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Targeted disruption of *fad24*, a regulator of adipogenesis, causes pre-implantation embryonic lethality due to the growth defect at the blastocyst stage

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ABSTRACT

In previous studies, we identified a novel gene, *factor for adipocyte differentiation 24 (fad24)*, which plays an important role during the early stages of adipogenesis in mouse 3T3-L1 cells. Moreover, overexpression of *fad24* increased the number of smaller adipocytes in white adipose tissue and improved glucose metabolic activity in mice, thus indicating that *fad24* functions as a regulator of adipogenesis *in vivo*. However, the physiological roles of *fad24 in vivo* are largely unknown. In this study, we attempted to generate *fad24*-deficient mice by gene targeting. No *fad24*-null mutants were recovered after embryonic day 9.5 (E9.5). Although *fad24*-null embryos were detected in an expected Mendelian ratio of genotypes at E3.5, none of the homozygous mutants developed into blastocysts. *In vitro* culture experiments revealed that *fad24*-null embryos develop normally to the morula stage but acquire growth defects during subsequent stages. The number of nuclei decreased in *fad24*-deficient morulae compared with that in wild-type ones. These results strongly suggested that *fad24* is essential for pre-implantation in embryonic development, particularly for the progression to the blastocyst stage.

1. Introduction

Obesity is a major risk factor for a variety of diseases, including type 2 diabetes, and results from an increase in the overall number and size of adipocytes [1]. To clarify the molecular mechanisms of adipogenesis, we previously used polymerase chain reaction (PCR)-subtraction methods to isolate 102 genes induced at the early stage of adipocyte differentiation [2,3]. One of these is a novel gene, *factor for adipocyte differentiation 24* (*fad24*). *Fad24* seems to be a mammalian homolog of yeast *nucleolar complex-associated protein 3* (*Noc3*), which functions in DNA replication and ribosome biogenesis [4,5].

We previously reported that *fad24* has an important role in the early stages of adipogenesis [6]. Furthermore, we revealed that FAD24 acts in concert with histone acetyltransferase binding to ORC1 (HBO1), a regulator of DNA replication, to promote 3T3-L1 adipogenesis by controlling a proliferating phase referred to as mitotic clonal expansion [7]. We also revealed that *fad24* assists the proliferation of C2C12 myoblasts, and NIH–3T3 fibroblasts, as well as 3T3-L1 pre-adipocytes [8]. To elucidate the role of *fad24 in vivo*, we generated *fad24* transgenic mice and reported that *fad24* overexpression increased the number of smaller adipocytes in white adipose tissue and improved glucose metabolic activity in the mice [9]. These accumulating results strongly suggested that *fad24* is involved in adipogenesis and glucose metabolism *in vivo*. However, the physiological contribution of *fad24 in vivo* remains unclear.

In this study, we attempted to generate *fad24*-deficient mice to determine the function of *fad24 in vivo*. Deletion of *fad24* led to embryonic lethality prior to implantation. Analyses of *fad24* homozygous mutants suggested that *fad24* is essential for development into blastocysts and that *fad24*-deficiency leads to a decreased number of nuclei in morulae due to the growth defect.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; E, embryonic day; *fad*, factor for adipocyte differentiation; HBO1, histone acetyltransferase binding to ORC1; IVF, *in vitro* fertilization; Neo, neomycin resistance; *Noc*, nucleolar complex-associated protein; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TK, thymidine kinase.

