Biochemistry

*Full paper*

**Dog Steroidogenic Factor-1: Molecular cloning and analysis of epigenetic regulation**

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Running head: MOLECULAR CLONING OF DOG SF-1
ABSTRACT
Steroidogenic factor 1 (SF-1) is a nuclear receptor that is important in steroid hormone production, and adrenal and gonad development. The SF-1 gene is highly conserved among most vertebrates. However, dog SF-1 registered in public databases, such as CanFam3.1, lacks the 5′ end compared to other mammals including mouse, human, bovine, and cat. Whether this defect is due to species differences or database error is unclear. Here, we determined the full-length dog SF-1 cDNA sequence and identified the missing 5′ end sequence in the databases. The coding region of the dog SF-1 gene has 1,386 base pairs, and the protein has 461 amino acid residues. Sequence alignment analysis among vertebrates revealed that the 5′ end sequence of dog SF-1 cDNA is highly conserved compared to other vertebrates. The genomic position of the first exon was determined, and its promoter region sequence was analyzed. The DNA methylation state at the basal promoter and the expression of dog SF-1 in steroidogenic tissues and non-steroidogenic cells were examined. CpG sites at the basal promoter displayed methylation kinetics inversely correlated with gene expression. The promoter was hypomethylated and hypermethylated in SF-1 expressing and non-SF-1 expressing tissues, respectively. In conclusion, we identified the true full sequence of dog SF-1 cDNA and determined the genome sequence around the first exon. The gene is under the control of epigenetic regulation, such as DNA methylation, at the promoter.

KEY WORD: DNA methylation, dog, molecular cloning, promoter, Steroidogenic factor 1
INTRODUCTION

Steroid hormones are bioactive substances synthesized in the adrenal cortex and gonads of vertebrates. These hormones are indispensable for homeostasis, metabolism, and sexual differentiation. Representative steroid hormones include glucocorticoid and mineralocorticoid produced in the adrenal cortex, testosterone produced in the testis, and estradiol and progesterone produced in the ovary. The orphan nuclear receptor steroidogenic factor 1 (SF-1, also called Ad4BP or NR5A1) is expressed mainly in steroidogenic tissues [24] and regulates transcription of genes associated with steroidogenesis by binding to DNA sequences commonly preserved in the promoter regions [3-5, 10, 16, 18, 28-30, 34]. The observations that SF-1 knockout mice lack adrenal glands and gonads and die soon after birth indicate that SF-1 plays an important role in fetal development and sex differentiation [15].

SF-1 contains several domains. An N-terminal DNA binding domain (DBD) includes two Cys2-Cys2 zinc fingers (ZF-I and ZF-II) and Fushi-tarazu factor 1 (FTZ-F1); a flexible hinge region involved in protein structural variability; a C-terminal ligand binding domain (LBD); and two activation function domains (AF-1 and AF-2) [9]. The amino acid sequence of SF-1 is highly conserved among most vertebrates including human, mouse, rat, bovine, pig, and cat. However, the dog SF-1 sequence recorded in CanFam3.1 (ENSCAFT0000032206) lacks the sequence equivalents of exon 1 and 2 of humans and mice (Fig. 1). Compared with the genomic sequence at the basal promoter regions, several transcription factor binding sites are conserved widely in mammals, but not in dog (based on ENSCAFT00000032206) [6, 20, 27, 31]. Whether the deficient structure of SF-1 specific to dog is species difference or database defect is unknown. However, considering the marked similarity of SF-1 among animals, except for dogs, and the fact that ENSCAFT00000032206 is computationally predicted, it is possible that the true exon1 and 2 of dog SF-1 gene are veiled.
In humans and mice, the basal promoter of SF-1 has been reported to contain a gene regulatory element with lower CpG density that exhibits dynamic DNA methylation patterns in cells or tissues [12]. DNA methylation generally occurs at the cytosine of CpG dinucleotides in higher vertebrates and is involved in tissue-specific and developmentally-regulated gene expression [25]. Expression of tissue-specific genes, such as Oct-4 [11] and Sry [19], are induced by transient demethylation during development. The human and mouse SF-1 gene is also considered to be under the control of DNA methylation. However, the regulation mechanism of dog SF-1 gene is still unknown. Identification of the true promoter region of the dog SF-1 gene will enable the analysis of the epigenetic regulation of the dog SF-1 gene. In this study, we identified the full-length dog SF-1 cDNA and promoter sequence. We also examined the relationship between the expression levels of dog SF-1 and the DNA methylation status in the basal promoter.

