

—Original—

# RNAi-mediated knockdown of mouse melanocortin-4 receptor *in vitro* and *in vivo*, using an siRNA expression construct based on the mir-187 precursor

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**Abstract:** RNA interference (RNAi) is a powerful tool for the study of gene function in mammalian systems, including transgenic mice. Here, we report a gene knockdown system based on the human mir-187 precursor. We introduced small interfering RNA (siRNA) sequences against the mouse melanocortin-4 receptor (*mMc4r*) to alter the targeting of miR-187. The siRNA-expressing cassette was placed under the control of the cytomegalovirus (CMV) early enhancer/chicken  $\beta$ -actin promoter. *In vitro*, the construct efficiently knocked down the gene expression of a co-transfected *mMc4r*-expression vector in cultured mammalian cells. Using this construct, we generated a transgenic mouse line which exhibited partial but significant knockdown of *mMc4r* mRNA in various brain regions. Northern blot analysis detected transgenic expression of *mMc4r* siRNA in these regions. Furthermore, the transgenic mice fed a normal diet ate 9% more and were 30% heavier than wild-type sibs. They also developed hyperinsulinemia and fatty liver as do *mMc4r* knockout mice. We determined that this siRNA expression construct based on mir-187 is a practical and useful tool for gene functional studies *in vitro* as well as *in vivo*.

**Key words:** mir-187, mMC4R, RNAi, Transgenic mice

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Supplementary Figure: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/expanim>

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## Introduction

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RNA interference (RNAi) is a sequence-specific gene-silencing mechanism widely conserved among eukaryotic cells [19]. RNAi is triggered in mammalian cells by small interfering RNAs (siRNAs) that are 21 nucleotides (nt) long and contain 2 nt 3' overhangs.

Animal cells contain numerous endogenous ~22 nt RNAs known as microRNAs (miRNAs) [16] that can cause mRNA degradation and/or translational inhibition when bound to partially complementary sites in the 3' untranslated region (3'-UTR) of mRNAs [32, 34]. Natural miRNAs are transcribed by RNA polymerase II (pol II) as long, primary, 5'-capped, and polyadenylated miRNAs (pri-miRNAs) [3, 17]. The pri-miRNA is cropped first to release a 70–90 nt hairpin-shaped precursor (pre-miRNA) by Drosha, a member of the ribonuclease III (RNase III) protein family, and its cofactor, DGCR8, in the context of the microprocessor complex [6]. The pre-miRNA is recognized by the exportin-5-RanGTP heterodimer, and the pre-miRNA is exported to the cytoplasm [18, 33]. In the cytoplasm, pre-miRNA is cut by the RNase III Dicer to generate a ~22 nt miRNA duplex [12, 14]. One strand of the Dicer product remains as a mature miRNA and is assembled into an effector complex known as the miRNA-induced silencing complex (miRISC), which has a gene-silencing effect [15, 26]. The RNA pol II-driven short hairpin RNA (shRNA) expression system was constructed by replacing the miRNA with siRNA.

RNAi has been used for the functional knockdown of specific proteins in several experimental systems, from cultured cells [7] to whole organisms, including mammals [25]. The RNAi technology represents an interesting tool in reverse genetic studies for several reasons. First, RNAi usually results in partial inhibition of gene expression and thus allows the generation of hypomorphic phenotypes, which are usually impossible to generate by complete knockout via gene targeting [9] or gene editing technologies [31]. Second, the expression of miRNA can be controlled using a large repertoire of RNA pol II-dependent promoters [35], including temporally and spatially specific and inducible promoters [20]. Consequently, the RNA pol II-mediated RNAi strategy can be a powerful alternative to conventional gene knockout technology.

miR-187, an miRNA that is highly expressed in neurons, has been intensively studied, and is considered a

potential biomarker in the early diagnosis of a wide range of human cancers [8]. We chose to engineer mir-187 because *DraIII* and *PshAI* recognition sites in its precursor sequence allow for the easy replacement of its siRNA.

