in vivo promoter analysis on refeeding response of hepatic sterol regulatory element-binding protein-1c expression.

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Abstract

Sterol regulatory element-binding protein (SREBP)-1c is the master regulator of lipogenic gene expression in liver.   The mRNA abundance of SREBP-1c is markedly induced when animals are refed after starvation, although the regulatory mechanism is so far unknown.   To investigate the mechanism of refeeding response of SREBP-1c gene expression *in vivo*, we generated a transgenic mouse model that carries 2.2kb promoter region fused to the luciferase reporter gene.   These transgenic mice exhibited refeeding responses of the reporter in liver and adipose tissues with extents essentially identical to those of endogenous SREBP-1c mRNA.   The same results were obtained from experiments using adenovirus-mediated SREBP-1c-promoter-luciferase fusion gene transduction to liver.   These data demonstrate that the regulation of SREBP-1c gene expression is at the transcription level, and that the 2.2kb 5'-flanking region is sufficient for this regulation.   Moreover, when these transgenic or adenovirus-infected mice were placed on insulin-depleted state by streptozotocin treatment, the reporter expression was upregulated as strongly as in control mice, demonstrating that this regulation is not dominated by serum insulin level.   These mice

are the first models to provide the mechanistic insight into the transcriptional regulation

of SREBP-1c gene *in vivo*.

Key words

Introduction

The conversion of carbohydrate into fat is known as *de novo* lipogenesis, which is catalyzed by series of lipogenic enzymes such as acetyl-CoA carboxylase and fatty acid synthase [1]. The activities of these enzymes are nutritionally regulated, *i.e.* downregulated when animals are starved and upregulated when they are refed. This regulation of these lipogenic enzymes has two remarkable features. First, their overall enzymatic activities largely depend on the amount of expressed protein that is primarily controlled at the transcriptional level. Second, their rates of transcription are coordinately regulated [2]. Therefore, it has been postulated that these genes share a regulatory sequence in their promoters that interacts with common trans-acting factors. In the liver, the most likely factor conducting this coordinate transcriptional regulation has been revealed to be sterol regulatory element-binding protein (SREBP) -1 [3, 4].

SREBPs are transcription factors that belong to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic

enzymes [5, 6].   The role of SREBP-1 in the regulation of lipogenesis in the liver has been well established by several lines of evidence, especially from transgenic and knockout mouse models [4, 7-9].   In these models, hepatic SREBP-1 protein levels dominate the mRNA expression levels for a battery of lipogenic genes.   Thus, SREBP-1 is now well established as the key transcription factor for the regulation of lipogenic gene expression and by extension triglyceride storage in liver [10, 11].

It has been well known that the mRNA and nuclear protein abundance of SREBP-1c, the main isoform of SREBP-1 in lipogenic organs such as liver and adipose tissue, is physiologically regulated by nutrient availability, *i.e.* it is downregulated when animals are starved and upregulated when they are refed, thereby adjusting lipogenic gene expression levels to the nutritional conditions [12].   Because the quantity of mRNA and nuclear protein of SREBP-1c goes up and down in parallel depending on dietary condition, the control at the mRNA expression level is considered primary at least in regard to refeeding response.   However, the mechanism of this regulation is yet to be clarified, including the point whether this regulation is at the transcription level or not.

It has been reported that elevated concentrations of glucose and insulin mimic the refeeding state *in vivo* and upregulate SREBP-1 expression in cultured hepatocytes [13, 14]. However, these responses induced by high dosage of glucose and insulin are within two to three-fold changes at best, and far weaker than the refeeding response observed in the *in vivo* liver. Therefore, to elucidate the precise mechanism of SREBP-1 regulation by nutrient availability, it is inevitable for one to study the *in vivo* mouse model.

These situations prompted us to generate transgenic mice that carry SREBP-1c-promoter-driven reporter gene cassette to investigate the nutritional regulation of the promoter activities *in vivo*. Moreover, based on the results from these experiments, we further assessed the promoter activities using adenovirus-mediated gene delivery to the liver, which complemented the data from transgenic mice by excluding the potential possibility of positional effects in transgenic mouse model.

