Development and identification of Set transgenic mice

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Received May 29, 2015; Accepted September 15, 2016

DOI: 10.3892/etm.2017.5612

Abstract. As a multifunctional protein involved in numerous biological processes, Set is expressed in several embryonic and adult organs. Furthermore, Set is overexpressed in numerous types of human cancers, including acute myeloid leukemia, breast cancer and pancreatic cancer. The expression of Set in germ cells is involved in gonad development, and the over-expression of Set has been observed in polycystic ovaries. In order to elucidate the physiological and pathological roles of Set, a Set transgenic mouse model was developed, in which the global overexpression of Set in adult tissues could be induced via the Cre/loxP system with the precise deletion of the Stop fragment in double-transgenic hybrids. This result was then confirmed by genotypical and protein analysis using polymerase chain reaction and bioluminescence imaging. In conclusion, the conditional Set transgenic mice carrying a reporter system were successfully generated. The transgenic mice open a new window for the further investigation of the function of Set using tissue-specific Cre mice and inducible Cre systems.

Introduction

Set, which is an inhibitor of protein phosphatase 2A (PP2A), is an evolutionarily conserved multifunctional oncoprotein involved in histone binding, nucleosome assembly, transcription control and cell apoptosis (1-5). In the gonadal system of adult rats, Set is expressed in spermatocytes, Leydig and Theca cells as well as oocytes (6,7). Furthermore, expression of Set during embryogenesis is much higher than that of adult gonads (6). These data indicate that Set participates in the early ontogenesis of the gonadal system, and may be important in oocyte development. Furthermore, Set is highly expressed in canine T-cell lymphoma, Wilms' tumors, ovarian cancer and lung tumor, and may contribute to liver carcinogenesis (8-10). Set also results in tumor metastasis by binding to Rac1 and activating Rac1-GTPase signaling during cell migration (11). As a transcriptional regulation factor, Set exerts its effect by binding to the transcriptional co-activators CBP/p300 and then cooperating directly to promote the transcription of CYP17A1 in human NT2 neuronal precursor, Leydig and Theca cells (11,12). Our previous experiments demonstrated that Set was one of the genes overexpressed in the ovaries of patients suffering from polycystic ovary syndrome (PCOS) when compared with normal ovaries using cDNA microarray technology (13). Set was also identified to be important in the positive regulation of ovarian androgen biosynthesis by enhancing the transcription of steroidogenic enzymes, HSD3B2 and CYP17A1, which may lead to hyperandrogenism in PCOS (11,12).

Thus far, the majority of studies (3,14,15) into the roles and functional mechanisms of Set have been performed at the cellular and molecular levels. In addition, a lack of animal models has limited the progress in determining Set function (16,17). Currently, the Cre/loxP response system is the most common approach to modulate gene expression in a temporal and tissue-specific manner (18) *in vivo* since no DNA topological requirements or accessory host factors are required for efficient Cre-mediated DNA recombination between pairs of loxP sites (19,20). These characteristics are key features that facilitated the initial determination of the suitability of Cre for genomic manipulation in eukaryotic cells (20). In the present study, based on the Cre/loxP system an animal model was developed first with the conditional Set transgenic mice. The model allowed Set global overexpression in transgenic mice, which helped study the Set function *in vivo* in tumorigenesis and the reproductive endocrine diseases.

Materials and methods

Reagents and instruments. Trichloromethane (Nanjing Zhongxu Chemical Industry Co., Ltd., Nanjing, China), agarose (Biowest Europe, Nuaillé, France), a nucleic acid dye (Goldview; Shanghai Saibaisheng Gene Technology Co., Ltd., Shanghai, China), Tris-phenol (Tianjin Haoyang Biological

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Key words: Set, Cre/loxP system, EIIa-Cre, transgenic model

Products Technology Co., Ltd., Tianjin, China), PrimeScript™ RT reagent kit with gDNA Eraser and SYBR Premix Ex Taq kits (Takara Bio, Inc., Otsu, Japan) were used. All other chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) unless otherwise stated. All primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The following machines and facilities were employed: ABI Prism 7300 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.), PE9600 PCR Amplification (Applied Biosystems; Thermo Fisher Scientific, Inc.), GeneQuant pro RNA/DNA quantitative analyzer (GE Healthcare, Fairfield, CT, USA), IVIS Spectrum Imaging System (Xenogen Corporation, Alameda, CA, USA), EPS2A/200 Electrophoresis System (Hoefer Pharmacia Biotech, Inc., Holliston, MA, USA) and Image Master VDS (Hoefer Pharmacia Biotech, Inc., San Francisco, CA, USA).

