FAD24, a Regulator of Adipogenesis, is Required for the Regulation of DNA Replication in Cell Proliferation

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A novel gene, *factor for adipocyte differentiation 24* (*fad24*), promotes adipogenesis by controlling DNA replication early on during a stage referred to as mitotic clonal expansion (MCE). MCE is considered distinct from the proliferation of pre-confluent cells, so we investigated the role of *fad24* in the process. First, the expression of *fad24* was examined in pre-confluent and post-confluent 3T3-L1 preadipocytes, NIH-3T3 fibroblasts, and C2C12 myoblasts. *fad24* was strongly expressed in the pre-confluent cells. The knockdown of *fad24* by RNA interference impaired the ability of the pre-confluent cells to proliferate. Moreover, bromodeoxyuridine labeling and chromatin immunoprecipitation experiments indicated that the knockdown inhibited DNA synthesis by preventing the recruitment of histone acetyltransferase binding to ORC1 (HBO1), a component of the pre-replicative complex, to origins. *fad24* plays positive roles in the proliferation of pre-confluent cells as well as adipogenesis.

Key words DNA replication; cell proliferation; adipogenesis; *fad24*; histone acetyltransferase binding to ORC1 (HBO1)

DNA replication, one of the most fundamental steps of the cell division cycle, is strictly regulated by a complex network of extracellular and intracellular signaling pathways that control cell proliferation, differentiation, and apoptosis. To ensure proper genome duplication and inheritance, eukaryotic cells control the initiation of DNA replication through the precise assembly of the pre-replicative complex (pre-RC) at origins once in the same cell cycle.^{1, 2)} The formation of the pre-RC, a prerequisite for the initiation of DNA replication, depends on the binding of the origin recognition complex (ORC) to origins.³⁾ ORC, in turn, recruits CDC6, CDT1, and eventually the minichromosome maintenance complex (MCM).^{4–7)} Recently, Iizuka *et al.* revealed that histone acetyltransferase binding to ORC1 (HBO1), a MYST family histone acetyltransferase (HAT), facilitates the recruitment of chromatin to the MCM necessary for the pre-RC's assembly.⁸⁾ However, the precise mechanism of replication initiation is still unclear, and some replication-initiation proteins may have not been discovered.

To elucidate the earliest step in adipocyte differentiation, we previously isolated 102 genes induced to express at the beginning of the differentiation of 3T3-L1 cells.^{9, 10)} One of them was a novel gene, *factor for adipocyte differentiation 24* (*fad24*). Gain-of-function and loss-of-function experiments indicated that *fad24* is a positive regulator of adipogenesis.¹¹⁾ Moreover, our recent findings indicated that FAD24 interacts with components of Pre-RC, localizes to origins of DNA replication, and recruits HBO1, a component of pre-RC, to origins. Consequently, FAD24 promotes adipogenesis by controlling DNA replication early on during a stage referred to as mitotic clonal expansion (MCE).¹²⁾

After the induction of differentiation, post-confluent, growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle and undergo approximately two rounds of mitosis, known as MCE.^{13, 14)} MCE appears to be necessary for adipocyte differentiation, and DNA replication and changes in chromatin structure during MCE might facilitate the transcription of genes leading to terminal differentiation.^{13, 14)} MCE is considered different from the proliferation of pre-confluent cells. In fact, some reports indicate that the tran-

scription factor CCAAT/enhancer-binding protein (C/EBP) β and *N*-acetyl-Leu-Leu-norleucinal (ALLN), a calpain inhibitor, play crucial roles in MCE but not in the proliferation of pre-confluent 3T3-L1 cells.^{15, 16)} Therefore, studying whether FAD24 acts in concert with HBO1 to control DNA replication only in MCE during adipogenesis or in the proliferation of pre-confluent cells is very important for elucidating the function of this novel gene.

In this study, we show that *fad24* is strongly expressed in pre-confluent 3T3-L1 preadipocytes, NIH-3T3 fibroblasts, and C2C12 myoblasts as well as at the early stages of adipocyte differentiation. Cell proliferation was inhibited when the expression of *fad24* was knocked down in these pre-confluent cells. Moreover, bromodeoxyuridine (BrdU) labeling and chromatin immunoprecipitation (ChIP) experiments indicated that FAD24 controls DNA replication in pre-confluent cells through the recruitment of HBO1 to origins in the same way as MCE. These results imply that *fad24* plays positive roles in the proliferation of pre-confluent cells as well as adipogenesis.