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2. Materials and methods

2.1. Construction of fad24 heterozygous mice

A 2.0 kb *Xho* I fragment located upstream of exon 5 and a 7.0 kb *Kpn* I fragment located downstream of exon 11 were amplified by PCR using genomic DNA as a template. The targeting vector was constructed by ligating these fragments to the pgk-Neo cassette of pNT1.1 (Fig. 1A). The pgk–tk expression cassette was used for negative selection against random integration. The constructed targeting vector was linearized with the *Not* I and electroporated into EGR-G01 ES cells. Three ES cell clones mutated correctly were screened by PCR analysis, and they were microinjected into C57BL/6NCr blastocysts purchased from Japan SLC (Shizuoka, Japan). These blastocysts were transferred to ICR pseudopregnant females to generate chimeras. Male chimeric offspring mice, which showed a high level of chimerism, were mated with C57BL/6J females, resulting in the birth of F1 *fad24*$^{+/-}$ mice. Heterozygote mice were backcrossed onto the C57BL/6J background for more than six generations. All experiments were performed in accordance with the guiding principles for the care and use of laboratory animals and were approved by the ethics committee of Nagoya City University.

2.2. ES cells screening

The primers used for screening PCR were as follows: primer a, 5′-AGCTGCCATTAGCATATAGCAGCC-3′, primer b, 5′-ACTTCA TCGGATCCGGAACCCTTA-3′, primer c, 5′-TAGTGAGACGTGCTACT TCCATTTG-3′, and primer d, 5′-GCTCCAACCAACCTAAGGGTAA-3′. PCR was performed with an amplification protocol as follows: primers a and b, 1 cycle of 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 3 min; primers c and d, 1 cycle of 94 °C for 2 min; 35 cycles of 98 °C for 10 s, 54.1 °C for 30 s, and 68 °C for 9 min.

2.3. Genotyping

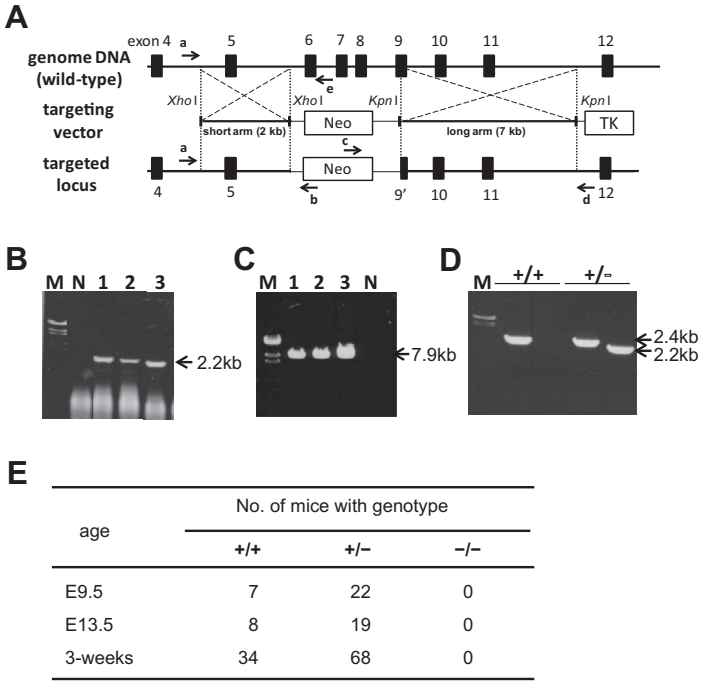
Pups and post-implantation embryos from *fad24* heterozygote intercrosses were characterized by PCR of genomic DNA. The tail snippets were lysed in lysis buffer (100 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, and 1% SDS) at 55 °C. The lysates were subjected to PCR with KOD FX (TOYOBO, Japan). The primer e, 5′-CAGGCTGAGTTTGAACTACTGGCA-3′, primer a, and primer b were used for PCR. PCR protocol was as follows: 1 cycle of 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 90 s.

2.4. Collection of pre-implantation embryos

fad24$^{+/-}$ female mice were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (Serotropin, Aska, Japan) followed by administration of 7.5 IU human chorionic gonadotropin (Gonatroqpin, Aska) 47.5 h later. They were then mated with *fad24*$^{+/-}$ male mice and the embryos were collected at E3.5 by flushing the uterus with M2 medium (Sigma). Individual embryos were lysed as previously described [10], and the lysates were subjected to PCR with KOD FX to determine the genotype as described above.