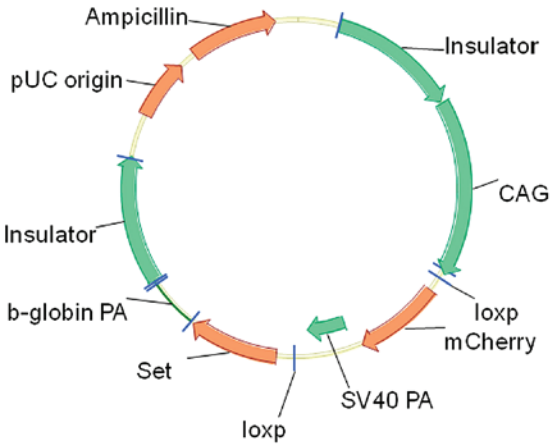

Figure 1. Map of pInsulator-CAG-loxp-mcherry-loxP-set transgenic vector.

Animals. Four EIIa-Cre transgenic mice (EIIa-Tg, C57BL/6N background; 2 male and 2 female; 8 weeks old; 18-24 g) were provided by Professor Xingxu Huang from the Model Animal Research Center of Nanjing University (Nanjing, China). hGFPA expression vector with a PCAG-promoter (pInsulator -CAG-mcherry-loxP-3xflag-hGFPA vector) was constructed by the Medical Animal Center of Jiangsu Province in Nanjing Medical University. The mice were housed under controlled daily lighting (12-h light/dark) at 20-22˚C, with water and food provided *ad libitum*. The mice were euthanized using a CO_2 flow at 3 l/min for 10-15 min in a clean cage. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu Province and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (21).

Development of the transgenic mice. The Set coding sequence from the mouse chromosome was cloned into the hGFPA expression vector with a pCAG-promoter (pInsulator -CAG-mcherry-loxP-3xflag-hGFPA) by replacing the coding sequence of hGFPA with the Set cassette. Next, the plasmid (pInsulator-CAG-mcherry-loxP-3Xflag-set; Fig. 1) was linearized with I-CeuI (New England Biolab, Inc.,Ipswich, MA,USA), purified using the classic phenol-chloroform method and used as a template for *in vitro* transcription. Independent lines of the founder mice (Set-Tg) carrying the Set transgene were separately generated by standard pronuclear injections (22) into fertilized eggs from C57BL/6N mice. The floxed SV40 stop signal and Mcherry sequence were located between a chicken β-actin promoter and the constitutively active intracellular domain of Set. Therefore, transgenic Set in founder mice is silent, but can be activated by the introduction of Cre recombinase and consequently the removal of the stop signal (Fig. 2). Furthermore, Cre excision can be monitored using the IVIS Spectrum Imaging System in which Mcherry is expressed prior to Cre excision.

Identification of transgenic mice. Genomic DNA was extracted from a 2-mm tail of the 7-day-old mice using phenol-chloroform and recovered by alcohol precipitation. In brief, samples were incubated overnight at 57˚C in a solution containing 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 100 mM Tris 8.5 and 10 mg/ml proteinase K. Following centrifugation at 11,000 x g (4˚C) for 10 min, DNA was precipitated with phenol-chloroform following 100 and 75% (v/v) ethanol, then air-dried and resuspended in double-distilled water. The purity of each DNA sample was determined by the absorbance ratio at an OD_{260}/OD_{280} using a NanoDrop 2000 instrument (Thermo Fisher Scientific, Inc.), and DNA with a ratio of 1.8-2.0 was used for polymerase chain reaction (PCR). The PCR products were evaluated by electrophoresis on a 1.5% (w/v) agarose gel containing 0.006% (v/v) Goldview. In order to identify the genotype of mice, the genomic DNA was dissolved in Tris-EDTA buffer [10 mM Tris/HCl, (pH 8.0), 0.1 mM EDTA] and the genotypes of all transgenic mice were analyzed by PCR analysis on genomic DNA with the primer pairs presented below. Each mouse was analyzed at least three independent times with separately prepared DNA samples.