Materials and Methods

Animals and treatment — Seven- to nine-week-old ICR and C57BL/6J male mice were purchased from CLEA (Tokyo, Japan). All animals were housed in a temperature-controlled environment with a 12h-light / dark cycle and free access to standard laboratory diet (MF from Oriental Yeast, Tokyo, Japan, composed of 60% carbohydrate, 13% fat and 27% protein on a caloric basis) and water. The protocol of dietary manipulation was as follows: for the fasting group, animals were starved 24h, and for the refeeding group, they were refed at 16h after a 24h starvation. To make insulin-depleted states, streptozotocin (100 mg/kg body weight, Sigma) was administered by two intraperitoneal injections with 1-day interval following an overnight fasting period as described previously [15]. Streptozotocin was dissolved in 50 mM sodium citrate buffer (pH 4.5) immediately before administration. Mice were considered diabetic when tail vain blood glucose levels exceeded 350 mg/dl, and euthanized within 8 days from the first injection. All the experiments were repeated more than twice and reproducibility was confirmed.

Production of Transgenic Mice — pBP1c2600Luc plasmid was constructed into pGL2

basic vector (Promega) as previously described [16].   The SmaI-SalI fragment of this

plasmid spanning -2.2k to +40 bp of the mouse SREBP-1c promoter fused to luciferase

gene was microinjected into BDF1 eggs.   Among the 55 offsprings, eight mice had the

integrated transgene as determined by Southern blot hybridization of the tail DNA after

digestion with BamHI or NheI.   The cDNA probe for luciferase gene was the 0.54kb

XbaI-EcoRI fragment of pGL2-basic plasmid and the cDNA probe for mouse

SREBP-1c promoter was the 1.1kb NheI fragment of SREBP-1c promoter cut out of the

pBP1c2600Luc plasmid.   Among eight founder mice, two were crossbred to C57BL/6J

background more than 5 times, and thus two lines of –2.2kb-SREBP-1c-promoter-Luc

transgenic mice were established.   The other founder mice were not bred and could not

be analyzed.

Generation of recombinant adenovirus — The SmaI-BglII fragment of

pBP1c2600bp-Luc containing -2.2k to +40 bp of the mouse SREBP-1c promoter was

subcloned into the pGL3 basic vector (Promega) at SmaI and BglII sites.   Then the

SREBP-1c promoter-luciferase gene cassette was cut out of the vector by NotI and SalI,

and after both ends were blunted with klenow DNA polymerase, it was subcloned into

the pENTR4 vector (Invitrogen) at the EcoRI site (blunted).   Then,

-2.2kb-SREBP-1c-promoter-Luc adenoviral plasmid was generated by homologous

recombination between the subcloned pENTR4 plasmid and pAd promoterless vector

using Clonase recombinase (Gateway system, Invitrogen).   Similarly,

SV40-promoter-Luc control adenoviral plasmid was generated from pGL3 promoter

vector (Promega).   After the transfection of the plasmid into 293A cells, recombinant

adenoviruses were collected by CsCl gradient centrifugation according to the

manufacturer's instructions.   Following titer determination by limiting dilution,

adenoviruses were injected intravenously into ICR male mice at the dose of 6.0×10^6

P.F.U./body unless otherwise indicated.

RNA isolation and Northern blotting — Total RNA from liver and subctaneous fat

pad was isolated with Trizol Reagent (Invitrogen), and 7.5µg RNA sample equally

pooled among each group was run on a 1% agarose gel containing formaldehyde and

transferred to a nylon membrane.   cDNA probes were cloned as previously described

[4, 17, 18].   The probes were labeled with [α-^{32}P]dCTP using Megaprime DNA

Labelling System (Amersham Biosciences).   The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65°C and washed in 0.1x SSC buffer with 0.1% SDS at 65°C.   Blots were exposed to imaging plate for BAS2000 BIO IMAGING ANALYZER (Fuji Photo Film, Tokyo, Japan).

Luciferase assays — Tissue samples (app. 50mg) were homogenized with a Polytron in 300μl of Reporter Lysis Buffer (Promega) and centrifuged at 15000 rpm for 15min, and luciferase activity in the supernatant was measured by standard kits (Promega) on a luminometer.   The luciferase activity was expressed as relative light units (RLU) / mg of used sample weight.

