Involvement of Forkhead Transcription Factor Foxa1 in Adipocyte Differentiation of Mouse Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) are able to differentiate to various cell types including adipocytes and osteoblasts. In this study, we examined the roles of forkhead transcription factor (Fox) a1 in the adipocyte differentiation in mouse ST2 MSCs. The expression of the Foxa1 gene was rapidly induced, reached its maximum level at 12 h after the initiation of adipocyte differentiation, and at 2 days decreased to almost the same level as that of undifferentiated MSCs. On the contrary, the Foxa1 gene expression level was not altered during osteoblastogenesis in MSCs. RNAi-mediated knockdown of Foxa1 mRNA increased the accumulation of lipid droplets in the adipocytes, and significantly enhanced the expression of peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer binding protein (C/EBP) α, which are key transcription factors in mid and late phases of adipogenesis, but not the transcription of C/EBPβ or C/EBPδ, critical transcription factors in the early phase of adipogenesis. Moreover, RNAi-mediated suppression and overexpression of Foxa1 demonstrated that Foxa1 was involved in the transcriptional suppression of adipogenic and lipogenic genes including PPARγ- and C/EBPα-target genes. These results suggest that Foxa1 plays a suppressive role in adipogenesis through the negative regulation of PPARγ and C/EBPα in MSCs.

Key words—forkhead transcription factor, adipogenesis, mesenchymal stem cells, PPARγ, C/EBPα

INTRODUCTION

Adipose tissue is an important organ for energy homeostasis as well as for energy storage. On the contrary, the excessive accumulation or increased size of adipocytes results in obesity and its associated diseases such as cardiovascular diseases, type 2 diabetes, hypertension, and dyslipidemia, and the prevalence of obesity is increasing in the developing countries. Adipocytes secrete various cytokines; termed adipocytokines, such as leptin, adiponectin, and resistin, which are known to influence food intake and insulin sensitivity. Obesity, a pathological accumulation of adipose tissue, results in an imbalance in the secretion of adipocytokines. Thus, understanding the molecular mechanisms underlying the regulation of obesity (adipogenesis) is critical in both scientific and clinical fields.

The molecular mechanisms of adipogenesis have been extensively studied. A number of transcription factors are involved in the regulation of adipocyte differentiation. Among them, peroxisome proliferator-
activated receptor (PPAR) γ and the CCAAT/enhancer binding protein (C/EBP) family have been identified as the master transcription factors in adipogenesis. C/EBPβ and C/EBPδ are the critical transcription factors in the early phase of adipogenesis. Whereas, C/EBPα and PPARγ are induced in turn by the activation of C/EBPβ and C/EBPδ, and regulate the transcriptions of a number of genes involved in the regulation of adipogenesis.

Forkhead transcription factor (Fox) is a transcription factor defined by a conserved DNA binding domain, and it comprises a family of transcription factors that bind to similar cis-regulatory elements. However, Fox proteins regulate diverse cellular processes including differentiation, metabolism, development, and cell proliferation. Some Fox proteins are associated with human diseases including insulin sensitivity and obesity. The Foxa protein subfamily (hepatocyte nuclear factor 3 [HNF-3]) consists of three subtypes, i.e., Foxa1 (HNF-3α), Foxa2 (HNF-3β), and Foxa3 (HNF-3γ). Foxa2 is expressed in adipose tissue only under conditions of obesity; whereas Foxc2, another member of the Fox family, is expressed in white and brown adipose tissues and blocks adipogenesis by inhibiting the expression of PPARγ-target genes. Moreover, Foxo1 plays a suppressive role in the insulin signaling in adipogenesis.

Mesenchymal stem cells (MSCs) have the potential to differentiate into many different cell types including osteoblasts, myoblasts, chondrocytes, and adipocytes. Often, an imbalance among these cell types is observed when bone loss occurs. In particular, an increased ratio of adipocytes to osteoblasts is associated with age-related osteoporosis. Thus, the regulatory factor involved in the commitment of MSCs to adipocytes and osteoblasts has the potential to be a novel therapeutic target for development of anti-obesity and anti-osteoporosis drugs. In this study, we investigated the roles of Foxa1 in the differentiation of adipocytes from MSCs, and found that Foxa1 is a novel suppressor of the adipogenesis in MSCs.