MATERIALS AND METHODS

Animals and sample collection
An adrenal gland from a male mixed breed dog, an ovary from a female beagle dog, a testis from a male beagle dog, and adipose tissues from a male chihuahua dog were collected during surgery at the University of Miyazaki Veterinary Teaching Hospital, Miyazaki, Japan, with the signed informed consent from dog owners and the ethical approval of the animal ethics committee of Faculty of Agriculture, University of Miyazaki, and the university’s research committee. All samples were grossly normal. The tissues used in this study were as follows; a part of the adrenal gland including capsule, cortex, and medulla; a part of the ovary including germinal epithelium, tunica albuginea, cortex, and medulla; and a part of the testis including tunica albuginea,
vascular layer, parenchyma, and interstitium. The adrenal gland, ovary, and testis were immediately frozen in liquid nitrogen and then stored at −80 °C until use.

Isolation and culture of dog adipose tissue-derived mesenchymal stem cells (AD-MSCs)

Intra-abdominal adipose tissues were aseptically collected from a 9-year-old male chihuahua dog. Adipose tissues were cut into pieces ≤0.2 mm³ and digested at 37°C with 0.1% (w/v) Trypsin-EDTA (FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan). After digestion, the cell suspension was centrifuged at 1,000 rpm for 3 min to collect the cells. The cells were cultured in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 2 mM GlutaMAX™ Supplement (Thermo Fisher Scientific), 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific), and 0.1% 2-mercaptoethanol (Thermo Fisher Scientific). After 48 hr, the medium was replaced, and non-adherent cells were removed. The medium was changed every 3 days. When the growth of the AD-MSCs was 70–80% confluent, the cells were detached by incubation in 0.05% trypsin-EDTA for 5 min at 37 °C. For demethylation assays, AD-MSCs were cultured for 96 hr in medium containing 0, 1, 5, or 10 μM 5-aza-2′-deoxycytidine (5-aza-dC; Merck Millipore, Billerica, MA, USA).

Cloning and sequencing of full-length cDNA of dog SF-1

Total RNA was extracted from tissues and cells using ISOGEN II (FUJIFILM Wako Pure Chemical Corp.) following the manufacturer’s instructions. Quality and concentration were measured using a NanoDrop® 2000C spectrophotometer (Thermo Fisher Scientific). Rapid amplification of cDNA ends (RACE) reactions were
performed using the GeneRacer™ Kit with SuperScript™ III RT (Thermo Fisher Scientific) and template cDNA from total RNA obtained from adrenal gland. The GeneRacer™ Kit provides a method to obtain full-length 5′ and 3′ ends of cDNA by removing the mRNA cap structure, ligating the GeneRacer™ RNA Oligo to the mRNA, and reverse transcribing the mRNA with oligo dT primer. Specific amplification products were obtained through polymerase chain reaction (PCR) performed under the following thermocycling conditions: 30 cycles of 98°C for 10 sec, 60°C for 5 sec, and 72°C for 3 min. The primers used are summarized in Table 1. All PCR products were extracted with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and were ligated into pBluescript II SK (-) by In-Fusion (TaKaRa Bio INC., Kusatsu, Japan). Ligated PCR products were sub-cloned and sequenced.

Genomic DNA extraction and genome walking

Genomic DNA was isolated from tissues and cells by phenol and chloroform separation, and ethanol precipitation. This DNA was suspended in TE buffer (nacalai tesque, Kyoto, Japan). Quality and concentration were measured using the aforementioned NanoDrop® 2000C spectrophotometer. Genomic DNA isolated from adrenal gland was amplified using Splinkerette PCR [26] with specific primers and sequenced to determine the genomic locations of dog SF-1. All Splinkerette PCR experiments were performed under the following thermocycling conditions: 30 cycles of 98°C for 10 sec, 60°C for 5 sec, and 72°C for 3 min. The primers used are summarized in Table 1. All PCR products were sub-cloned and sequenced described above.
Bioinformatics analysis

Ensemble IDs of transcriptions used in this study were as follows: *Homo sapiens* (ENST00000373588.8), *Mus musculus* (ENSMUST00000112883.7), *Rattus norvegicus* (ENSRNOT0000017651.3), *Bos taurus* (ENSBTAT00000011869.3), *Sus scrofa* (ENSSSCT0000034748.2), *Felis catus* (ENSFCA00000026158.3), and *Canis lupus familiaris* (ENSCAFT00000032206.3). Multiple alignments of SF-1 protein sequences were analyzed using clustalW (https://clustalw.ddbj.nig.ac.jp). The open reading frame (ORF) of dog SF-1 was identified with ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The amino acid sequence deduced from the cDNA sequence was obtained with the EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq). Calculated molecular weights and predicted isoelectric points were obtained with EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats). Sequence identity of cDNA and basal promoter was analyzed using EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher) and EMBOSS Water (https://www.ebi.ac.uk/Tools/psa/emboss_water), respectively. TFBIND (http://tfbind.hgc.jp) was used to search for transcription factor binding sites in the dog SF-1 basal promoter.