Primers and conditions for identification of the Set-Tg mice. Three pairs of primers were used to PCR amplify the transgene: i) F1: 5'-CTGACGCAGGTGCTGATGA-3' and R1: 5'-CTG ACGCAGGTGCTGATGA-3', which flank the exogenous Set region. The product size was 366 bp and the PCR conditions were 94˚C for 5 min; 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec and 30 cycles, and 72˚C for 10 min; ii) loxP1-F: 5'-GCA ACGTGCTGGTTATTGTG-3' and loxP1-R: 5'-CCTTGG TCACCTTCAGCTTG-3', which flank the front loxP site in the transgene (product size, 314 bp); and iii) loxP2-F: 5'-GTC TGGATCTGACATGGTAAG-3' and loxP2-R: 5'-GTAGTC CATGGTGGCTTAAG-3', which flank the later loxP site in the transgene (product size, 171 bp). The PCR conditions for the later two primers were as follows: 95˚C for 5 min; 95˚C for 40 sec, 58˚C for 40 sec, 72˚C for 45 sec and 35 cycles, and 72˚C for 8 min.

Primers and conditions for identification of the EIIa-Tg mice. The following primer pair that recognized the Cre coding region was used: 5'-ATCAACGTTTTCTTTTCGG-3' and 5'-ATTTGCCTGCATTACCGGTC-3', and the product size was 350 bp. The PCR conditions were as follows: 95˚C for 5 min, 95˚C for 30 sec, 55˚C for 45 sec, 72˚C for 45 sec and 30 cycles, and 72˚C for 8 min.

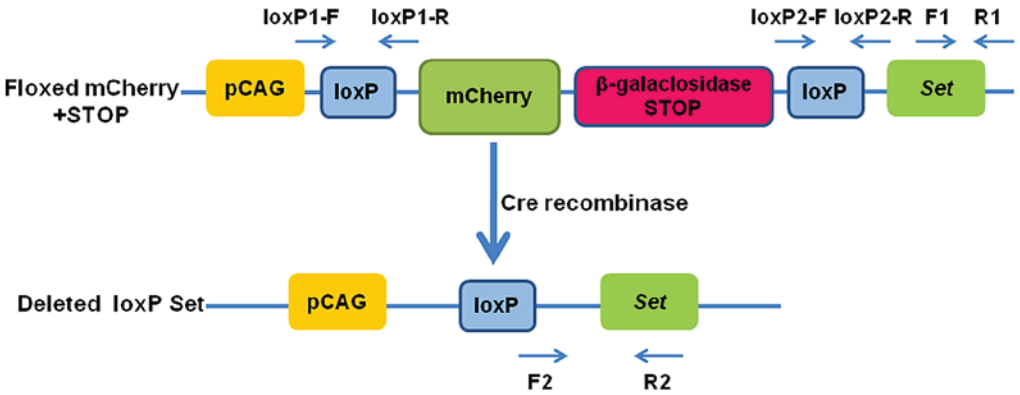

Figure 2. Gene targeting scheme for Set transgenic mice construction. The native chicken β-actin promoter (pCAG) drives transcription of *Set* after Cre-mediated removal of the STOP cassette (floxed SV40 stop signal) with Mcherry sequences. The arrows indicate the primers for genomic polymerase chain reaction analysis.

Primers and conditions for identification of the Set-EIIa-Tg mice. The primers were as follows: F2: 5'-GCAACGTGC TGGTTATTGTG-3' and R2: 5'-CGACTTGTCCTCCAGCTT CG-3', which flank the region produced by homologous recombination (product size, 358 bp). The PCR conditions were as follows: 95˚C for 5 min; 95˚C for 40 sec, 58˚C for 40 sec, 72˚C for 45 sec and 35 cycles, and 72˚C for 8 min.