RESULTS AND DISCUSSION

Expression of Foxa1 during adipogenesis in MSCs
At first, we caused ST2 MSCs to differentiate into adipocytes by incubating the former 2 days in medium containing a differentiation cocktail composed of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), and insulin and thereafter incubating them further 6 days in medium containing insulin alone. The differentiation of MSCs into adipocytes was confirmed by the accumulation of lipid droplets in the cells, as shown by Oil Red O staining (Fig. 1A). The Foxa1 gene was expressed in MSCs, and its mRNA level increased at 1 day, and then decreased at 10 days after the initiation of adipocyte differentiation (Fig. 1B). On the other hand, the expression of PPARγ was enhanced at 10 days after the initiation of adipogenesis in MSCs (Fig. 1B). In the case of differentiation into osteoblasts, when MSCs were treated with bone morphogenetic protein (BMP) 2, alkaline phosphatase (ALP) activity, a marker of osteoblasts was clearly increased in the cells, as demonstrated by ALP staining (Fig. 1C). Foxa1 gene expression was not altered during the differentiation of MSCs into osteoblasts, although the mRNA levels of osteocalcin and Runx-related gene 2 (Runx2), marker...
Fig. 1. Expression of Foxa1 during differentiation of adipocytes and osteoblasts from MSCs

A. Adipocyte differentiation of ST2 MSCs. MSCs were caused to differentiate into adipocytes by treatment with insulin, IBMX, and DEX for 2 days, followed by incubation with insulin alone for 2 days. Medium was changed every 2 days to 6 days. Lipid droplets were stained with Oil Red O. U: undifferentiated cells, D: differentiated cells. B, Expression of Foxa1, C/EBPβ, and PPARγ genes in adipocytes differentiating from MSCs. MSCs were made to differentiate into adipocytes for 1 or 10 days. Quantitative PCR analysis was performed to measure the mRNA levels. The data represent the means ± S.D. from 3 independent experiments. IDX; insulin, DEX, and IBMX. C, Osteoblast differentiation in MSCs. MSCs were caused to differentiate into osteoblasts by treatment with BMP2 (100 ng/ml) for 14 days. The medium was changed every 3 days. ALP staining was carried out as described in EXPERIMENTAL. U: undifferentiated cells, D: differentiated cells. D, Expression of Foxa1, osteocalcin, and Runx2 genes in osteoblasts differentiated from MSCs. MSCs were made to differentiate from MSCs into osteoblasts for 8 or 14 days. RNA extraction and quantitative PCR analysis were carried out as described above. The data represent the means ± S.D. from 3 independent experiments. E, Expression of Foxa1 during the adipogenesis of MSCs. Expression level of Foxa1 gene was measured by quantitative PCR. The data are presented as the mean ± S.D. from 3 independent experiments.
genes of osteoblastogenesis, were up-regulated during osteoblastogenesis (Fig. 1D).

Next, we investigated the expression profile of Foxa1 gene during adipogenesis in MSCs. The Foxa1 mRNA level was increased just after the initiation of adipogenesis, reached its maximum level at 12 h, and then decreased to lower level that was almost the same as that detected in undifferentiated MSCs (Fig. 1E). These results, taken together, indicate that Foxa1 mRNA was expressed in MSCs and that its expression was enhanced during the early phase of adipogenesis, but not during osteoblastogenesis in MSCs.

RNAi-mediated suppression of Foxa1 accelerates adipogenesis in MSCs

To investigate the roles of Foxa1 during the adipogenesis in ST2 MSCs, we carried out RNAi-mediated knockdown to suppress the expression level of the Foxa1 gene. MSCs were transfected with either of two independent Foxa1 siRNAs and caused to differentiate into adipocytes for 6 days. Both Foxa1 siRNAs reduced the Foxa1 mRNA level to less than 20% of that obtained with the negative control (N.C.) siRNA (Fig. 2A). Thus, we decided to use more effective Foxa1 siRNA(#2) in further experiments.

To examine the effect of Foxa1 on the accumulation of lipid droplets in the cells, we transfected MSCs with Foxa1 siRNA or N.C. siRNA, and caused to differentiate for 6 days. Lipid droplets accumulated during the adipocyte differentiation in MSCs, and the Foxa1 siRNA enhanced the lipid accumulation approximately 1.8-fold as compared with that in N.C. siRNA-transfected MSCs (Fig. 2B and C). These results indicate that Foxa1 acted as a suppressor in the lipid accumulation during the adipogenesis in MSCs.