Adenoviral DNA isolation and quantification by real-time PCR — The pellets obtained in the process described above were digested in SNET buffer (1% SDS, 400 mM NaCl, 5 mM EDTA and 20 mM Tris-HCl at pH 8.0) containing proteinase K (0.01 mg/ml).   After extraction with phenol / chloroform and RNase treatment, the pellets were dissolved in TE buffer and DNA concentrations were measured.   The amount of adenovirus DNA in liver was determined with Sybr-Green Dye (ABgene) -based real-time PCR, the protocol of which was 50°C for 2 min and 95°C for 10 min,

followed by 60 cycles of 95°C for 15 sec and 60°C for 1 min using 50ng genomic DNA

as a template on an ABI 7000 PCR instrument (Applied Biosystems). The primers

used   for   the   quantification   of   luciferase   gene   were

5'-GTCCGGTTATGTAAACAATCC-3' and 5'-ATGAAGAAGTGTTCGTCTTCG-3'.

Results and Discussion

Generation of transgenic mice carrying SREBP-1c promoter-luciferase fusion gene.

To examine the transcriptional regulation of SREBP-1c gene *in vivo*, we have generated transgenic mice carrying the luciferase reporter gene driven by the 2.2kb SREBP-1c 5'-flanking sequence that we have studied *in vitro* previously [16, 19]. The genomic structure of SREBP-1 gene has been previously clarified in detail [20], and the SREBP-1c promoter is located at 10kb downstream of the SREBP-1a promoter. Because refeeding manipulation selectively upregulates SREBP-1c rather than SREBP-1a [21], the responsible element was expected to be within the SREBP-1c 5'-flanking region. Since expression of reporter genes in transgenic animals might be affected by the location of genomic integration, we analyzed two independent founder lines, designated as line A and B. From Southern blotting analyses of genomic DNA (Fig. 1B), line A and B mice were estimated to harbor 2 and 6 copies of transgene per allele, respectively.

Transcriptional activation of the SREBP-1c promoter-driven transgene by refeeding.

For these lines of transgenic mice, the expression of reporter gene in liver and white adipose tissue was assessed by luciferase activity. As shown in Fig. 2, the luciferase activities in the livers of both lines were markedly upregulated by refeeding manipulation, which paralleled the responses of endogenous SREBP-1c expression. This result means that the SREBP-1c expression is regulated at the transcription level in this situation, rather than through the variable stability of mRNA, which is reported to be involved in the regulation by polyunsaturated fatty acids [22].

The response of reporter gene in adipose tissue was apparently weaker than that of intrinsic gene. This might result from weaker expression of transgene and relatively higher background level in adipose tissue compared to liver.

SREBP-1c induction by refeeding without insulin.

We have previously reported that the refeeding response of SREBP-1c does not require insulin [15]. To examine the role of insulin in this transcriptional upregulation

of the SREBP-1c by refeeding, we placed the transgenic mice on insulin-depleted state

with streptozotocin treatment.   As shown in Fig. 3, increase in plasma insulin by

refeeding was essentially abolished, but the SREBP-1c promoter activities as assessed

by luciferase reporter expression were vigorously induced in the liver accompanied by

elevation in blood glucose levels.   This result indicates that insulin is not inevitable for

the upregulation of SREBP-1c gene transcription by refeeding, consistent with our

previous report [15].

Adenovirus-mediated transduction of SREBP-1c promoter-luciferase fusion gene

into the mouse liver and assessment of refeeding response.

We further took another strategy for *in vivo* assay of SREBP-1c promoter

activity using an adenovirus vector for introduction of the reporter gene into the liver.

We generated an adenovirus harboring the 2.2kb promoter region of SREBP-1c fused to

the luciferase reporter gene, and validated that intravenously administered adenoviruses

are uniformly introduced into the mouse liver as shown in Fig. 4A.   Next we examined

the   refeeding   responses   of   these   adenovirus-infected   mice   expressing

SREBP-1c-promoter-luciferase fusion gene in the liver. At the initial estimation, the absolute luciferase activities observed varied among individual mice in a wide range despite the same number of adenovirus particles for injection. As shown in Fig. 4A, it was indicated that the variation among different portions of one liver was negligibly small, thus most of the variation in measured luciferase activities was considered to originate from inter-mouse variation in viral transduction efficiency. Two approaches were taken to cancel this dispersion: 1. The amount of transducted adenoviral DNA was quantified by real-time PCR technique on each mouse and luciferase activity was adjusted based on this quantification (Fig.4B). 2. Liver samples on fasted and refed states were taken from the same mouse by biopsy under brief anesthesia and data of fasting and refeeding conditions from one mouse are analyzed consecutively (Fig.4C). Both approaches produced essentially the same results, and refeeding responses of twenty to thirty-fold increase in luciferase activities were consistently observed in a dose-independent way, which paralleled the increases in the endogenous SREBP-1c mRNA (Fig. 4E, F). In contrast, when SV40-promoter-Luc adenovirus was used as a negative control instead of -2.2kb-SREBP-1c-promoter-Luc adenovirus, no refeeding

responses were observed (Fig. 4D).