To confirm the knockdown efficacy of an endogenous gene *in vivo*, we selected the mouse melanocortin-4 receptor (*mMc4r*) gene as the target. The mMC4R is a seven-transmembrane G protein-coupled receptor that is predominantly expressed in the hypothalamic nuclei, and is implicated in the regulation of food intake and body weight [2]. Homozygous *mMc4r* knockout mice are known to express an obese phenotype caused by excess food intake. On the other hand, heterozygous *mMc4r* knockout mice express an intermediate phenotype, indicating that obesity is tightly coupled to gene dosage [11]. Thus the reduction of *mMc4r* expression in knockdown mice can be conveniently followed by monitoring body weight.

To test the utility of an mir-187-based shRNA construct driven by an RNA pol II promoter, we used a cytomegalovirus (CMV) early enhancer/chicken  $\beta$ -actin promoter (CAG)-driven expression vector with the potential for expression in a range of tissues and cells [23].

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## Materials and Methods

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### Plasmids

An siRNA-expressing construct was generated as follows. The human miR-187 expression region, containing the mir-187 stem-loop region and 5' and 3' flanking regions (271 bp in total), was PCR-amplified from HeLa genomic DNA using the primers 5'-CGCGGATCCATC-GGGATGCACAGCAAGT-3' and 5'-GCTC-TAGACCCACCAGAGCCTGGACTTTC-3', digested with *BamHI/XbaI* and ligated into the same restriction sites in the pCAG expression vector (pCAG-miR-187). Next, an EF1- $\alpha$ /EGFP expression cassette and an SV40/blastocidin expression cassette were introduced to visualize the efficacy of transfection and to establish stable cell lines (pGKD-miR-187). Hairpin sequences specific to the targeted mRNAs were inserted into the *DraIII/PshAI* site of either the pCAG-miR-187 or pGKD-miR-187 vector (pCAG-miR-mMC4R, pCAG-miR-Luc, and pGKD-miR-mMC4R). The inserted oligonucleotide sequences were as follows: mMC4R 5'-GTGACCCCTCCAGGATGCTATGAGCAACTTTTTTGTGAAGCCACAGATG-GAAAAAAGTTGCTCATAGCATCCTGAGGGAC-



cDNAs were produced using SuperscriptII Reverse Transcriptase (Invitrogen). The relative expression of *mMc4r* was estimated by qPCR using SYBR Premix Ex TaqII reagents (TaKaRa; Kyoto, Japan) under standard reaction conditions. The primer sequences were as follows: mouse MC4R-F, 5'-CGCCAGGGTACCAACATGAAG-3'; mouse MC4R-R, 5'-ACATGAAGCACACGCAGTATGG-3'; mouse  $\beta$ -actin-F, 5'-GGCCAACCGT-GAAAAGATGA-3'; mouse  $\beta$ -actin-R, CACAGCCTG-GATGGCTACGTA-3'. Primers for human *GAPDH* were purchased from TaKaRa.

#### Northern blotting for small RNA

In brief, 15% polyacrylamide-8M urea gels were used to isolate the low-molecular-weight RNA-enriched fraction. Band sizes were estimated by comparing them to a 10 bp DNA Marker (NEB; Ipswich, MA). Gels were transferred to Hybond-N+ membranes (GE Healthcare; Uppsala, Sweden) by capillary transfer. After cross-linking, membranes were placed in DIG-easy Hyb (Roche; Indianapolis, IN). DIG-labeled riboprobes were synthesized using an mirVana miRNA probe construction kit (Ambion) using the following DNA oligonucleotide as a template: miR-mMC4R sense probe (for guide strand siRNA detection), 5'-AAAAAGTTGCTCATAG-CATCCCCTGTCTC-3'. After hybridization at room temperature overnight, membranes were washed twice at 37°C for 15 min in  $0.1 \times$  SSC, 0.1% SDS. Probe detection was performed using a DIG Luminescent Detection Kit (Roche) according to the manufacturer's protocol.

Stripping of the riboprobe was performed at 68°C for 30 min in 0.1% SDS, followed by washing in  $2 \times$  SSC at room temperature. Then membranes were reprobbed with miR-16 riboprobe (Ambion) in the same manner as described above.