Expression of adipogenic transcription factors in Foxa1 siRNA-transfected MSCs

Next, we examined the effects of Foxa1 on the expression of key transcription factors, i.e., C/EBPα, C/EBPβ, C/EBPδ, and PPARγ during adipogenesis in ST2 MSCs. MSCs were transfected or not with Foxa1 siRNA or N.C. siRNA, and caused to differentiate or not into adipocytes for 6 days. The transcription levels of C/EBPα and PPARγ, which are master transcription factors operating in the mid and late phases of adipogenesis, were increased about 1.8- and 1.7-fold, respectively, in the differentiated MSCs as compared with those in the undifferentiated cells (Fig. 3). Moreover, C/EBPα and PPARγ mRNA levels were up-regulated approximately 1.4- and 1.3-fold, respectively, by the transfection with Foxa1 siRNA as compared with those obtained with N.C. siRNA (Fig. 3). On the contrary, the expression levels of C/EBPβ and C/EBPδ were almost unchanged, even when the MSCs were transfected with Foxa1 siRNA (Fig. 3). These results suggest that Foxa1 suppressed the expression of adipogenic transcription factors that function in MSCs during the mid and late phases, but not during the early phase, of adipogenesis.

Transcription levels of adipogenic and lipogenic genes in the Foxa1-knockdown MSCs

Next, we examined the effects of Foxa1 on the expression of adipogenic and lipogenic genes during adipogenesis in ST2 MSCs. MSCs were transfected or not with Foxa1 siRNA or N.C. siRNA, and made to differentiate or not into adipocytes for 6 days. The expression levels of all adipogenic and lipogenic genes examined in this study increased approximately 1.4 to 143-fold during adipogenesis (Fig. 4). Moreover, when the cells were transfected with Foxa1 siRNA, the mRNA levels of fatty acid binding protein 4 (aP2),
Fig. 2. SiRNA-mediated suppression of Foxa1 in adipocyte differentiation from MSCs

A, SiRNA-mediated suppression of Foxa1 gene expression in adipocytes in ST2 MSCs. MSCs were transfected with either of two Foxa1 siRNAs or N.C siRNA. One day after the transfection, the cells were caused to differentiate into adipocytes for 6 days and then harvested for extraction of RNA. The expression of Foxa1 gene was measured by quantitative PCR. The data are presented as the means ± S.D. from 3 independent experiments. *p<0.01 as compared with value obtained with N.C. siRNA. B, Accumulation of lipid droplets in MSCs. MSCs (U) were made to differentiate into adipocytes for 6 days (D). Lipid droplets were stained with Oil Red O. C, Measurement of Oil Red O dye extracted from lipid droplet-laden cells. U: undifferentiated cells, D: differentiated cells.
Fig. 3. Foxa1 siRNA-mediated suppression of adipogenic transcription factors during adipogenesis in MSCs

MSCs were caused to differentiate (D) or not (U) into adipocytes for 6 days. Expression levels of PPARγ, C/EBPα, C/EBPβ, and C/EBPδ genes were measured by quantitative PCR after treatment or not with Foxa1 siRNA or N.C. siRNA. Data are the means ± S.D. from 3 independent experiments. *p<0.01 and **p<0.05 as compared to N.C. siRNA or undifferentiated cells.
Fig. 4. Expression of adipogenic and lipogenic genes during adipogenesis in Foxa1 siRNA-transfected MSCs

ST2 MSCs were transfected or not with Foxa1 or N.C. siRNA, and caused to differentiate or not for 6 days. Messenger RNA levels of adipogenic and lipogenic genes were measured by quantitative PCR. Data are the means ± S.D. from at least 3 independent experiments. *p<0.01 and **p<0.05 as compared with value for N.C. siRNA or undifferentiated cells. U: undifferentiated cells, D: differentiated cells.
stearoyl-CoA desaturase (SCD), CD36, acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, and lipoprotein lipase (LPL) were increased about 1.2 to 1.7-fold as compared with those of cells transfected with N.C. siRNA (Fig. 4). On the contrary, the expression levels of liver X receptor (LXR) α and LXRα-target genes, i.e., sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS), were not altered by the transfection with Foxa1 siRNA (Fig. 4). These results reveal that Foxa1 was involved in the suppression of the PPARγ and C/EBPα-mediated pathway, but not the LXRα-mediated pathway, during adipocyte differentiation in MSCs.