These results are in complete accordance with those from transgenic mouse model described above.   Moreover, the adenoviral approach can avoid the potential involvement of positional effects that might influence transgenic mouse data.   It is intriguing that whether the promoter-reporter fusion gene is inserted in the genome (i.e. in the case of transgenic mouse) or not (i.e. in the case of adenovirus-mediated gene transfer) does not affect the regulation of the SREBP-1c promoter activity.

Evaluation of insulin involvement based on adenovirus-mediated reporter gene transfer technique.

The involvement of insulin was also evaluated using this adenovirus-mediated introduction of SREBP-1c-promoter-luciferase fusion gene into the liver.   As shown in Fig. 4G, streptozotocin-treated diabetic mice exhibited strong refeeding responses of luciferase reporter activities in a similar manner to the responses of intrinsic SREBP-1c mRNA, fully consistent with the data from transgenic mice.

Regarding the role of insulin in the upregulation of lipogenesis, many previous

reports indicate that insulin increases the expression of SREBP-1c and thereby upregulates lipogenesis [23-31]. However, our present methods clearly support that insulin signaling pathway does not directly control SREBP-1c expression. This suggests that the insulin response *in vitro* might not be identical to the refeeding response *in vivo*, thus previous knowledge on *in vitro* promoter analyses of SREBP-1c regulation by insulin might not fully hold true of *in vivo* regulation. For better understanding of the *in vivo* physiology, further studies of *in vivo* promoter analyses based on our reporter gene transfer technique utilizing adenovirus vector are currently ongoing.

In summary, we generated two *in vivo* systems to estimate the SREBP-1c promoter activity. It was demonstrated that the fasting-refeeding regulation of SREBP-1c expression in the liver is exerted at the transcription level, and that 2.2kb of the 5'-flanking sequence is sufficient for this regulation. Our adenoviral strategy provides a feasible approach to identify the promoter regions responsible for refeeding response of SREBP-1c expression, which is currently under going.

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Figure legends

Fig. 1 Transgene construct and Southern blot. A. Construct used to generate

-2.2kb-SREBP-1c-promoter-Luc transgenic mice. The mouse SREBP-1c promoter

region spanning -2.2k to +40bp was fused to a luciferase reporter gene and SV40 late

polyadenylation sequence. **B**. Southern blotting of genomic DNA from transgenic

mice. Genomic DNA prepared from tail samples of wild-type (WT) controls and two

lines (line A and B) of transgenic mice were analyzed by Southern blotting using two

distinct probes. For probe 1 (shown in upper panel), a cDNA probe for luciferase gene

was used. DNA samples were digested with BamHI. For probe 2 (shown in lower

panel), a cDNA probe for mouse SREBP-1c promoter region was used to determine the

copy number of transgene. DNA samples were digested with NheI.

Fig. 2 Refeeding response of transgenic mice. A. Transgene expression as

assessed by luciferase activities of liver and white adipose tissue (WAT) from two lines

of transgenic mice. Transgenic mice were either fasted for 24 h or refed for 16 h after

a 24 h fast before euthanized.   Each group consists of 4 to 7 male mice.   Results are

mean ± SE.   * and ** denote significance at P<0.05 and P<0.01, respectively.   **B**.

Intrinsic gene expression as assessed by Northern blot analyses of wild type (WT)

control and transgenic (Tg) mice.   Total RNA (7.5 μg) from livers and white adipose

tissues pooled equally among each group was subjected to Northern blotting analysis to

determine SREBP-1 and 36B4 (used as a loading control) mRNA levels.   The

quantification results were obtained with BAS2000 system and normalized to the signal

generated from 36B4 mRNA, and the fold-changes between refed *vs.* fasted states are

shown.   Wild type and transgenic mice were littermates.