Gene expression analysis

Total RNA was extracted from tissues and cells using ISOGEN II (FUJIFILM Wako Pure Chemical Corp.) following the manufacturer’s instructions. For the reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized using total RNA (1 μg) with random hexamers and ReverTra Ace reverse transcriptase (TOYOBO Co., Ltd., Osaka, Japan). The cDNA template was amplified using BIOTAQ™ HS DNA Polymerase (Bioline Ltd; London, UK) and specific primers for
dog SF-1 and dog glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All PCR experiments were performed under the following thermocycling conditions: 95°C for 10 min; 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. Quantitative real-time PCR (qPCR) was performed using SYBR® Green PCR master mix (Applied Biosystems, Woburn, MA, USA). Data were normalized to GAPDH expression. Gene expression levels are presented as the fold-change in expression, which was calculated using the Pfaffl method [22]. The sequences of the primers used in this study are summarized in Table 1.

**Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing**

Sodium bisulfite treatment of genomic DNA was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA). PCR amplification was performed using BIOTAQ™ HS DNA Polymerase (Bioline Ltd.) and specific primers for dog SF-1 promoter. The sequences of primers used in this study are summarized in Table 1. All PCR experiments were performed under the following thermocycling conditions: 95°C for 10 min; 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. For COBRA [32], the PCR product was treated by HpyCH4IV (New England Biolabs Inc., Ipswich, MA, USA) or TaqI (New England Biolabs Inc.). Concentration of the treated PCR products was measured using MultiNA (SHIMADZU, Kyoto, Japan). To determine the methylation states of individual CpG sites at the dog SF-1 promoter, the PCR product was gel-extracted, sub-cloned into the pGEM-T Easy vector (Promega), and sequenced. Methylation sites were visualized and quality control was performed using the QUMA web-based tool (http://quma.cdb.riken.jp/) [14].
Differences between two independent samples were evaluated by performing two-tailed Student’s t test. All error bars represent the standard error of the mean. Linear regression and Pearson product-moment correlation coefficient were used to analyze correlations between gene expression and DNA methylation.

RESULTS

Identification and sequence analysis of full-length dog SF-I gene.

To identify the true full-length sequence of dog SF-I mRNA, we extracted total RNA from dog adrenal grand and performed 5′- and 3′-RACE (Fig. 2a). In 5′-RACE PCR, five PCR products were obtained and sequenced. One of the five PCR fragments contained the dog SF-I sequence. The other fragments were non-specific products. In 3′-RACE PCR, four PCR products were obtained and sequenced. One of the four PCR fragments contained dog SF-I sequence. The other fragments were non-specific products. Combined the results of 5′- and 3′-RACE, a true full-length dog SF-I cDNA sequence was determined. No other mRNA variant was detected. The full-length dog SF-I cDNA was consisted of 3,016 base pairs (bp) including a 162 bp 5′ untranslated region (UTR), 1,386 bp ORF, and 1,468 bp 3′ UTR (Fig. 2b). The sequence was submitted to the DNA Data Bank of Japan (DDBJ) and assigned an accession number (ID: LC494495).

Since the full-length sequence of dog SF-I cDNA was clarified, we attempted to identify the dog SF-I position in the *Canis lupus familiaris* genome database (CanFam 3.1) using BLAT. However, most of the dog SF-I sequence identified in this study was not present in the database, indicating that the genomic sequence immediately upstream of the transcription start site (TSS) of ENSCAFT00000032206 is not registered. Therefore, we performed genome walking using Splinkerette PCR. Sequencing analysis
of Splinkerette PCR products determined that the dog SF-1 is located on chromosome 9 (estimated position: 58,460,020 - 58,484,999) and contains seven exons (Fig. 2c). The identified genomic sequence was submitted to DDBJ and assigned an accession number (ID: LC494496).

We compared the nucleic acid sequence between the full-length dog SF-1 determined in this study and ENSCAFT0000032206 (Fig. 2c). The full-length dog SF-1 cDNA was 300 bp long upstream of the predicted start codon of ENSCAFT0000032206. The 3’ UTR of the full-length dog SF-1 cDNA was 1,716 bp, which was shorter than that of ENSCAFT0000032206. Other parts of the full-length dog SF-1 cDNA sequence almost matched to those of ENSCAFT0000032206, except for some single nucleotide polymorphisms.

Based on the cDNA sequence, the dog SF-1 protein is composed of 461 amino acids, with an estimated molecular weight of 51.6 kDa and theoretical isoelectric point of 7.66. When the amino acid sequence of the full-length dog SF-1 was aligned to the SF-1 of several mammals, the sequence in DBD completely matched, demonstrating that SF-1 is also highly conserved in dogs (Fig. 3). The protein sequence of the full-length dog SF-1 shared 95.0 and 94.8% identity to the sequence of human and mouse, respectively. These data indicated that the full-length cDNA identified in this study is probably an original and normal sequence of dog SF-1.