2.5. In vitro fertilization (IVF) and in vitro culture of pre-implantation embryos

IVF was performed according to the method described by Egashira et al. [11]. The eggs were transferred into KSOM medium under mineral oil and incubated for up to 48 h after insemination at 37 °C in 5% CO_2. The embryos were then placed into 96-well



E

age	No. of mice with genotype		
	+/+	+/−	−/−
E9.5	7	22	0
E13.5	8	19	0
3-weeks	34	68	0

Fig. 1. Generation of *fad24* KO mice. (A) Schematic representation of the *fad24* locus, the targeting vector, and the targeted locus. The black boxes represent exon 4–12 of *fad24* gene. Arrows show the primers used for PCR analyses. The targeting vector includes a neomycin resistance gene (Neo) and thymidine kinase gene (TK). (B) PCR analysis to detect short arm recombination in transfected ES cells using primers a and b. The length of PCR products from the targeted allele is 2.2 kb. (C) PCR analysis to detect long arm recombination in transfected ES cells using primers c and d. The length of PCR products from the targeted allele is 7.9 kb. Genomic DNA prepared from untransfected ES cell was used as a negative control (N). M; size marker. (D) Genotyping of *fad24*$^{+/+}$ and *fad24*$^{+/-}$ mice by PCR analysis. The length of PCR products in wild-type allele when used the primers a and e, and in the targeted allele when used the primers a and b are 2.4 and 2.2 kb, respectively. (E) Genotypes of progeny aged E9.5, E13.5, and 3 weeks after birth from *fad24* heterozygote intercrosses were determined by PCR.

plates, cultured individually, and observed with stereoscopic microscope (MZ95, Leica, Germany) at the interval of 12 h.

2.6. Nuclear staining of the morula with 4′,6-diamidino-2-phenylindole (DAPI)

The embryos obtained by IVF were cultured until they developed into morulae. The embryos were then washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C. They were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and washed thrice with PBS. Nuclear DNA staining was performed with 1 μg/ml DAPI in PBS for 10 min at room temperature. The DAPI signal was detected by using microscope (LSM5 PASCAL, Carl Zeiss, Germany), and images were captured (AxioVision 4.8, Carl Zeiss). For deconvolution analysis, serial optical section data (40–45 focal phases at 2.2 μm intervals) were collected and computationally processed by a 3D deconvolution method (AxioVision 4.8, Carl Zeiss). Further, the genotypes were determined as described above.

3. Results

3.1. Targeted disruption of fad24 results in early embryonic death

To better understand the function of *fad24 in vivo*, we attempted to generate *fad24*-deficient mice. Murine *fad24* is located on chromosome 19 and is comprises 21 exons [6]. The initiation ATG codon is located in exon 1. Although we previously attempted to generate mice with a targeted deletion of the first exon of *fad24*, we were unable to obtain targeted ES colonies. In the current study, we attempted to generate *fad24*-null mice by designing a targeting vector that excised exons 6–8, and a part of 9 in which a frame shift mutation was introduced (Fig. 1A).

The targeting vector was introduced by electroporation into ES cells. Positive cells were selected using G418 and ganciclovir. Colonies were selected and verified by PCR for homologous recombination on the 5′ and 3′ ends, resulting in three targeted clones (Fig. 1B and C). These targeted ES clones were injected into C57BL/6NCr blastocysts to produce chimeric male mice. Chimeras were backcrossed onto strain C57BL/6J mice to generate *fad24* heterozygous mutants, and the genotypes of the resulting offspring were determined by PCR of genomic tail DNA (Fig. 1D). All three lines exhibited germline transmission. No abnormalities were detected in *fad24* heterozygous ($fad24^{+/-}$) mice during a 12-month observation period (data not shown).