Examination of transgene copy number in transgenic mice. Quantitative PCR methods, with the highly conserved mouse housekeeping gene β-actin used as a reference control, were used to calculate the transgene copy number with PrimeSTAR Max DNA Polymerase kit (Takara Bio, Inc.), as previously reported (23). Two sets of PCR reactions were set up, each containing a dilution series of the positive DNA plasmid. One set of reactions was prepared to amplify the gene of interest and the other to amplify the β-actin sequence. Each reaction was run in triplicate and the cycling parameters were as follows: 95˚C for 5 sec, 95˚C for 10 min, 40 cycles of 95˚C for 30 sec, 55˚C for 60 sec and 72˚C for 30 sec. The PCR efficiency was calculated with the following equation: Efficiency = $10^{(-1/slope)}$ - 1. The ratio of the copy number of the set gene β-actin was calculated by applying the following equation: Ratio = (1 + $Efficiency^{Ct\text{-}Set}$) / (1 + $Efficiency^{Cr\beta\text{-}actin}$). In addition, the DNA primers for the exogenous set and β-actin were used at a final concentration of 0.5 μM. The following primers were used: cSet-F, 5'-GGACCTACAAAGACCATG ACGG-3' and cSet-R: 5'-CGACTTGTCCTCCAGCTTCG-3'; β-actin-F, 5'-GAGCAACCTTCTAGGTTTAAGG-3' and β-actin-R, 5'-GCTTGCTGATCCACATCTGC-3'.

Detection of Set mRNA expression in transgenic mice. Different tissues were harvested from mice using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), including the heart, kidney, liver, brain and ovary/testis from Set-EIIa-Tg mice and control littermates. To extract and purify total RNA, an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was applied in accordance with the manufacturer's protocol. RNA integrity was assessed by 0.8% agarose gel electrophoresis and the concentration and purity of the isolated RNA were evaluated using the NanoDrop 2000 with an absorbance ratio of 1.8-2.0. Using the RT reagent kit, the gDNA was erased using eraser buffer at 42˚C for 2 min, and then 1 μg RNA was reverse transcribed into cDNA at 37˚C for 15 min, and then 85˚C for 5 sec in order to inactivate the RT enzyme according to the manufacturer's protocol. The extracted RNA containing ribosomal 28S and 18S RNA that had an absorbance ratio of 1.85-2.0 at OD_{260}/OD_{280} was used for quantitative PCR (qPCR). The qPCR system consisted of 10 μl SYBR Premix Ex Taq, 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 0.4 μl ROX Reference Dye (50X), 2 μl cDNA and 6.8 μl ddH_2O. The primers for total Set and for GAPDH were as follows: Set-t-F, 5'-GAAGAGGTCAGAATTGATCGCC-3' and Set-t-R, 5'-GTCACCACTCTCGTTCAGATG-3' (product size, 244 bp); GAPDH-PF, 5'-AGGTTGTCTCCTGCGACT TCA-3' and GAPDH-PR, 5'-GGGTGGTCCAGGGTTTCT TACT-3'. Melting curve analysis was performed in order to ensure the specificity of the products. Fold changes in the expression of the total Set in different tissues were compared with Cre-negative littermate mice and calculated according to the $2^{-\Delta\Delta Ct}$ method (24) and presented as the relative fold to the GAPDH internal reference gene.

Detection of exogenous Set protein expression by bioluminescence imaging. To illuminate the expression and distribution of exogenous Set in positive transgenic mice, Mcherry bioluminescence imaging was observed in a double-positive mouse and in control littermate after Cre-mediated recombination. The mice were fasted for a whole day prior to imaging and then anesthetized by isoflurane inhalation (4%, v/v). For *in vivo* imaging of whole animals, mice were shaved from the neck to the lower torso. Animals were next placed in the IVIS Imaging System and analyzed for fluorescence using excitation and emission wavelengths of 405-485 and 625 nm, respectively (25). Data were collected in units of photons/sec/cm^2 and visualized using Living Image software v2.50 (Xenogen Corporation).

Statistical analysis. Data are expressed as the mean ± standard deviation from at least three independent experiments. Independent samples and Student's t-tests were used for statistical comparisons. Analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA), and $P<0.05$ was used to indicate a statistically significant difference.

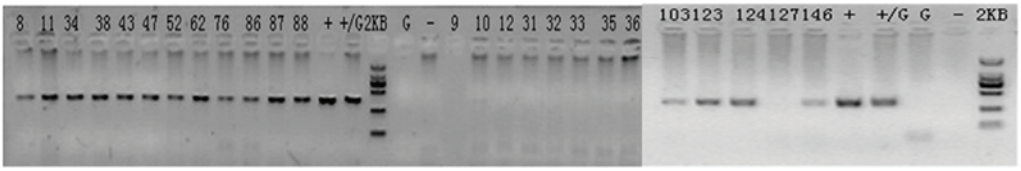

Figure 3. Analysis of the founder mice genotype by polymerase chain reaction. Positive mice numbers: 8, 11, 34, 38, 43, 47, 52, 62, 76, 86, 87, 88, 103, 123, 124, 146 and 34 (died before sexual maturity); negative mice numbers: 9,10,12,31,32,33,35,36 and 127. +, pInsulator-CAG-loxp-mcherry-loxP-set plasmid as positive control; +/G, pInsulator-CAG-loxp-mcherry-loxP-set: B6 genomic DNA as 1:10,000; G, B6 genomic DNA as negative control; -, H_2O as blank control.