#### Measurement of body weight and food consumption

Body weight was regularly measured, beginning at 3–4 weeks of age. Food intake was measured for male transgenic and non-transgenic mice at 37 to 41 weeks of age. A sufficient amount of food for a 9-day period was weighed and provided to the mice *ad libitum*. Each weekday morning, the remaining food was weighed, for a total of six measurements.

#### Determination of metabolic parameters

Fasting male transgenic and non-transgenic mice at 41 weeks of age were sacrificed under anesthesia, and

blood, liver, pancreas, and mesenteric fat were collected and weighed. Plasma glucose concentrations were determined using a Glucose-CII Test Kit (Wako Pure Chemical Industries, Osaka, Japan). Insulin concentrations were measured with an enzyme-linked immunosorbent assay (Morinaga Institute of Biologic Science, Yokohama, Japan). Triglyceride levels were measured using a Triglyceride E-test Kit (Wako). Plasma free fatty acid levels were measured using an NEFA C-test Kit (Wako). Plasma leptin levels were measured using Mouse Leptin ELISA kits (Morinaga).

#### Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student's *t*-tests.

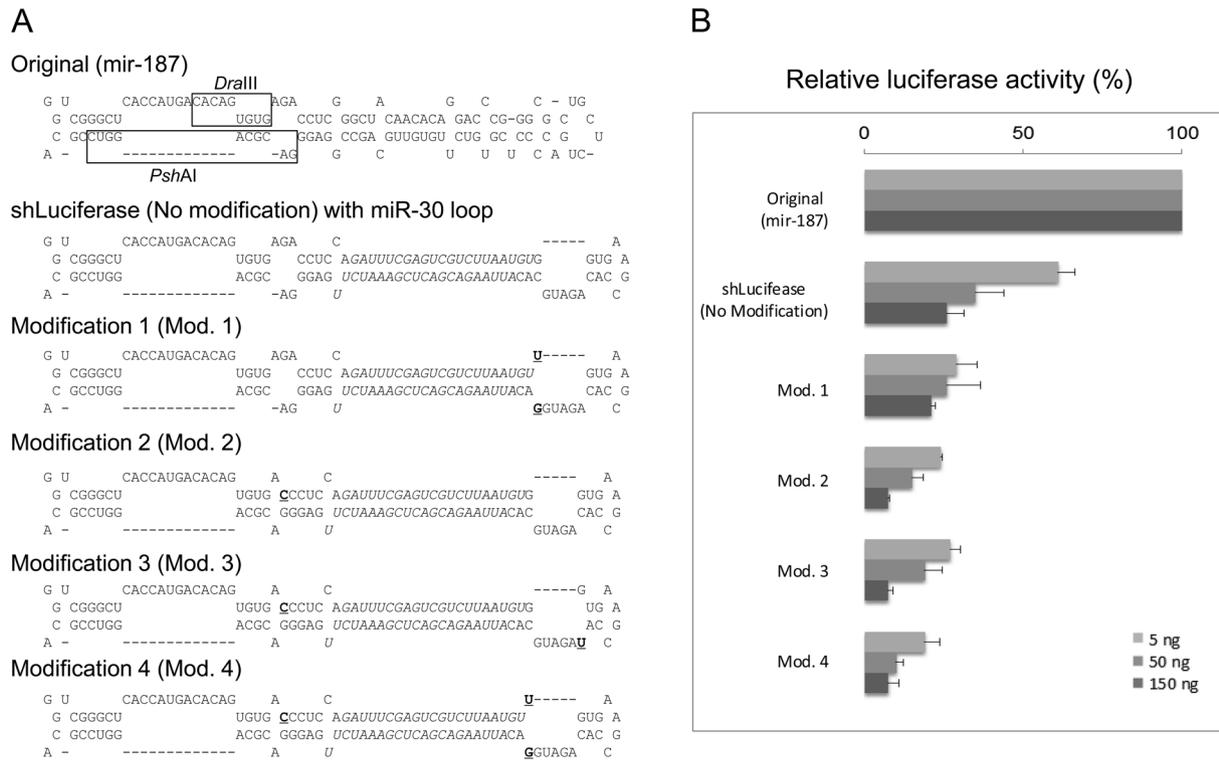
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## Results