To establish *fad24* homozygous mutants ($fad24^{-/-}$), $fad24^{+/-}$ mice were intercrossed and the genotypes of the resulting offspring were determined 3 weeks after birth. However, as shown in Fig. 1E, none of the 102 offspring were found to be *fad24*-deficient. In addition, no increase in neonatal mortality was observed in the initial 3 weeks after birth. These results indicate that inactivation of functional *fad24* alleles leads to embryonic lethality.

To assess the time of the presumptive embryonic mortality in $fad24^{-/-}$ mice, we analyzed the genotypes of the embryos from $fad24^{+/-}$ intercrosses. Post-implantation embryos at E9.5 or E13.5 (E0.5 corresponds to the morning of vaginal plug detection) were collected and genotyped. No homozygous mutants were identified in either E13.5 or E9.5 embryos, indicating that *fad24* is essential for either pre-implantation or early post-implantation (Fig. 1E).

3.2. Development of $fad24^{-/-}$embryos arrests at the morula stage

To further define the defects in peri-implantation development of $fad24^{-/-}$ mutants, E3.5 embryos from heterozygous intercrosses were recovered and genotyped. Table 1 shows that $fad24^{-/-}$ mutants were detected in an expected Mendelian ratio of genotypes. However, when all the embryos were divided into four categories according to their appearance, an interesting difference was observed between wild-type embryos and $fad24^{-/-}$ mutants. Homozygous mutant embryos did not developed into blastocysts, whereas most wild-type embryos did develop to form blastocysts.

Table 1
Appearance of embryos from intercrosses of fad24 heterozygous littermates recovered at E3.5.

	+/+	+/−	−/−	Total
8 cells	0	1	3	4
>8 cells[a]	0	4	3	7
Morula	2	8	5	15
Blastocyst	8	14	0	22
Total	10	27	11	48

The embryos were divided into four categories according to their appearance.
[a] Embryos having more than eight blastomeres.

To further observe the development of *fad24*-null embryos, we performed IVF using oocytes and sperm collected from $fad24^{+/-}$ mice. Two days after fertilization, all the embryos developed into 8-cell stage (Fig. 2A, a–d) and were placed into individual wells in 96-well plates for observation over a 2-day incubation period. Subsequently, the embryos were lysed and genomic DNA was extracted for PCR genotyping. Both wild-type and $fad24^{-/-}$ embryos developed into morulae at day 3 after IVF (Fig. 2A, e–h). At day 4, the wild-type morulae continued normal development into blastocysts (Fig. 2A, m and n). However, $fad24^{-/-}$ embryos ceased growing and appeared abnormal (Fig. 2A, o and p). They exhibited significant cellular degradation and appeared to be non-viable. The appearance of 27 embryos obtained from IVF are summarized in Fig. 2B. All $fad24^{-/-}$ mutants failed to develop into blastocysts. These results indicate that the development of *fad24*-null embryos arrest at the morula stage.

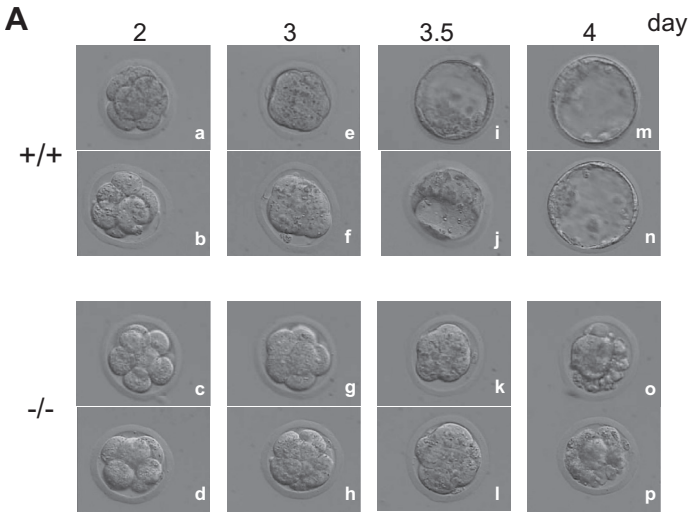
3.3. fad24 deficiency results in a decrease in the number of nuclei in the morula stage