Analysis of dog SF-1 basal promoter

Clarifying the TSS of dog SF-1 identified the genome position and sequence of the basal promoter. The DNA sequence of the dog SF-1 basal promoter (from –120 bp to +120 bp) shared 88.9% and 81.8% identity to that of human and mouse, respectively. Similar to human and mouse SF-1, the basal promoter of dog SF-1 contains regulatory elements including the SOX9 binding site, E box, CCAAT box, and Sp1/Sp3 site (Fig.
DNA methylation at the basal promoter in the \textit{SF-1} gene has been reported to regulate tissue-specific expression in humans and mice. Dog \textit{SF-1} also has 15 CpG sites around exon 1 (Fig. 4a). Thus, dog \textit{SF-1} may be also under the control of DNA methylation.

**Relationship between DNA methylation at dog \textit{SF-1} promoter and gene expression**

To investigate whether DNA methylation is involved in the regulation of dog \textit{SF-1} expression, we performed demethylation assay with the demethylating reagent 5-aza-dC using dog AD-MSCs. Dog \textit{SF-1} mRNA was not detected in the AD-MSCs. However, 5-aza-dC treatment induced the dose-dependent expression of dog \textit{SF-1} (Fig. 4b and 4c). This result suggested that DNA methylation affects the expression of dog \textit{SF-1}.

We next analyzed the expression of dog \textit{SF-1} gene and DNA methylation around exon 1 in steroidogenic tissues, including adrenal, ovary, and testis. The gene expression was detected in adrenal, ovary, and testis, but the expression level was tissue-dependent (Fig. 5a and 5b). The DNA methylation rates of three CpG sites around the dog \textit{SF-1} promoter, -82 bp, +286 bp, and +426 bp from TSS, was analyzed using COBRA. DNA methylation rates were 0-3% in ovary, 30-39% in adrenal, 63-69% in testis, and 74-100% in AD-MSCs (Fig. 5c). There was a clear inverse correlation between the gene expression and the DNA methylation rates among samples (Fig. 5d). Bisulfite sequencing analysis was performed to investigate the DNA methylation state of individual CpG sites around the dog \textit{SF-1} promoter from positions -93 bp to +426 bp, which contains 30 CpG sites (Fig. 5e). As expected, based on the COBRA results, methylation levels were low throughout the promoter in ovary, while AD-MSCs were highly methylated. In addition, the inverse correlation of methylation...
pattern to expression extended downstream of exon 1. These results indicated that the
promoter activity of dog SF-1 is under the control of DNA methylation.

**DISCUSSION**

The dog SF-1 mRNA recorded in the database (CanFam3.1) lacks the 5′-end
sequence coding for the DBD, which is conserved in other mammals. In this study, we
identified the true full-length cDNA of dog SF-1. It possessed the 5′-end sequence
coding DBD and shared high similarities with sequences in human, mouse, bovine, pig,
and cat. In addition, determination of the 5′-end sequence of dog SF-1 mRNA enabled
us to identify the genomic location and the genomic sequence of the promoter. There
are many unclarified genomic sequences in the dog genome database [13]. Most of the
RefSeq genes in dog have been computationally predicted based on CanFam3.1. In fact,
3,181 RefSeq genes in CanFam3.1 have unclarified regions within 5,000 upstream from
TSS, suggesting that the correct 5′-end sequences of those genes have been veiled.

Many previous studies have reported that point mutations of the human SF-1
gene cause adrenal insufficiency and disorders of sex development (DSD). In particular,
mutations in DBD, such as p.G35E or p.R92Q, led to severe phenotype of those
diseases [8]. The 46, XY DSD, which is the most common SF-1-related disease
featuring a DBD mutation, includes clitoral enlargement, small inguinal testes, and
absent or rudimentary Müllerian structures as the typical phenotype. In the field of
veterinary medicine, dog 78, XY DSD exhibits symptoms including testicular
hypoplasia with clitoral enlargement, persistent Müllerian duct syndrome,
cryptorchidism, and hypospadias, similar to human 46, XY DSD [17, 23]. However,
dog SF-1 gene mutations have not been detected in the dog 78, XY DSD. One of the
reasons is that the genome sequence of dog SF-1 DBD have not been identified. Our
results provide the sequence of dog SF-1 DBD and information that will be useful in veterinary medicine diagnosis and research.

In this study, the basal promoter sequence of dog SF-1 gene was identified. Epigenetic analyses of the dog SF-1 promoter revealed that the expression of dog SF-1 gene is under the control of DNA methylation. These results indicate that epigenetic mutation influences gene expression of SF-1 in dog. Aberrant hypomethylation at the basal promoter of the SF-1 gene has been reported to induce ectopic gene expression in human endometriosis [33]. In dogs, ectopic-endometrium and endometrioma have been reported, which are homologous diseases to human endometriosis [1, 2, 7, 21]. However, the relationship between those dog diseases and SF-1 has not been clarified. Based on our findings, it is possible that ectopic SF-1 gene is overexpressed in dog endometriosis by epigenetic mutation in the promoter.