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#### Assembly of an siRNA-expression construct based on *mir-187* precursor sequences

We constructed an siRNA-expressing cassette containing *mir-187* precursor sequences with *mir-30* loop sequences (Fig. 1A). Minor modifications to the flanking and loop sequences were introduced to obtain the highest knockdown efficacy by using Dual-Luciferase Reporter Assay system (Fig. 2). In the original shLuciferase cassette with no modification, the G:C pair at the neck of the *mir-30* loop was changed to UG wobble pair to open easily in modified siRNA-expressing cassette, Modification (Mod) 1, and 4. As shown in our siRNA design algorithm (22, 29), the 5' terminal residue of functional siRNA guide strand is preferable to be A or U, and Dicer cleavage site is considered to be at two nucleotides inside from the double strand terminal. Thus, the relative luciferase activity is evidently reduced in Mod.1 cassette compared to the original one, probably because the guide strand is correctly cleaved at A or U at two nucleotides inside from the double strand terminal by this nucleotide modification. Furthermore, AG residues at the two nucleotides downstream of *DraIII* site in the original *mir-187* sequence were changed to C residue in Mod.2, 3, and 4. This region is the binding site of DGCR8, a partner of Droscha, which is a double-stranded RNA binding protein. The accessibility of DGCR8 may increase, since the double stranded region is enlarged by this modification. Indeed, the luciferase activity was also reduced compared to the original one. Then, the expression cassette was inserted into the pCAG



**Fig. 2.** Modification of mir-187 precursor and mir-30 loop sequences for optimizing the knockdown efficacy. A: The modified nucleotide (s) in original mir-187 sequence was shown in underlined bold character, and siRNA sequence against firefly luciferase is shown in italic. The loop region of mir-30 was essentially used except for Mod.3. B: The knockdown efficacy was measured by Dual-Luciferase Reporter Assay System (Promega) by co-transfection of each of RNA polymerase II-driven miR-187-based siRNA expression construct (5, 50, 150 ng) with target firefly luciferase-expressing pGL3-Control vector (1.0  $\mu$ g) and *Renilla* luciferase-expressing pRL-SV40 vector (10 ng) using human HeLa cells. Luciferase activities were measured in two to four wells, respectively. Each error bar represents the SD.

expression vector (pCAG-miR-187, Fig. 1B). Subsequently, an EF1- $\alpha$ /EGFP expression cassette was inserted to visualize the efficiency of transfection *in vitro*. Furthermore, an SV40/bleomycin gene was inserted for the establishment of stable cell lines. Consequently, the RNAi vector could be utilized for both *in vitro* and *in vivo* studies (pGKD-miR-187, Fig. 1C). The shRNA sequences against *mMc4r* or control firefly luciferase (*Luc*) genes were designed using an algorithm reported previously [22, 29]. The annealed oligonucleotides containing the miR-30 loop sequence were inserted into the pCAG-miR-187 or pGKD-miR-187 vector in the site created by a double digest with *PshAI* and *DraIII* (pCAG-miR-mMC4R, pCAG-miR-Luc, and pGKD-miR-mMC4R).