Functional expression of Foxa1 in MSCs
To further investigate the roles of Foxa1 in the regulation of adipogenesis in ST2 MSCs, we overexpressed Foxa1 heterologously in MSCs, and caused the transfected cells to differentiate into adipocytes for 4 days. When MSCs were transfected with pFLAG-mFoxa1 (Fig. 5A), the accumulation of lipid droplets was decreased to about 57% as compared with that in MSCs transfected with the empty vector (FLAG-CMV2; Fig. 5B and C). Moreover, when Foxa1 was overexpressed, the transcription levels of PPARγ and C/EBPα genes were decreased to approximately 74 and 63%, respectively as compared with those obtained with the empty vector. However, the mRNA levels of C/EBPβ and C/EBPδ were not affected by this overexpression of Foxa1 (Fig. 5D). Furthermore, we investigated the expression levels of adipogenic and lipogenic genes during adipogenesis in MSCs overexpressing Foxa1. Overexpression of Foxa1 decreased the transcription levels of aP2, SCD, CD36, ACC, HMG-CoA reductase, and LPL to approximately 78-88% of those obtained with the empty vector-transfected MCSs (Fig. 6). On the contrary, the mRNA levels of LXRα, SREBP-1c, and FAS, which are regulated by LXRα, were not affected by Foxa1 overexpression. These results also suggest that Foxa1 suppressed the expression of the C/EBPα and PPARγ-mediated pathway, but not that of the LXRα-mediated pathway, and are quite consistent that the data obtained by Foxa1 siRNA-mediated knockdown (Fig. 4).

Several clinical and experimental studies suggest that the differentiation program of MSCs is regulated by various factors such as hormones, cytokines, and transcription factors. These factors are important for the regulation of the balance between osteoblastogenesis and adipogenesis in MSCs. Previous studies suggest the reciprocal roles of Runx2 and PPARγ during osteoblastogenesis and adipogenesis from a common MSCs. Noteworthily, activators of differentiation often inhibit other types of differentiation and vice versa; e.g., PPARγ acts as an inducer of adipogenesis, but inhibits osteoblastogenesis.

Our present results demonstrated that Foxa1 was not associated with the transcriptional regulation of LXRα and LXRα-target genes in MSCs. However, LXRα activates adipocyte differentiation via regulation of lipogenesis and adipocyte-specific gene expression through PPARγ activation in 3T3-L1 cells. The discrepancy between our current results and this previous report may be due to the cell specificity of 3T3-L1 cells and MSCs. Thus, the regulatory mechanism of Foxa1-mediated suppression of C/EBPα and PPARγ and their target genes, but not that of LXRα-related genes, should be further elucidated in adipogenesis in MSCs.

In summary, Foxa1 was preferentially expressed in early-phase adipogenesis in MSCs. RNAi-mediated suppression or overexpression of Foxa1 during adipogenesis of MSCs impaired the expression of
Fig. 5. Functional expression of Foxa1 in MSCs

A, Heterologous expression of Foxa1 in ST2 MSCs. MSCs were transfected with pFLAG-mFoxa1 or empty (FLAG-CMV2) vector, and caused to differentiate for 4 days. Expression of recombinant FLAG-tagged Foxa1 was detected by Western blot analysis. B, Foxa1 suppresses accumulation of lipid droplets in differentiated MSCs. MSCs were transfected with FLAG-mFoxa1 or empty vector, and then made to differentiate into adipocytes for 2 days, after which they were incubated in medium with insulin alone for 2 more days. Lipid droplets were stained with Oil Red O. C, Oil Red O was extracted from lipid droplet-laden cells and quantified by colorimetric intensity. D, Expression of adipogenic transcription factors in Foxa1-transfected MSCs. Expression levels of PPARγ, C/EBPα, C/EBPβ, and C/EBPδ genes during adipocyte differentiation in MSCs were measured by quantitative PCR. Data are the means ± S.D. from three independent experiments. *p<0.01 as compared with value obtained with the empty vector.
Fig. 6. Adipogenic and lipogenic gene expression in Foxa1-overexpressed adipocytes in MSCs

ST2 MSCs were transfected with Foxa1 or empty vector and caused to differentiate into adipocytes for 4 days. Transcription levels of adipogenic and lipogenic genes were measured by quantitative PCR. Data are the means ± S.D. from at least 3 independent experiments. *p<0.01 and **p<0.05 as compared with value for the empty vector.
C/EBPα and PPARγ, and that of their target genes. Therefore, Foxa1 is a novel suppressor of adipogenesis in MSCs.

**EXPERIMENTAL**

**Cell Culture**

Mouse ST2 MSCs (bone marrow stroma cell-derived; Riken Bioresource Center, Ibaraki, Japan) were cultured in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum and antibiotics, and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Adipocyte differentiation and Oil Red O staining

MSCs were made to differentiate into adipocytes by being incubated in RPMI1640 containing insulin (10 μg/ml; Sigma), 1 μM DEX (Sigma), and 0.5 mM IBMX (Sigma). After 2 days, the medium was replaced with RPMI1640 containing insulin (10 μg/ml) alone and changed every 2 days. Oil Red O staining was carried out as described earlier. For quantification of Oil Red O staining, stained cells were treated with isopropanol for 10 min to extract the Oil Red O, and the colorimetric intensity of the extract was measured by spectrometry at 520 nm.