In conclusion, the complete sequences of mRNA and the promoter region of dog SF-1 were identified. Expression of dog SF-1 is under the control of DNA methylation at the promoter. Our results provide a molecular biological basis for a better understanding of developmental and metabolic mechanisms for dogs.
CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

1. Bartel, C., Berghold, P., and Walter, I. 2011. Ectopic endometrial tissue in mesonephric duct remnants in bitches. Reprod. Domest. Anim. 46: 950-956.

2. Bartel, C., Schönkypl, S., and Walter, I. 2010. Pseudo-placentational endometrial cysts in a bitch. Anat. Histol. Embryol. 39: 74-80.

3. Bassett, M. H., Suzuki, T., Sasano, H., White, P. C., and Rainey, W. E. 2004. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. Mol. Endocrinol. 18: 279-290.

4. Bassett, M. H., Zhang, Y., Clyne, C., White, P. C., and Rainey, W. E. 2002. Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. J. Mol. Endocrinol. 28: 125-135.

5. Clyne, C. D., Zhang, Y., Slutsker, L., Mathis, J. M., White, P. C., and Rainey, W. E. 1997. Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements. Mol. Endocrinol. 11: 638-649.

6. Daggett, M. A., Rice, D. A., and Heckert, L. L. 2000. Expression of steroidogenic factor 1 in the testis requires an E Box and CCAAT Box in its promoter proximal region. Biol. Reprod. 62: 670-679.

7. Demirel, M. A. 2017. A case of spontaneous abortion related to ovarian endometriosis in a Golden Retriever dog. Iran J. Vet. Res. 18: 63-66.

8. El-Khairi, R., and Achermann, J. C. 2012. Steroidogenic factor-1 and human disease. Semin. Reprod. Med. 30: 374-381.

9. Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel. N. L., and Inghram, H. A. 1999. Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. Mol. Cell 3: 521-526.

10. Hanley, N. A., Rainey, W. E., Wilson, D. I., Ball, S. G., and Parker, K. L. 2001.
Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. *Mol. Endocrinol.* **15**: 57-68.

11. Hattori, N., Nishino, K., Ko, Y. G., Ohgane, J., Tanaka, S., and Shiota, K. 2004. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J. Biol. Chem.* **279**: 17063-17069.

12. Hoivik, E. A., Aumo, L., Aesoy, R., Lillefosse, H., Lewis, A. E., Perrett, R. M., Stallings, N. R., Hanley, N. A., and Bakke, M. 2008. Deoxyribonucleic acid methylation controls cell type-specific expression of steroidogenic factor 1. *Endocrinology* **149**: 5599-5609.

13. Holden, L. A., Arumilli, M., Hytönen, M.K., Hundi, S., Salojärvi, J., Brown, K.H., and Lohi, H. 2018. Assembly and analysis of unmapped genome sequence reads reveal novel sequence and variation in dogs. *Sci. Rep.* **8**: 10862.

14. Kumaki, Y., Oda, M., and Okano, M. 2008. QUMA: quantification tool for methylation analysis. *Nucleic Acids Res.* **36**: W170-5.

15. Luo, X., Ikeda, Y., and Parker, K. L. 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77**: 481-490.

16. Martin, L. J., and Tremblay, J. J. 2005. The human 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase type 2 promoter is a novel target for the immediate early orphan nuclear receptor Nur77 in steroidogenic cells. *Endocrinology* **146**: 861–869.

17. Meyers-Wallen, V. N. 2012. Gonadal and sex differentiation abnormalities of dogs and cats. *Sex. Dev.* **6**: 46–60.

18. Morohashi, K., Honda, S., Inomata, Y., Handa, H., and Omura, T. 1992. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J. Biol. Chem.* **267**: 17913-17919.
19. Nishino, K., Hattori, N., Tanaka, S., and Shiota, K. 2004. DNA methylation-mediated control of Sry gene expression in mouse gonadal development. *J. Biol. Chem.* 279: 22306-22313.

20. Nomura, M., Bärtsch, S., Nawata, H., Omura, T., and Morohashi, K. 1995. An E box element is required for the expression of the ad4bp gene, a mammalian homologue of ftz-f1 gene, which is essential for adrenal and gonadal development. *J. Biol. Chem.* 270: 17453-7561.

21. Paiva, B. H., Silva, J. F., Ocarino, N. M., Oliveira, C. A., Assis, W. A., and Serakides, R. 2015. A rare case of endometrioma in a bitch. *Acta Vet. Scand.* 57: 31.

22. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: No. 9e45.

23. Poth, T., Breuer, W., Walter, B., Hecht, W., and Hermanns, W. 2010. Disorders of sex development in the dog—Adoption of a new nomenclature and reclassification of reported cases. *Anim. Reprod. Sci.* 121: 197-207.

24. Ramayya, M. S., Zhou, J., Kino, T., Segars, J. H., Bondy, C. A., and Chrousos, G. P. 1997. Steroidogenic factor 1 messenger ribonucleic acid expression in steroidogenic and nonsteroidogenic human tissues: Northern blot and in situ hybridization studies. *J. Clin. Endocr. Metab.* 82: 1799–1806.

25. Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N., and Cedar, H. 1984. Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. *Proc. Natl. Acad. Sci. USA* 81: 2275-2279.

26. Shao, H., and Lok, J. B. 2014. Detection of piggyBac-mediated transposition by splinkerette PCR in transgenic lines of Strongyloides ratti. *Bio. Protoc.* 4: e1015.

27. Shen, J. H., and Ingraham, H. A. 2002. Regulation of the orphan nuclear receptor steroidogenic factor 1 by Sox proteins. *Mol. Endocrinol.* 16: 529-540.

28. Sugawara, T., Kiriakidou, M., McAllister, J. M., Kallen, C. B., and Strauss, J. F. 3rd.
1997. Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36**: 7249–7255.

29. Ueda, H., and Hirose, S. 1991. Defining the sequence recognized with BmFTZ-F1, a sequence specific DNA binding factor in the silkworm, Bombyx mori, as revealed by direct sequencing of bound oligonucleotides and gel mobility shift competition analysis. *Nucleic Acids Res.* **19**: 3689-3693.

30. Wang, X. L., Bassett, M., Zhang, Y., Yin, S., Clyne, C., White, P. C., and Rainey, W. E. 2000. Transcriptional regulation of human 11beta-hydroxylase (hCYP11B1). *Endocrinology* **141**: 3587-3594.

31. Woodson, K. G., Crawford, P. A., Sadovsky, Y., and Milbrandt, J. 1997. Characterization of the promoter of SF-1, an orphan nuclear receptor required for adrenal and gonadal development. *Mol. Endocrinol.* **11**: 117-126.

32. Xiong, Z., and Laird, P. W. 1997. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* **25**: 2532-2534.

33. Yamagata, Y., Nishino, K., Takaki, E., Sato, S., Maekawa, R., Nakai, A., and Sugino, N. 2014. Genome-wide DNA methylation profiling in cultured eutopic and ectopic endometrial stromal cells. *PLoS One* **9**: e83612.

34. Zeitoun, K., Takayama, K., Michael, M. D., and Bulun, S. E. 1999. Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same cis-acting element. *Mol. Endocrinol.* **14**: 239-253.
FIGURE LEGENDS

Fig. 1. Schematic overviews of Steroidogenic factor 1 (SF-1) registered in public databases of several species. In most species, SF-1 contains two domains: a DNA binding domain (DBD) and ligand binding domain (LBD) that are linked via a hinge region. Dog SF-1 (ENSCAFT00000032206) lacks the N-terminal aa sequence containing the major part of the DBD.

Fig. 2. (a) Representative electrophoresis image. All polymerase chain reaction (PCR) fragments (arrows) were sub-cloned and sequenced. Red arrows indicate the fragments including dog Steroidogenic factor 1 (SF-1) mRNA sequence. Black arrows represent non-specific products. M: Marker. (b) The full-length cDNA sequence of dog SF-1 identified in this study. Numbers on the left represent nucleotide positions. The 5’ and 3’ untranslated regions (UTRs) are indicated in lower case. The open reading frame is indicated in upper case. The start codon and the stop codon are underlined. Sequences in red represent the region newly identified in this study. Sequences in blue represent the polyadenylation signal. (c) Comparison of exon-intron structure of ENSCAFT00000032206 and the full-length dog SF-1. Exons are shown as boxes and are numbered. The coding region newly identified in this study are filled with red.

Fig. 3. Multiple sequence alignments of deduced amino acid (aa) sequence of full-length dog Steroidogenic factor 1 (SF-1) with other species. Alignments were performed with clustalW. Conserved aa sequences are indicated by a dark background. Highly similar aa sequences are indicated by a dark grey background. Weakly similar aa sequences are indicated by a light grey background. Numbers on the right show the position of the aa sequence. DNA binding domain is boxed in red.
Fig. 4. (a) Overview of the CpG sites in the Steroidogenic factor 1 (SF-1) basal promoter (-120 pb ~ +120 bp) in dogs, humans, and mice. Lollipops indicate the position of individual CpG sites. Horizontal lines indicate transcription factor binding sites. Sx, a binding site for SRY-box9 (SOX9); E, an E box; C, a CCAAT box; Sp, a binding site for Sp1 or Sp3 transcription factors. (b, c) Effect of a demethylating reagent (5-aza-dC) on the expression of dog SF-1 gene in adipose tissue-derived mesenchymal stem cells (AD-MSCs). (b) Expression of the dog SF-1 and dog glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes by reverse transcription-polymerase chain reaction (RT-PCR). Expression of the dog SF-1 increased depending on 5-aza-dC concentration. GAPDH was used as an internal control. (c) Relative SF-1 gene expression levels measured by quantitative RT-PCR, with normalization to GAPDH expression using the Pfaffl method. The data shown represent the mean ± standard error (SE) (n = 3). *p < 0.05, **p < 0.01. N.D.: not detected.