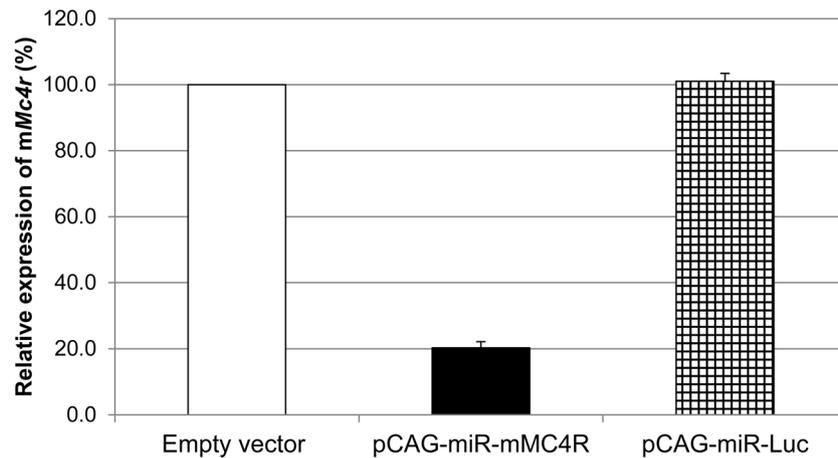
#### Knockdown efficacy of pCAG-miR-mMC4R in cultured cells

We estimated the knockdown efficacy of the pCAG-miR-mMC4R construct against transiently expressed

*mMc4r* in HEK293 cells. The pcDNA3.1/mMC4R expression construct was transiently co-transfected into HEK293 cells with an empty pCAG vector without the miR-187 cassette, the pCAG-miR-mMC4R construct, or the pCAG-miR-Luc construct. One day after transfection, qPCR analyses were performed to measure *mMc4r* and human  $\beta$ -actin expression. Co-transfection of the pCAG-miR-mMC4R construct reduced the expression of *mMc4r* mRNA by 80%, while co-transfection with pCAG-miR-Luc had no effect (Fig. 3). These results indicate that the pCAG-miR-187 expression cassette effectively induced RNAi and specifically repressed target gene expression *in vitro*.

#### Generation of GKD-miR-mMC4R transgenic mice

We generated four transgenic founder mice carrying the GKD-miR-mMC4R gene (Suppl. Fig. 1A), and investigated whether the miR-mMC4R construct could knock down endogenous expression of *mMc4r* *in vivo*.

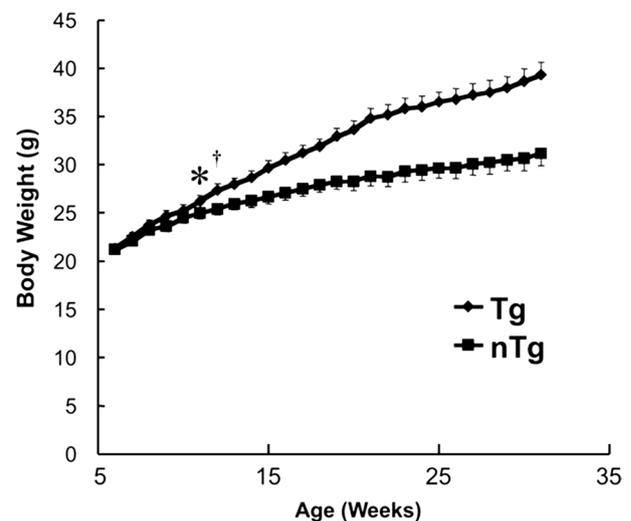


**Fig. 3.** Knockdown efficacy of pCAG-miR-mMC4R in HEK293 cells. HEK293 cells were co-transfected with pcDNA3.1-mMC4R and either pCAG-miR-187 empty vector, pCAG-miR-mMC4R, or pCAG-miR-Luc. One day after transfection, total RNA was extracted and qPCR analyses for mouse *Mc4r* and human *GAPDH* were performed. The mean of three independent assays were shown. Each error bar represents the SE.

Of these transgenic mice, weight gain was observed in only one transgenic male (10L, Suppl. Fig. 1B). Correspondingly, the highest expression among the founder mice of the miR-mMc4r precursor RNA was detected in the hypothalamus of mouse 10L (Suppl. Fig. 2). From these results, we concluded that the obese phenotype observed in 10L can be explained by the greater expression of the miR-mMc4r precursor RNA in the hypothalamus (Suppl. Fig. 2). The body weight of the transgenic mouse line (10L) was further compared to those of wild-type sibs from 6 to 31 weeks of age. A significant increase in the body weight of the transgenic mice started at 11 weeks, and transgenic mice were 30% heavier than non-transgenic littermates at 31 weeks (Fig. 4).