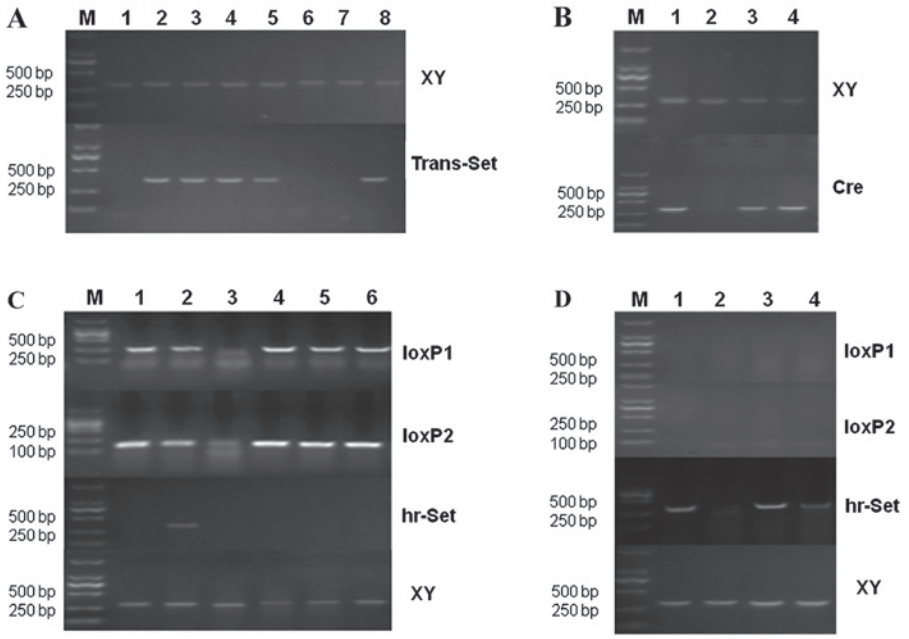

Figure 4. Identification of the transgenic mice by polymerase chain reaction of the genomic DNA. (A) Identification of Set-Tg mice: Lanes 1, 6 and 7 represent the control mice while lanes 3, 4, 5 and 8 represent the Set-Tg mice, respectively. (B) Identification of EIIa-Tg mice: Lanes 1, 3 and 4 represent for EIIa-Tg mice while lanes 2 represents the control. (C) Verification of the STOP cassette removal in Set-EIIa-Tg mice: Lane 2 represents a double-transgenic mouse, lanes 1 4, 5 and 6 represent the littermates with the Set-Tg genotype, while lane 3 represents double-negative littermate. Coexistence of panels for the inserted sequence and the homologous recombination can be seen in lane 2. (D) Verification of the STOP cassette removal in Set-EIIa-Tg mice. Lanes 1, 3 and 4 represent double-transgenic mice and lane 2 represents control littermate. M: DNA marker 2000. XY (*Mus musculus* strain C57BL/6J chromosome X/Y, GRCm38.p2 C57BL/6J), for gender identification used as an internal reference. The primer pair was: 5'-CCGCTGCCAAATTCTTTGG-3' and 5'-TGAAGCTTTTGGCTTTGAG-3' (the size of the product in males was 301 bp, while in the females it was 330 bp). trans-Set: the exogenous Set region in Set-Tg mice; hr-set: the Set region produced by homologous recombination.

Results

Generation of Set-EIIa-Tg mice. After inserting the murine Set cDNA into a vector with a chicken β-actin promoter (pCAG), the construct was injected in zygote pronuclei resulting in 15 founders (eight females and seven males; Fig. 3) that were backcrossed to C57BL/6N mice in order to generate transgenic lines. The transgene Set was transmitted to their offspring in five lines (confirmed by PCR analysis). Among them, three lines of founder mice were propagated, and the other two founder mice were infertile.