Fig. 5. Analysis of dog Steroidogenic factor 1 (SF-1) expression and DNA methylation in the promoter. (a) Expression of the dog SF-1 gene in adrenal gland, ovary, testis and adipose tissue-derived mesenchymal stem cells (AD-MSCs) by reverse transcription-polymerase chain reaction (RT-PCR). The upper and lower panels show dog SF-1 and dog glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, respectively. (b) Relative dog SF-1 gene expression levels measured by quantitative RT-PCR with normalization to GAPDH expression using the Pfaffl method. The data shown represent the mean ± SE (n = 3). (c) DNA methylation level measured by combined bisulfite restriction analysis (COBRA) at the dog SF-1 promoter region in tissues and cultured cells. Numbers on the left indicate the position of CpG site from TSS. The data shown represent the mean ± SE (n = 3). (d) Scatter plot of dog SF-1 gene expression and DNA methylation levels in the promoter, defined by quantitative RT-PCR and COBRA. Red
line indicates linear regression line. (e) Bisulfite sequencing analysis around exon 1 of dog SF-1 gene. (Top) Diagram of the dog SF-1 gene. Exon 1 is shown as a white box. Vertical lines indicate the position of individual CpG sites. Black arrowheads represent the position of the CpG sites measured in COBRA (d). (Bottom) The open and closed circles indicate the unmethylated and methylated states of each CpG site, respectively.
| Primers            | Sequence (5’-3’)                  | Application         |
|--------------------|-----------------------------------|---------------------|
| 5’_dSF1-F          | CGACTGGAGCACGGAGGACCTGA           | RACE                |
| 5’_dSF1-R          | GTCCACGATGGAGATGGAAGG            | RACE                |
| 5’_Nested_dSF1-F   | GGACACTGACATGGACTGAGAGTA         | RACE                |
| 5’_Nested_dSF1-R   | GCTCTGGGTACTCAGACTTGGTA          | RACE                |
| 3’_dSF1-F          | TCCAGAAGTGCCGAGCAGTG            | RACE                |
| 3’_dSF1-R          | GTGTCGAAACGATGCTACGTAACG         | RACE                |
| 3’_Nested_dSF1-F   | AGCATCTGGCAACGAGGATG            | RACE                |
| 3’_Nested_dSF1-R   | CGCTACGTAAACGGCATGACAGTG         | RACE                |
| CDS_dSF1-F         | ATGGACTTTGGTACGACGAG            | RACE                |
| CDS_dSF1-R         | TCAAGTCTGCTGGCTTGGCA            | Splinkerette PCR    |
| Sp_adaptor         | CGAAGAGTAAACGGTGGCTAGGAGAGACC   | Splinkerette PCR    |
| Sp_Nested_adaptor  | GTGGCTGAATGAGACTGAGTGTGCAC       | Splinkerette PCR    |
| Sp_dSF1-F          | CATGGACTATTTCTGACGAGGACCTG       | Splinkerette PCR    |
| Sp_Nested_dSF1-F   | GCTACCACTACGGACTGCTCACG          | Splinkerette PCR    |
| Sp_dSF1-R          | ACCTGCGACTCTCGCAGTG             | Splinkerette PCR    |
| Sp_Nested_dSF1-R   | ACGTGAGCAGTCCGTAGGCTAGGC         | Splinkerette PCR    |
| M13-F              | TGTTAAACACGCGACGCTAAGG          | Sequence            |
| M13-R              | CGAAGAACACGTATGACGACG           | Sequence            |
| Seq_dSF1-F         | ACCGACCGGCCTGTATAG              | Sequence            |
| Seq_dSF1-R         | ACGCCAAAACCCCGATCTGAG           | Sequence            |
| GAPDH-F             | AATGCCCTTGGCACCAACCAAC          | qPCR                |
| GAPDH-R             | GAAGGGCATGCCATGAGGTCTTCA        | qPCR                |
| dSF1-F              | TCCAGAAGTGCCGAGCAGTG            | qPCR                |
| dSF1-R              | TGAAGCATTGGCTGAAATCTG           | qPCR                |
| Bis_dSF1-F         | GATTTAAATGAAAGAAAAATTTAATAAGAGAAG | Bisulfite PCR       |
| Bis_dSF1-R         | ACCATAAACACATTCAAAACTAC         | Bisulfite PCR       |

**Table 1.** Primers used in this study
| Species     | DNA Binding Domain | Hinge Region | Ligand Binding Domain |
|-------------|--------------------|--------------|-----------------------|
| human       | ![Diagram](human.png) |             | ![Diagram](human.png)  |
| mouse       | ![Diagram](mouse.png) |             | ![Diagram](mouse.png)  |
| rat         | ![Diagram](rat.png)  |             | ![Diagram](rat.png)    |
| bovine      | ![Diagram](bovine.png)|             | ![Diagram](bovine.png) |
| pig         | ![Diagram](pig.png)  |             | ![Diagram](pig.png)    |
| cat         | ![Diagram](cat.png)  |             | ![Diagram](cat.png)    |
| **dog**     | ![Diagram](dog.png)  |             | ![Diagram](dog.png)    |