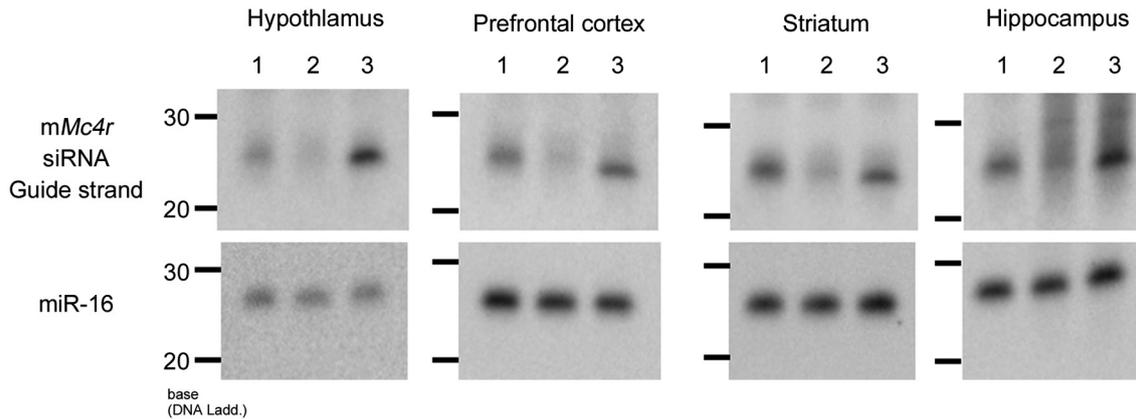
#### RNA expression in GKD-miR-mMC4R transgenic mice

Small RNAs were purified from the hypothalamus, striatum, hippocampus, and pre-frontal cortex regions of the brains of both the GKD-miR-mMC4R transgenic mice (10L) and wild-type sibs, and were examined by Northern blot analysis. Although a faint background signal was also detected in the brain of wild type mouse, a sense probe detected the strong signals of the guide strand of mMc4r siRNA in all of the brain regions extracted from the transgenic mice (Fig. 5). In addition, the mMc4r transcripts in these four brain regions of the transgenic mice were compared to those in the wild-type sibs using qPCR. A significant reduction in the expres-



**Fig. 4.** Weight gain of GKD-miR-mMC4R transgenic mice. The symbols indicate the first week that body weight differed significantly between transgenic (Tg; n=9) and non-transgenic (nTg; n=15) mice (\*;  $P < 0.05$ , †;  $P < 0.01$ ). Error bars indicate SE.

sion of mMc4r mRNA (20–30%) was obtained in all four brain regions of the transgenic mouse line (Table 1). This partial decrement of mMc4r mRNA in the brains of the transgenic mice strongly indicated that the siRNA produced from the GKD-miR-mMC4R transgene attenuated the expression of mMc4r mRNA *in vivo*.



**Fig. 5.** Small RNA analyses of GKD miR-mMC4R mice. Northern blots for guide strand of mMC4R siRNA and miR-16 in various brain regions of miR-mMC4R transgenic mice. Either 6  $\mu$ g (prefrontal cortex, striatum, or hippocampus) or 2  $\mu$ g (hypothalamus) of the small RNA fraction was separated on 15% polyacrylamide-urea gels. 1: miR-mMC4R transgenic, 2: wild-type, 3: wild-type + 1 ng synthetic siRNA for *mMc4r*.

**Table 1.** qPCR analyses of GKD-miR-mMC4R transgenic mice

	Relative expression of <i>mMc4r</i> (mouse <i>Mc4r</i> / mouse $\beta$ -actin)	
	non-Tg (n=9)	Tg (n=15)
Hypothalamus	100 $\pm$ 2.47	80.6 $\pm$ 4.34 **
Prefrontal cortex	100 $\pm$ 4.21	80.2 $\pm$ 8.30 *
Striatum	100 $\pm$ 3.51	70.1 $\pm$ 5.32 **
Hippocampus	100 $\pm$ 3.73	73.9 $\pm$ 5.53 **

Mean  $\pm$  SE, \*,  $P < 0.05$ , \*\*,  $P < 0.01$  (*t*-test).