MATERIALS AND METHODS

Cell Culture and Cell Counting Mouse 3T3-L1 preadipocyte cells, mouse NIH-3T3 fibroblastic cells, and mouse C2C12 myoblasts were cultured as previously described.¹¹⁾ For cell counting, cells were trypsinized and replated into 12-well plates at a density of 1×10^4 cells/well. The cell numbers at different time points were measured with the WST-8 assay (TetraColor One, SEIKAGAKU CORPORATION) following the manufacturer's recommended protocols.

RNA Interference (RNAi) The shRNA expression plasmids for *fad24* and the control plasmid were used as described.¹¹⁾ Transfection of the shRNA expression plasmids into 3T3-L1 and C2C12 cells was performed as previously described,¹¹⁾ except that the C2C12 cells were transfected using program 'B-32' of Nucleofector (Amaxa).

RNA Isolation, Real-Time Quantitative PCR (Q-PCR), and Northern Blot Analyses Total RNA was extracted

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with TRIzol (GIBCO BRL Life Technologies) according to the manufacturer's instructions. The reverse transcription and Q-PCR were performed as previously described.¹¹⁾ The pre-designed primers and probe sets for *fad24* and 18S rRNA were obtained from Applied Biosystems. Northern blot analyses were also performed as described.¹¹⁾

BrdU Labeling and Immunofluorescence For BrdU labeling and immunofluorescence microscopy, 3T3-L1 cells were plated on coverslips. At 24 h after plating, cells were labeled for 2 h with 30 μ g/ml BrdU (BD Biosciences). Coverslips were fixed in 70% ethanol for 30 min and incubated in 100% methanol for 10 min at room temperature. The fixed cells on coverslips were then treated for 30 min with 1.5 M HCl, blocked with 0.5% Tween 20 in phosphate-buffered saline (PBS) for 5 min, incubated with fluorescein isothiocyanate (FITC)-anti-BrdU monoclonal antibody (BD Biosciences) for 30 min at room temperature, and incubated with 7-amino-actinomycin D (BD Biosciences) for 5 min at room temperature. At each step described above, the cells were washed with PBS three times after treatment.

ChIP Assay Approximately 1.0×10^7 cells were incubated with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated by the addition of the stop solution (10 mM Tris-HCl, pH 8.0, and 2 M glycine). The cells were washed with PBS and then scraped into 1 ml of the cold lysis buffer (25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, and 150 mM NaCl) containing the protease inhibitor mixture. These cell lysates were sonicated 10 times for 10 s each time to generate DNA fragments that ranged in size from 200 to 1000 bp. The sheared chromatin-lysed extracts were incubated with either 2 μ g of the anti-HBO1 antibody (N-18, Santa Cruz Biotechnology) or 2 μ g of normal goat IgG (Santa Cruz Biotechnology) overnight at 4 °C. These extract-antibody mixtures were incubated for an additional 1 h with protein G-Sepharose beads (Amersham Bioscience). The immunoprecipitates were washed according to the conventional method,¹⁷⁾ suspended in the extraction buffer (TE, pH 8.0, and 1% SDS), and incubated overnight at 65 °C and for 2 h at 37 °C with 100 μ g of proteinase K to reverse protein/DNA cross-links. Finally, these samples were processed for DNA purification by the phenol-chloroform extraction method and ethanol precipitation. PCRs were performed in 20 μ l with 1 : 1000 and 1 : 2000 of input DNA and 1 : 10 of the immunoprecipitates as described using the primers amplicon 15 and amplicon 19.¹⁸⁾ The number of PCR cycles yielding products within the linear range was determined using 2-fold serial dilutions of the input DNA. PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide (EtBr). The intensities were determined with a fluorimager (FluorImager 595, Amersham Biosciences).

RESULTS

***Fad24* Is Strongly Expressed in Pre-confluent Cells as Well as at the Early Stages of Adipogenesis** We recently showed that *fad24* stimulates MCE and adipogenesis by positively regulating DNA replication through the recruitment of HBO1 to origins,¹²⁾ but it is unclear whether *fad24* is required for DNA replication and cell proliferation in pre-confluent cells or not. First, we determined the expression of

Fig. 1. Expression Profile of *fad24* in Pre-confluent and Post-confluent Cells

Total RNA was isolated from pre-confluent (proliferating) and post-confluent (growth-arrested) 3T3-L1, C2C12, and NIH-3T3 cells, or post-confluent 3T3-L1 cells at 3 h after adipogenic induction. Twenty-five micrograms of RNA was loaded in each lane and subjected to Northern blot analysis of *fad24*. Staining with EtBr for ribosomal RNA is shown as a loading control. This experiment was conducted twice and the reproducibility was confirmed.

fad24 in pre-confluent (proliferating) and post-confluent (growth-arrested) 3T3-L1 preadipocytes, C2C12 myoblasts, and NIH-3T3 cells by conducting Northern blots analyses. *Fad24* was strongly expressed in the pre-confluent cells rather than post-confluent cells, although its expression was weak compared to that at 3 h after adipogenic induction (Fig. 1). These results indicate that the expression of *fad24* correlates with cell proliferation.