(ENSCAFT00000032206)
**Fig. 3**

**DNA binding domain**

- **Canis lupus familiaris**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK
- **Felis catus**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK
- **Sus scrofa**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK
- **Homo sapiens**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK
- **Mus musculus**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK
- **Rattus norvegicus**: DYSDEDEELPVCGDKVSYYGTLTCCESCKFFKRTVONNKHYCTGKTSKIDKTQRKCFRFOKCLTVGMRELAVRDMMRGRNNK

**Canis lupus familiaris**

- **Felis catus**: EKSEYEPVYASPPPPLYPEPSGGPVQELILLEPELEQRARAGCLOEPKRDGPGRDERLLCRMADOTFSIVDWARC
- **Sus scrofa**: EKSEYEPVYASPPPPLYPEPSGGPVQELILLEPELEQRARAGCLOEPKRDGPGRDERLLCRMADOTFSIVDWARC
- **Homo sapiens**: EKSEYEPVYASPPPPLYPEPSGGPVQELILLEPELEQRARAGCLOEPKRDGPGRDERLLCRMADOTFSIVDWARC
- **Mus musculus**: EKSEYEPVYASPPPPLYPEPSGGPVQELILLEPELEQRARAGCLOEPKRDGPGRDERLLCRMADOTFSIVDWARC
- **Rattus norvegicus**: EKSEYEPVYASPPPPLYPEPSGGPVQELILLEPELEQRARAGCLOEPKRDGPGRDERLLCRMADOTFSIVDWARC

**Canis lupus familiaris**

- **Felis catus**: VKELEAVDOMTLCWSELVELDHYRDOIGKGDIILTYGEVETSYYAGSLLVELRAELVELQDDLROFVCLFLFLS379
- **Sus scrofa**: VKELEAVDOMTLCWSELVELDHYRDOIGKGDIILTYGEVETSYYAGSLLVELRAELVELQDDLROFVCLFLFLS379
- **Homo sapiens**: VKELEAVDOMTLCWSELVELDHYRDOIGKGDIILTYGEVETSYYAGSLLVELRAELVELQDDLROFVCLFLFLS379
- **Mus musculus**: VKELEAVDOMTLCWSELVELDHYRDOIGKGDIILTYGEVETSYYAGSLLVELRAELVELQDDLROFVCLFLFLS379
- **Rattus norvegicus**: VKELEAVDOMTLCWSELVELDHYRDOIGKGDIILTYGEVETSYYAGSLLVELRAELVELQDDLROFVCLFLFLS379

**Canis lupus familiaris**

- **Felis catus**: DXFLNNDLXVDAEKLNALDLYTCHYPGCGKQOLLCLVYRALSBOAOEYLYHUKGNGNEPRNLLLELGAKE461
- **Sus scrofa**: DXFLNNDLXVDAEKLNALDLYTCHYPGCGKQOLLCLVYRALSBOAOEYLYHUKGNGNEPRNLLLELGAKE461
- **Homo sapiens**: DXFLNNDLXVDAEKLNALDLYTCHYPGCGKQOLLCLVYRALSBOAOEYLYHUKGNGNEPRNLLLELGAKE461
- **Mus musculus**: DXFLNNDLXVDAEKLNALDLYTCHYPGCGKQOLLCLVYRALSBOAOEYLYHUKGNGNEPRNLLLELGAKE461
- **Rattus norvegicus**: DXFLNNDLXVDAEKLNALDLYTCHYPGCGKQOLLCLVYRALSBOAOEYLYHUKGNGNEPRNLLLELGAKE461
Fig. 4

(a) Sx E C Sp

dog

human

mouse

Exon 1

Exon 1

Exon 1

(b) Sx E C Sp

SF-1

GAPDH

5-Aza-dC (µM)

M 0 1 5 10

(c) Relative dog SF-1 mRNA expression

5-Aza-dC

0µM 1µM 5µM 10µM

N.D. * **

0.00e+00 2.50e−04 5.00e−04 7.50e−04 1.00e−03 1.25e−03

0 µM 1 µM 5 µM 10 µM
Fig. 5

(a) Western blot images showing SF-1 and GAPDH expression in Ovary, Adrenal, Testis, and AD-MSCs.

(b) Bar graph showing relative dog SF-1 mRNA expression in Ovary, Adrenal, Testis, and AD-MSCs.

(c) Graph comparing DNA methylation levels in Ovary, Adrenal, Testis, and AD-MSCs.

(d) Scatter plot with linear regression line showing DNA methylation level versus expression, with $r^2 = 0.906$.

(e) DNA methylation pattern for Exon1 in Ovary, Adrenal, Testis, and AD-MSCs.