Set positive transgenic offspring, which were obtained from founder mice crossing with wild type mice, carried a 366 bp Set fragment (Fig. 4A). Set-Tg mice were crossed with EIIa-Tg (Fig. 4B) mice to obtain Set-EIIa-Tg mice that widely express Set. The recombination resulted in a 358 bp fragment, while the offspring with the Set-Tg genotype resulted in 314 and 171 bp fragments, and the negative control had no fragment detected (Fig. 4C and D).

The coexistence of 358-, 314- and 171-bp fragments in double transgenic offspring suggests the insufficient efficiency of Cre recombinase. Therefore, the STOP sequence can not be completely excised, leading to the coexistence of an exogenous sequence and the homologous recombination (Fig. 4C).

Copy number in transgenic mice. The slope of the line was used as a function of the PCR efficiency, with a slope of -0.301 indicating that the PCR was 100% efficient. The slope for the set line was 0.285, while the slope for the β-actin line was 0.290. Overall, the PCR efficiencies were >90%. A template was selected at a concentration in which both set and β-actin amplified efficiently in order to calculate the copy number. The copy numbers of the transgenic mice were 4, 7 and 14 in the founders 1, 7 and 9, respectively.

mRNA expression and tissue distribution of total Set in Set-EIIa-Tg mice. Compared with the wild type littermates, the mRNA expression levels of the total Set in different tissues

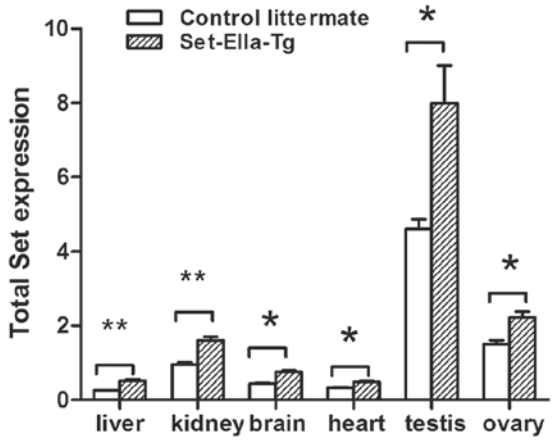

Figure 5. Overexpression of Set mRNA in the Set-EIIa-Tg mice. Fold changes for the expression of total Set in different tissues were measured by the quantitative polymerase chain reaction. Significant differences were shown (compared with the control. *P<0.05 and **P<0.001). Error bars indicate the standard deviation.

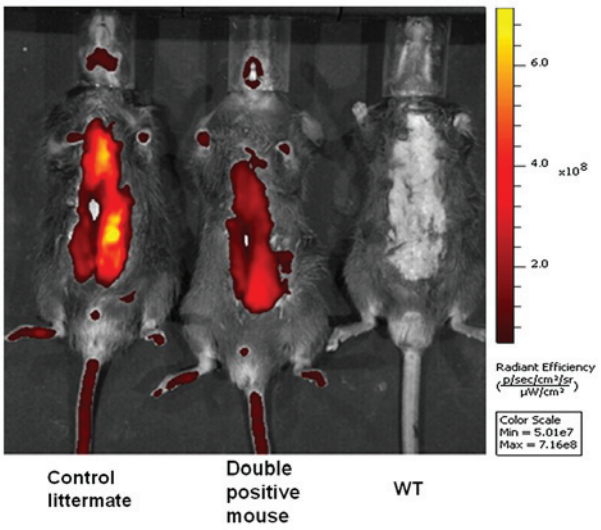

Figure 6. Expression of Set protein in Set-EIIa-Tg mice. Mcherry biofluorescence for founder 9 with fluorescence intensity of 10^8 photons per second per cm^2. Double-positive mouse with a Set-EIIa-positive genotype; control littermate with the Set genotype; WT, wild-type.

were nearly doubled in Set-EIIa-Tg mice (P<0.05) in the liver, kidney, brain, heart, ovary and testis (Fig. 5). Furthermore, the original expression level of Set was markedly higher in the testis compared to other tissues.

Protein expression of Set in Set-EIIa-Tg mice. The systemic, specific recombination was induced by the EIIa-Cre, double-positive offspring (8-10-weeks-old) from crossing between Set-Tg and EIIa-Tg mice. Together with their control littermates, they were all imaged using an IVIS Spectrum Imaging System (Fig. 6). The expression of fluorescent Mcherry was observed to be decreased in the double-positive (Set-EIIa-Tg) compared with the control littermate (Set-Tg). Thus, the results elucidated that the loxP stop codon cassette was deleted mediating by the Cre recombinase, and the Set in Set-EIIa-Tg mice was actively transcribed and translated.