***Fad24* Is Required for the Proliferation of Pre-confluent Cells** As shown in Fig. 1, *fad24* is thought to be involved in the proliferation of pre-confluent cells as well as MCE during adipocyte differentiation. Next, we examined the effect of knocking down *fad24* with RNAi on the proliferation of pre-confluent cells. The expression level of *fad24* in 3T3-L1 and C2C12 cells transfected with the *shfad24* expression plasmid declined compared with that in control cells transfected with a scrambled shRNA expression plasmid (Fig. 2A). These cells were plated at a low density, and cell numbers were determined every 24 h by the WST-8 assay. The numbers of *shfad24*-treated cells were lower than control values from 48 to 96 h after plating (Fig. 2B). These results indicate that *fad24* is required for the proliferation of pre-confluent cells as well as MCE.

The Knock Down of *Fad24* Causes an Impairment of DNA Replication in Pre-confluent Cells Moreover, to explore the role of *fad24* in cell proliferation, DNA synthesis was evaluated using BrdU labeling. Pre-confluent *shfad24*-treated 3T3-L1 cells and control cells were pulse-labeled for 2 h with BrdU, and immunostained with the anti-BrdU antibody. The DNAs in the cells were also detected by 7-amino-actinomycin D. As shown in Fig. 3A, the incorporation of BrdU was detected in almost all control cells. On the other hand, less BrdU was incorporated into *shfad24*-treated cells than control cells, indicating that the knock down of *fad24* impaired DNA replication in pre-confluent cells (Figs. 3A, B).

The Knock Down of *Fad24* Inhibits the Recruitment of HBO1 to Origins To limit replication to a single round per cell cycle, DNA replication can begin only when sufficient

Fig. 2. Effect of the Knock Down of *fad24* on the Proliferation of Pre-confluent Cells

(A) Q-PCR analyses of the expression of *fad24*. Total RNA was obtained from pre-confluent 3T3-L1 cells (left panel) and C2C12 cells (right panel) transfected with the plasmids expressing shRNA for *fad24* (*shfad24*; white bar) or with plasmids expressing scrambled shRNA as a control (Control; black bar). The expression level of *fad24* was determined by Q-PCR, and normalized with 18S rRNA expression. (B) Effect of shRNA for *fad24* on the proliferation of pre-confluent 3T3-L1 and C2C12 cells. *Shfad24*-treated cells (white bars) or control cells (black bars) were plated at a low density. Cell numbers were determined at different time points after plating with the WST-8 assay. Each value represents the mean $\pm$ standard deviation ($n=3$). The asterisk and the double asterisks indicate significant differences when compared with the values of control cells at different time points ($p<0.05$ and $p<0.01$, respectively).

Fig. 3. The Inhibition of DNA Replication by the Knockdown of *fad24*

(A) Effect of shRNA for *fad24* on the incorporation of BrdU in pre-confluent 3T3-L1 cells. Pre-confluent *shfad24*-treated cells or control cells were labeled with BrdU, and stained with the FITC-conjugated anti-BrdU monoclonal antibody and 7-amino-actinomycin D (DNA). The fluorescence of BrdU (green) and DNA (red) was detected with a fluorescence microscope. (B) The cells labeled with BrdU in three microscopic view fields in (A) were counted for each experiment, and the results from three experiments were averaged. The value represents the mean $\pm$ standard deviation ($n=3$). The asterisk indicates significant difference when compared with the value for control cells ($p<0.05$).

DNA replication initiation machinery is recruited to origins.¹⁹⁾ As mentioned above, we revealed that *fad24* positively regulates MCE through the recruitment of HBO1 to origins.¹²⁾ Then, we examined the effect on HBO1's localization to origins of *fad24*'s knock down in pre-confluent cells. ChIP analyses were performed using pre-confluent *shfad24*-treated 3T3-L1 cells and control cells. The immunoprecipitated DNA was amplified by PCR with primer pairs against a mouse origin (ORI), or an outside region (non-ORI) as a negative control. The signal against ORI was decreased in *shfad24*-treated cells compared with control cells (Figs. 4A, B). Consistent with these results, *fad24* seems to be required for the recruitment of HBO1 to origins in pre-confluent cells.