Based on our observation of pre-implantation embryonic development above, we further observed the nuclear morphologies of $fad24^{-/-}$ embryos by DAPI staining. The eggs obtained from IVF were cultured and all the embryos showed normal development into morulae as observed by phase contrast microscopy (Fig. 3A). Morulae were then stained with DAPI and fluorescence microscopy (Fig. 3A) was used to count the number of nuclei. Fig. 3B shows that the number of nuclei decreased significantly in *fad24*-deficient morulae compared with those in wild-type. This result indicates that loss of *fad24* function results in the deteriorated growth of the embryos, probably due to the inhibition of proliferation.

4. Discussion

To analyze the function of *fad24 in vivo*, we attempted to generate *fad24*-null mice by gene targeting. No $fad24^{-/-}$ embryos were recovered after E9.5 (Fig. 1E) and no homozygous mutants reached blastocyst stage at E3.5, in contrast to the majority of wild-type and heterozygous embryos (Table 1). In addition, IVF experiments demonstrated that all $fad24^{-/-}$ embryos exhibited an abnormal appearance and died prior to blastocyst formation. These results strongly suggested that the absence of *fad24* prevents the developmental progression from morulae into blastocysts.

The finding that homozygous mutants were unable to develop into blastocysts prompted us to examine growth rate differences between wild-type and $fad24^{-/-}$ embryos prior to E3.5. In order to accomplish this, IVF was performed and the embryo development was observed *in vitro*. As shown in Fig. 2A, both wild-type



B

apperance	+/+	+/–	–/–	unknown[a]	total
blastocyst	7	13	0	0	20
abnormal morphology	1	0	5	1	7

[a]Genotype could not be determined because of PCR failure.

Fig. 2. *fad24* deficiency results in early embryonic lethality. (A) Pre-implantation development of *fad24* deficient embryos from the 8-cell to the blastocyst stage. Embryos were obtained from heterozygous IVF at 2-days cultivation, and cultured *in vitro* for two more days. The number shows the day after IVF. (B) Genotypes of 27 morulae that developed into blastocysts or exhibited abnormal morphology at day 4 after IVF were determined by PCR.