Fig. 3   Refeeding response of streptozotocin-treated transgenic mice.   A.

Transgene expression in streptozotocin-treated mouse liver as assessed by luciferase

activity.   At two days after second administration of streptozotocin, transgenic mice

were either fasted for 24 h or refed at 16 h after a 24 h starvation, and euthanized to

obtain liver samples.   Luciferase activities of liver samples were determined as

indicated in material and methods.   Results are mean ± SE of 4 to 6 male mice.   * and
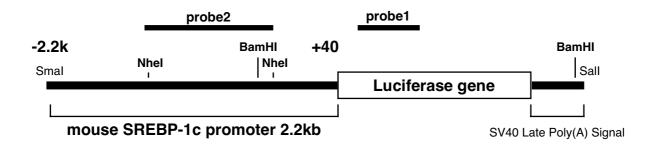
** denote significance at P<0.05 and P<0.01, respectively. **B**. Northern blot analyses visualizing endogenous SREBP-1 mRNA expression pattern in streptozotocin-treated mouse liver. Total RNA (7.5 μg) from livers pooled equally among each group was subjected to Northern blotting to determine SREBP-1 and 36B4 (used as a loading control) mRNA levels. The quantification results were obtained with BAS2000 system and normalized to the signal generated from 36B4 mRNA, and the fold-changes between refed *vs.* fasted states are shown. **C**. Blood insulin and glucose levels of streptozotocin-treated transgenic mice are shown.

Fig. 4   Adenovirus-mediated transduction of -2.2kb-SREBP-1c-promoter-Luc into liver and assessment of refeeding response. **A**. Validation of homogenous transfer of reporter gene into various portions of liver. Variation within a mouse liver was estimated by sampling 5 pieces of liver from distinct lobes after three days of -2.2kb-SREBP-1c-promoter-Luc adenovirus injection (6.0 x 10^6 P.F.U./body). Adenoviruses were intravenously injected into ICR male mouse. **B**. Estimation of refeeding response of luciferase activity adjusted by real-time PCR. Three days after

the administration of -2.2kb-SREBP-1c-promoter-Luc adenovirus, luciferase activities

were measured and at the same time, the amount of transducted adenoviral DNA was

quantified by real-time PCR technique on each mouse of fasting and refeeding group

and luciferase activities per transducted adenoviral DNA were calculated based on this

quantification.　50 ng genomic DNA was used as templates for real-time PCR.

Results are mean ± SE of 4 to 5 mice.　** denotes significance at $P<0.01$.　**C.**

Pairwise　estimation　of　refeeding　response　using　biopsy　method.

-2.2kb-SREBP-1c-promoter-Luc adenovirus-administered mice were fasted for 24 h and

then liver samples were taken by biopsy under inhalation anesthesia.　After refed for

16 h, mice were euthanized to obtain refed liver samples.　Luciferase activities were

determined and fold changes of luciferase activities were calculated by dividing data on

a refed state by those of the same mouse on a fasted state.　Results are mean ± SE of 5

mice.　** denotes significance at $P<0.01$.　**D**. Negative control for data shown in C.

SV40-promoter-Luc　control　adenovirus　was　used　instead　of

-2.2kb-SREBP-1c-promoter-Luc adenovirus.　Results are mean ± SE of 4 mice.　**E.**

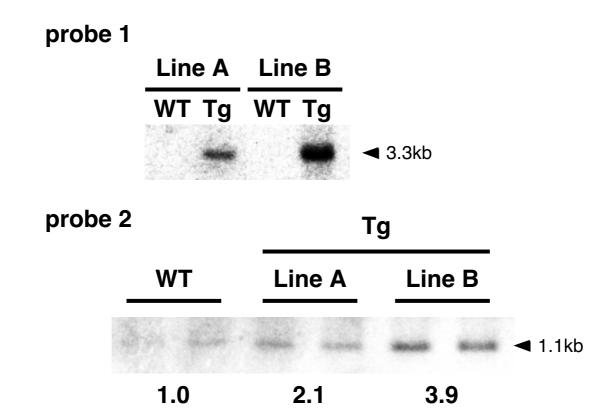Dose　independence　of　refeeding　response.　　Indicated　amounts　of