#### Food consumption and metabolic phenotypes of GKD-miR-mMC4R transgenic mice

Next, we examined the hyperphagic phenotype in male litters of transgenic mice. The total food consumption of 37- to 41-week-old transgenic mice was determined over 9 days. The knockdown of *mMc4r* resulted in a significant increase (9%) in normal diet consumption over that of non-transgenic littermates (Table 2). To further understand the obese phenotype of transgenic mice, we performed a number of assays of parameters related to obesity: oral glucose tolerance tests (OGTT); measurements of glucose, triglyceride (TG), insulin, free fatty acid (FFA), total cholesterol, and leptin in serum; measurement of tissue weight for mesenteric fat, pancreas, and liver; and measurement of leptin and TG levels in the latter two tissues, respectively. There were no significant differences in glucose, TG, FFA, or total cholesterol levels between fasting transgenic and non-transgenic mice. However, insulin and leptin were significantly elevated in transgenic mice, by 6-fold and 2.5-fold, respectively.

In transgenic mice, the tissue weights of pancreas, liver, and mesenteric fat were significantly increased. The content of insulin in pancreas showed a 3.5-fold increase in transgenic mice. Furthermore, liver TG levels were also significantly elevated in transgenic mice (Table 2). The  $AUC_{0-2h}$  of the glucose response during OGTT tended to increase in transgenic mice compared to non-transgenic mice but was not significantly different (Suppl. Fig. 3).

From these observations, we concluded that GKD-miR-mMC4R transgenic mice had a phenotype of hyperinsulinemia, fatty liver, functional disorder of the pancreas, and the accumulation of mesenteric fat.

In conclusion, we have determined that a mir-187-based expression construct is practically useful for both *in vitro* and *in vivo* RNAi. The expression cassette introduced into mice could express functional siRNAs against target genes in tissues. While transgenic miR-mMC4R mice had the obese phenotype previously seen in knockout mice, this has revealed that such a phenotype may be the result of as little as a 20–30% partial knockdown of *mMc4r* expression *in vivo*.

## Discussion

We have described the development of a new siRNA-expressing cassette, and applied it in RNAi *in vitro* and *in vivo*. The method depends on the expression of specific siRNAs within mir-187 precursor sequences. Because mir-187 has recognition sites for the restriction enzymes *Dra*III and *Psh*AI in its flanking regions, syn-

**Table 2.** Food consumption and metabolic phenotypes of GKD-miR-mMC4R transgenic mice

	non-Tg (n=9)	Tg (n=15)	
Food consumption			
Total food intake for 9 days (g)	35.6 ± 3.4	38.4 ± 3.3	*
Serum			
Glucose (mg/dl)	85.12 ± 3.20	89.12 ± 3.76	NS
Triglyceride (mg/dl)	71.8 ± 1.8	73.6 ± 3.6	NS
Insulin (ng/ml)	3.2 ± 0.3	20.0 ± 6.7	*
Free fatty acid (uEq/l)	1,369.3 ± 44.4	1,483.4 ± 98.0	NS
Total cholesterol (mg/dl)	181.9 ± 10.8	199.4 ± 11.6	NS
Leptin (ng/ml)	19.8 ± 2.9	50.8 ± 15.7	*
Pancreas			
Insulin (ng/ml/g tissue)	58,387.2 ± 3,246.2	203,192.5 ± 28,998.7	**
Tissue weight (g)	0.372 ± 0.013	0.399 ± 0.016	*
Liver			
Triglyceride (mg/g tissue)	28.3 ± 1.3	38.1 ± 2.0	**
Tissue weight (g)	1.387 ± 0.060	2.258 ± 0.174	**
Mesenteric Fat			
Tissue weight (g)	0.990 ± 0.067	1.653 ± 0.070	**

Mean ± SE, \*,  $P < 0.05$ , \*\*,  $P < 0.01$  ( $t$ -test).

thetic hairpin DNA sequences can be directly inserted into the vectors (Fig. 1A). This enables high-throughput construction of siRNA-expressing libraries.