DISCUSSION

In this study, we revealed that *fad24* is strongly expressed in pre-confluent cells as well as at the early stages in adipocyte differentiation. Our previous studies reported that the induction of *fad24* expression during adipogenesis is mainly dependent on insulin which is essential for MCE.¹²⁾ Therefore, the expression of *fad24* seems to correlate with cell proliferation. Moreover, we revealed that human *fad24* is

ubiquitously expressed in various tissues.¹¹⁾ These results suggest that *fad24* is necessary for the proliferation of many types of cells.

It was revealed that the knock down of *fad24* expression inhibits the proliferation of pre-confluent cells as well as MCE, an early and essential step in the differentiation program. It is clear that the cyclin-dependent kinase inhibitor p27 and the transcription factor C/EBP β play crucial roles in MCE.^{15,16)} The inhibition of p27's degradation with ALLN and dominant-negative C/EBP blocked MCE, but not the proliferation of pre-confluent 3T3-L1 cells.^{15,16)} Considering that C/EBP β is not expressed in pre-confluent 3T3-L1 cells,²⁰⁾ whether each gene product functions as a specific regulator for MCE might be determined mainly in the context of the expression pattern.

This study indicates that the knock down of *fad24* expression in pre-confluent 3T3-L1 cells impaired DNA replication by reducing the localization of HBO1 to origins in the same way as MCE. Nucleolar complex-associated protein 3 (Noc3p), a yeast ortholog of FAD24, plays a direct role in the initiation of DNA replication by interacting with MCM and ORC, as a cofactor for the recruitment of Cdc6p and MCM.²¹⁾ Moreover, Doyon *et al.* reported that HBO1 plays

Fig. 4. Effect of the Knockdown of *fad24* on the Localization of HBO1 to Origins of DNA Replication

(A) A ChIP assay was performed using pre-confluent 3T3-L1 cells transfected with the *shfad24* expression plasmids or the control plasmids using the anti-HBO1 antibody (lanes 3, 7), and normal goat IgG was used as a negative control (lanes 4, 8). Input DNA (1 : 1000 and 1 : 2000; lanes 1, 2, 5, 6) and immunoprecipitated DNA were amplified by PCR with primer sets against the mouse DNA origins (ORI) or an outside region (non-ORI). PCR products were resolved on an 8% polyacrylamide gel and stained with EtBr. (B) The intensities in (A) were determined with a fluorimeter. This experiment was conducted three times, and ChIP efficiency against ORI was calculated as a percentage of immunoprecipitated material. Each value represents the mean $\pm$ standard deviation ($n=3$). The asterisk indicates a significant difference when compared with the value for control cells ($p<0.05$).

essential roles in DNA replication in MCF7 and 293T cells.²² Therefore, FAD24 may act as a general cofactor for the recruitment of pre-RC including HBO1 to origins. At present, how FAD24 regulates pre-RC assembly and DNA replication with HBO1 remains largely unknown. HBO1 is the major histone H4 histone acetyltransferase, and chromatin structure modulated by histone modifications including acetylation has been linked to the control of pre-RC assembly and initiation of DNA replication in *Saccharomyces cerevisiae*, *Xenopus*, and *Drosophila*.^{23–25} Consistent with these reports, FAD24 might act in concert with HBO1 to modulate chromatin structure around origins of DNA replication. In this article, we observed the effect of knocking down *fad24* on cell proliferation, but not the effect of over-expressing *fad24*. Recently, a growing body of data indicates that over-expression of individual pre-RC proteins results in uncontrolled cell cycle and tumorigenesis.¹⁹ Therefore, it might be possible that over-expression of *fad24* in quiescent cells promotes cell proliferation. Further studies on the functions of *fad24* in cell proliferation are definitely needed.

In summary, our findings indicate that *fad24* has important roles in cell proliferation as well as adipogenesis. It is possible that *fad24* is required for DNA replication and subsequent cell proliferation by recruiting HBO1 to origins in pre-confluent cells. However, the precise regulation mechanisms of cell proliferation by *fad24* and also the contribution of

fad24 to cell cycle are still unclear. Further analyses of the mechanism for the regulation of DNA replication by this novel gene are required and seem to provide insight into the molecular basis of replication initiation as well as adipogenesis. In addition, the abrogation of the exquisite control mechanisms that maintain the precise duplication of DNA replication results in detrimental gains and losses of genomic DNA commonly seen in cancer and developmental defects.¹⁹ To elucidate the biological and biochemical roles of *fad24* in these processes, the development and analyses of knockout mice and transgenic mice are ongoing.

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