-2.2kb-SREBP-1c-promoter-Luc adenoviruses were intravenously injected into ICR male mice, and refeeding responses were assessed by pairwise method. F, fasted; R, refed. Results are mean ± SE of 3 mice. **F**. Endogenous SREBP-1 expression is not affected by adenovirus administration. Total RNA (7.5 μg) from livers pooled equally from each group (n=3) was subjected to Northern blotting analysis to determine SREBP-1 and 36B4 (used as a loading control) mRNA levels. **G**. Refeeding response of luciferase reporter activity in adenovirus-infected and streptozotocin-treated mouse liver. At two days after second administration of streptozotocin, -2.2kb-SREBP-1c-promoter-Luc adenovirus (6.0 x 10^6 P.F.U./body) was intravenously injected into ICR male mice. Three days thereafter, mice were fasted for 24 h and then liver biopsies were performed under inhalation anesthesia. After refed for 16 h, mice were euthanized to obtain liver sample. Luciferase activities were measured and refeeding responses were determined pairwise. Results are mean ± SE of 4 to 5 mice. ** denotes significance at $P<0.01$. **H**. Refeeding responses of endogenous SREBP-1 mRNA expression in streptozotocin-treated mice. Total RNA (7.5 μg) from livers pooled equally among each group was subjected to Northern blotting analysis to

determine SREBP-1 and 36B4 (used as a loading control) mRNA levels. **I**. Blood

insulin and glucose levels of the control and streptozotocin-administered mice are
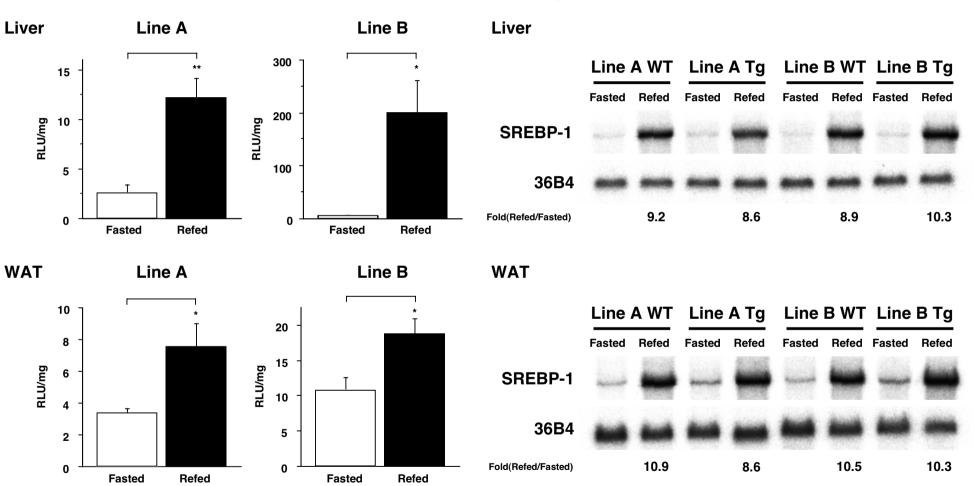
shown.

Figure 1

Fig. 1

A. Transgene construct

B. Southern blot

probe 1

probe 2

Fig. 2

A. Luciferase activities

Liver

Line A

Line B

WAT

Line A

Line B

B. Intrinsic gene expression

Liver

Line A WT		Line A Tg		Line B WT		Line B Tg	
Fasted	Refed	Fasted	Refed	Fasted	Refed	Fasted	Refed

SREBP-1

36B4

Fold(Refed/Fasted) 9.2 8.6 8.9 10.3

WAT

Line A WT		Line A Tg		Line B WT		Line B Tg	
Fasted	Refed	Fasted	Refed	Fasted	Refed	Fasted	Refed

SREBP-1

36B4

Fold(Refed/Fasted) 10.9 8.6 10.5 10.3

Fig. 3

A. Luciferase activities

Liver

B. Intrinsic gene expression

Liver

C. Blood parameters

Fig. 4

A

B

C

D

E

x10⁶ P.F.U./body

F

x10⁶ P.F.U./body

SREBP-1

36B4

Fold(Refed/Fasted)   9.4   6.7   7.9   7.8   7.2   7.3

G

H

SREBP-1

36B4

Fold(Refed/Fasted)   6.2   2.8

I

Insulin

Glucose