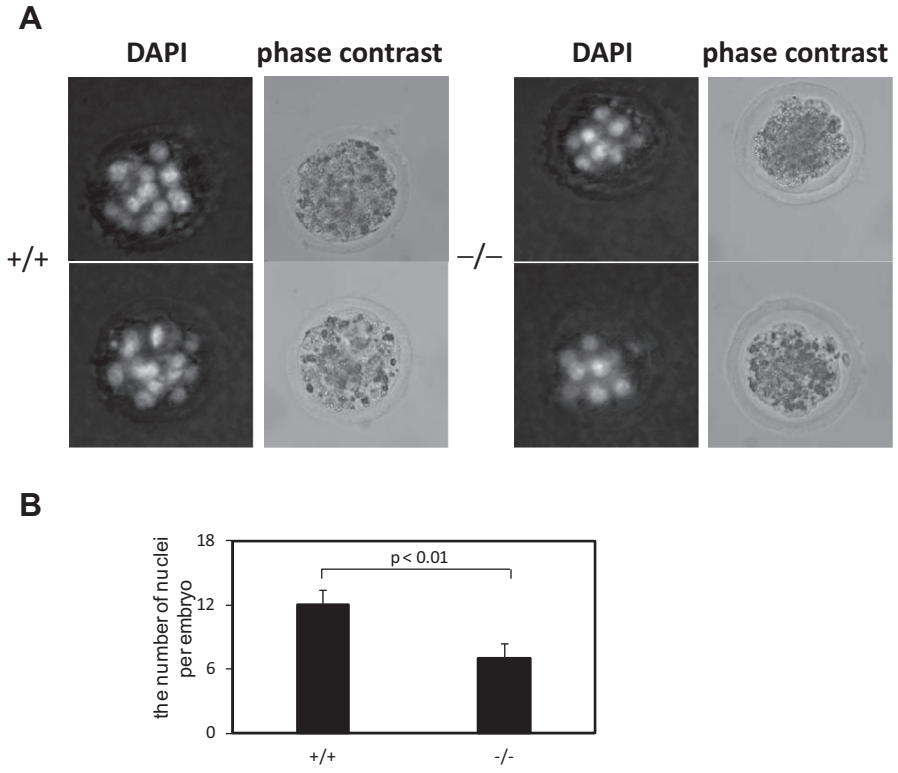


Fig. 3. Analysis of preimplantation embryos from *fad24*$^{+/-}$ intercrosses. (A) Nuclear staining with DAPI of *in vitro*-cultured embryos 3-days after IVF. The phase contrast pictures and DAPI-stained pictures are shown in right- and left side in each panel, respectively. (B) A series of z-plan images was stacked and analyzed by an image processing software to quantify the number of nuclei in *fad24*$^{+/+}$ and *fad24*$^{-/-}$ embryos. *P*-value was determined by a *t*-test.

and homozygous mutants developed into morulae at day 3 after IVF, and no significant differences were observed at this stage. However, *fad24*$^{-/-}$ morulae were unable to develop into blastocysts, and appeared non-viable at day 4. These results strongly indicate that *fad24* is a stage-specific regulator critical for the progression of morulae into blastocysts.

We isolated *fad24* as a novel gene and identified it as a mammalian homolog of yeast *Noc3* [6], which is required for DNA replication and ribosome biogenesis [4,5]. Our previous study also indicated that *fad24* was required for DNA replication *in vitro* [7,8]. Several DNA replication factors have been reported to be essential for mouse embryogenesis. For example, *Mcm10*, a regulator of initiation and elongation of DNA replication, plays an important role in peri-implantation embryonic development [12]. Therefore, we speculated that *fad24* may contribute to DNA replication during early embryogenesis. The result of nuclear staining of *fad24*-null morulae supports this speculation, because the observed decrease in the number of nuclei is indicative of defective cell proliferation (Fig. 3). In 3T3-L1 pre-adipocytes, FAD24 may act as a cofactor for the recruitment of HBO1 to origins of replication, and thus exert control of DNA replication (7). It is also possible that FAD24 acts in concert with HBO1 during embryogenesis. However, Kueh et al. recently reported that *hbo1*-deficient mouse died at E10.5 [13] indicating that *hbo1* is essential for post-gastrulation embryonic development, but not for pre-implantation embryogenesis. Thus, *fad24* may act in an *hbo1*-independent manner during early embryonic development. The isolation of factors that act in concert with *fad24* during peri-implantation embryonic development is an important focus for future studies.

fad24 might function in ribosome biogenesis as well as DNA replication, akin to *Noc3* in yeast [4,5]. Like *Rpo1-2*, some regulators for ribosome biogenesis are also known to be essential for early mouse development [14], although further analyses are necessary to elucidate the involvement of *fad24* in ribosome biogenesis.

Although our study revealed that *fad24* is involved in development of early mouse embryos, the function of *fad24* in adult is still unknown. Our previous studies suggest that *fad24* plays important roles in adipogenesis and cell proliferation *in vivo* [7,8]. In addition, it has been reported that *fad24* is important for the muscle development in zebrafish [15]; thus, *fad24* may contribute to mammalian skeletal muscle development as well as adipogenesis. Further analysis of *fad24*-conditional knockout mouse may provide useful information regarding the physiological roles of *fad24*.

In conclusion, we generated *fad24*-deficient mice using a gene targeting strategy and revealed that *fad24* is essential for pre-implantation embryos to develop into blastocysts. Further analysis of *fad24* will help us understand the molecular mechanisms of mammalian embryogenesis.

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