, we identified and analyzed the structure of suncus leptin, and identified the difference compared with human leptin. cDNA cloning confirmed that suncus has leptin protein that is highly homologous to other mammals. We speculated that the insertion of the three amino acids, VPQ, which is not found in other mammalian leptin, is a result of slippage-like microindels. The predicted 3D structure was similar to that of human

leptin, but the VPQ region slightly protruded outward. The expression of the *Lep* gene was restricted to WAT and BAT, similarly to other mammals. Detailed analysis of the function of leptin by experiments using recombinant proteins will reveal the physiological role of suncus leptin and the reasons why normal suncus does not show insulin resistance.

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## Conflict of Interest

The authors have no conflicts of interest to declare.

## Author Contribution

SS led the experiments, and wrote, reviewed, and edited the manuscript. NS contributed to the main design of this study, supported the experiments, analyzed the data, and reviewed and edited the manuscript. TA contributed to the design of this study, and reviewed and edited the manuscript. All authors approved the final version of the manuscript and agreed to publish it.

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