Discussion

A previous study showed that placement of recombination sites into the genome and subsequent targeted expression of recombinase have allowed for the development of genetic switches that can either turn on or ablate any desired gene in gene-modified or transgenic mice (19). A specific application of this technique is the adaptation of a Cre/loxP site-specific recombinase system that allows for an exact recombination between two loxP sites (26,27). The bacteriophage P1 Cre recombinase recognizes the loxP site, which is composed of an 8-bp sequence flanked on either side by two 13-bp inverted repeats, forming a 13-8-13 structure (26). Besides, Cre recombinase can inverse or excise the inserted sequences surrounded by two loxP sites depending on the orientation of the two loxP sites relative to each other (28).

In order to investigate the physiological roles of Set, a conditional Set transgenic mouse model was established to allow the inducible expression of Set. The EIIa-Cre transgene can induce systemic and stable Cre excision from the early embryo stage and can be transmitted to their offspring (29). In order to stabilize the genotype, the founder mice were bred with wild-type mice for several generations to acquire Set transgenic mice with a clear genome background. In addition, multiple primers were used to confirm the insertion and the integrity of the exogenous Set sequence in transgenic mice. Total Set expression was doubled in different tissues of Set-EIIa-Tg mice, including the liver, kidney, heart, brain, ovary and testes when compared with that of the control littermates. The higher expression level of Set in the testis and ovary is consistent with previously published articles (7,30). Our former evidence suggested that in the ovary and testis, Set regulated both the promoter activity of CYP17 and the biological activity of P450c17 (7,11,12). Furthermore, we identified that the increased expression of Set protein in adult male mice is in accord with continuous spermatogenesis. In brief, Set is a key regulator for spermatogenesis in the testes and in androgen production (31).

Determining the transgene copy number is an important step in transgenic mice characterization. This knowledge can be important when determining what future experiments and which transgenic lines it can be utilized for. In the present study, quantitative PCR was used to determine the transgene copy number in the genome of founder mice. Both specific and nonspecific PCR products will bind SYBR green, but a simple dissociation curve analysis at the end of the PCR run will determine whether the binding is nonspecific. This method measures the relative transgene copy number by comparing it with an endogenous gene with a known copy number (23,32). This method is a quick and effective alternative to the Southern blot, a method that is time consuming, laborious and uses hazardous radioisotopes in a number of cases.

Previously, bioluminescence imaging technology was widely used in the investigation of almost all facets of mammalian biology (33-36). In the present study it was applied to indirectly detect the efficiency of EIIa-Cre induced global recombination. The data revealed that Mcherry biofluorescence in double-positive mice was decreased compared with the control littermate, indicating effective recombination. However, PCR analysis indicated the coexistence of 358-, 314- and 171-bp *Set* fragments in double-transgenic offspring,

suggesting the insufficient efficiency of Cre recombinase that could not completely excise the STOP sequence. Conditional gene targeting based on the inversion or excision of loxP-flanked DNA segments by Cre recombinase is a powerful tool for investigating gene function (33-35). At the same time, the variability of recombination efficiency, transgenic random insertion and copy number, the unexpected expression pattern of Cre transgenes and the potential toxicity of Cre recombinase, as well as the complicated pleiotropic effect also cause serious challenges for the experimenter (37,38) and limit the applications of these technologies in regulating gene expression. In order to exclude the discordance of the target gene with its product, the present study tested Set expression at different DNA, RNA and protein levels.

In summary, a conditional Set of transgenic mice was created that may provide a useful tool to study Set function. To date, a variety of Cre transgenic mouse lines incorporated with tissue specific promoters have been created in order to target specific organs, tissues or cell types. By crossing tissue-specific Cre mice (39) with our Set-Tg mice, the effects of Set in particular tissues would be demonstrated (40). In the future, the focus will be on finding more powerful and stable tissue-specific Cre systems that could effectively activate Set in different tissues. Furthermore, we will also try to investigate human diseases related with changes on tumor marker expression and hormone secretion by regulating Set expression in the Set-EIIa-Tg mice.

Acknowledgements

This study was supported by the China 973 Program (grant nos. 2012CB944703 and 2012CB944902) and the National Natural Science Foundation (grant nos. 81370754 and 31301182).

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