Osteoblastic differentiation and ALP staining

MSCs were caused to differentiate into osteoblasts by treating them with BMP2 (100 ng/ml; R&D Systems, Minneapolis, MN, USA) as described previously. ALP staining was performed as follows: Cells were washed twice with PBS, and then fixed with 10% (v/v) formaldehyde for 10 min at room temperature. The fixed cells were sequentially washed with PBS, incubated in 100 mM Tris-Cl (pH 9) containing 2 mM MgCl₂, 0.1 mg/ml Naphthol AS-MX phosphate (Nacalai Tesque, Kyoto, Japan), 0.6 mg/ml Fast Blue BB salt (Sigma), 5% (v/v) N,N-dimethylformamide at 37°C, washed again with PBS and observed the cells microscopically.

RNA extraction, cDNA synthesis, and quantitative PCR analysis

Total RNA was extracted by the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and further purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand cDNAs were synthesized from total RNA (1 μg) by using ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan) and random-hexamer (Takara-Bio, Kyoto, Japan) at 42°C for 60 min after denaturation of the RNA at 72°C for 3 min, followed by heat-inactivation of the enzyme at 99°C for 5 min.

Quantification of mRNA levels was measured by using a real-time PCR system (LightCycler, Roche Diagnosis, Mannheim, Germany) and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnosis). Gene-specific primer sets (sequences available upon request) were designed by using a Universal ProbeLibrary (Roche Diagnostics). The expression level of the genes was normalized to that of TATA-binding protein (TBP).

**RNAi study**

Stealth siRNAs for mouse Foxa1 and Stealth Negative Control siRNA were obtained from Invitrogen. The following mouse Foxa1 siRNAs were used: Foxa1 siRNA#1, 5’-GAGGUAAUAGCUAGGUAGAUAGCAU-3’ and Foxa1 siRNA#2, 5’-CAGUGUUAAUGCAGCUUCCACAGUU-3’. Cells were transfected with Foxa1 siRNA or Negative Control siRNA (20 nM) by using Lipofectamine RNAiMAX (Invitrogen) according to the protocol prescribed by the manufacturer, and cultured in RPMI1640 supplemented with insulin (10 μg/ml)
for 2 days. When medium containing with insulin was replaced into new one, siRNA was transfected together every 2 days. RNA was extracted at the indicated times and measurement of mRNA level was performed as described above.

**Foxa1 expression vector and transient transfection**
The coding region of mouse Foxa1 was cloned into the FLAG-CMV2 expression vector (Sigma) to obtain pFLAG-mFoxa1, which produces Foxa1 as a fusion protein with a FLAG tag sequence. MSCs were transfected with pFLAG-mFoxa1 vector was transfected into MSCs by using FuGene 6 Transfection Reagent (Roche Diagnostics) according to the manufacturer’s instruction, and cultured in the presence of IBMX, DEX, and insulin. Two days after transfection, the medium was removed; and then fresh medium with insulin alone, was added, after which the cells were transfected again with pFLAG-mFoxa1 vector as described above. Two days after the second transfection, RNA was prepared and mRNA levels were quantified as described above.

**Western blot analysis**
The cells were lysed with RIPA buffer [50 mM Tris-Cl, pH 8.0 containing 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) NP-40] supplemented 1% (v/v) Triton X-100 with Protease Inhibitor Cocktail (Roche Diagnosis). Protein concentrations were measured with Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA). Proteins were separated by SDS-PAGE (e-PAGEL, 10% (w/v); ATTO, Tokyo, Japan) and transferred onto PVDF membranes (Immobilon P; Millipore, Bedford, MA, USA). Western blot analysis was performed with a SNAP i.d. Protein Detection System (Millipore). Blots were blocked with 0.5% (w/v) skim milk (0.2 μm iltered; Becton, Dickinson, Franklin Lakes, NJ, USA), incubated with anti-FLAG (1:2000; M2, Sigma) or anti-actin (1:1000; AC-15; Sigma) monoclonal antibody, and then incubated with anti-mouse IgG antibody conjugated with horseradish peroxidase (1:1000; GE Healthcare, Buckinghamshire, UK). Immunoreactive signals were detected by the use of an ECL Plus Western Blotting Detection System (GE Healthcare).

**Statistical analysis**
The data were presented as means ± S.D. and statistically analyzed by the use of the unpaired t test or Welch t test when variances were heterogeneous. p values < 0.05 considered significant.

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