A previous study showed that the body weights of homozygous *mMc4r* mutant mice were 50% heavier than those of wild-type mice at 15 weeks of age, and heterozygous mice showed an intermediate weight gain of approximately 25% [11]. In the present study, miR-mMC4R transgenic mice, whose mRNA level of *mMc4r* decreased by 20~30% (Table 1), body weights were 15% heavier than those of wild-type littermates at 15 weeks of age (Fig. 4). Furthermore, in the abovementioned previous study, food consumption of homozygous *mMc4r* knockout mice increased by 40% over that of wild-type mice [11], whereas the rate of increase in miR-mMC4R transgenic mice in our study was 9% (Table 2). We conclude that the degree of *mMc4r*-downregulation and the phenotypes of obesity and hyperphagia are directly proportional.

Although the miR-mMC4R expression construct mediates effective (around 80%) knockdown in cell culture (Fig. 3), the knockdown efficiency in transgenic mice was limited to about 20~30% (Table 1). One potential reason for this discrepancy is that the expression level of transgenic siRNA against *mMc4r* was not enough to achieve effective knockdown *in vivo*. In Northern blot analysis, approximately 1 ng siRNA was detected in 6  $\mu$ g small RNA extracted from striatum (Fig. 5). The 6

$\mu$ g small RNA was obtained from about 80 mg striatal tissue (data not shown). The concentration of 1 ng siRNA / 80 mg tissue is comparable with ~1 nM siRNA. There are two main reasons that explain the low-level expression of transgenic siRNA. One possibility is that a large amount of shRNA expressed from the CAG promoter could saturate the activity of exportin-5, a rate-limiting factor required for nuclear export of pre-miRNAs [5]. Such high shRNA expression may inhibit endogenous miRNA functions required for normal embryogenesis and development [30]. Consequently, only transgenic mice with low shRNA expression would survive to birth. In fact, after microinjection of the CAG-EGFP transgene, bright EGFP expression in embryos can be observed at early developmental stages, from the 2-cell stage to blastocysts [13]. To overcome this possible problem, conditional expression constructs, with inducible or stage-specific RNA pol II promoters, may be valuable. Another possible reason for the low-level expression of transgenic siRNA is that the silencing of transgene by the vector sequences or by the random and multi-copy integration [4, 24, 28]. Especially, the vector used in this study (Fig. 1C) contained prokaryote-derived elements from pBluescript, therefore, it's plausible to remove such bacterial sequences before injection into mice eggs.

Although the obese phenotypes of miR-mMC4R transgenic mice were similar to those of *mMc4r* knockout mice in terms of hyperinsulinemia [1] and fatty liver

[27], the serum levels of glucose were normal in miR-mMC4R transgenic mice (Table 2). In previous studies, pre-obese *mMc4r* homozygous knockout mice did not display hyperglycemia and hyperinsulinemia, although they were already significantly heavier than heterozygous knockout and wild-type mice [1]. *mMc4r* knockout mice develop both hyperglycemia and hyperinsulinemia [11], whereas the miR-mMC4R transgenic mice in our study exhibited only hyperinsulinemia (Table 2). Re-expression of *mMc4r* specifically in the lateral hypothalamic area (LHA) improves glucose metabolism in obese *mMc4r* knockout mice, without affecting body weight or circulating insulin levels [21]. It is possible that a partial knockdown of *mMc4r* (20% to 30%) did not affect LHA signaling in the regulation of glucose homeostasis. The partial knockdown by the mir-187 expression construct *in vivo* may be a powerful tool to investigate the relationship between the level of gene expression and the phenotype of interest.

In conclusion, we developed a new siRNA-expression construct based on the mir-187 precursor. Transgenic mice with partial knockdowns offer valuable functional information. Such mir-187 precursor-based constructs will provide new tools for validating drug target genes *in vitro* and *in vivo*, and for generating hypomorphic animal models.

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