# A transcription factor of lipid synthesis, sterol regulatory element-binding protein (SREBP)-1a causes G₁ cell-cycle arrest after accumulation of cyclin-dependent kinase (cdk) inhibitors

Masanori Nakakuki[1], Hitoshi Shimano[1,2], Noriyuki Inoue[1], Mariko Tamura[1], Takashi Matsuzaka[1], Yoshimi Nakagawa[1,2], Naoya Yahagi[2], Hideo Toyoshima[1], Ryuichiro Sato[3] and Nobuhiro Yamada[1]

1 Department of Internal Medicine (Endocrinology and Metabolism), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Japan
2 Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Japan
3 Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan

Sterol regulatory element-binding protein (SREBP)-1a is a unique membrane-bound transcription factor highly expressed in actively growing cells and involved in the biosynthesis of cholesterol, fatty acids, and phospholipids. Because mammalian cells need to synthesize membrane lipids for cell replication, the functional relevance of SREBP-1a in cell proliferation has been considered a biological adaptation. However, the effect of this potent lipid-synthesis activator on cell growth has never been explored. Here, we show that induction of nuclear SREBP-1a, but not SREBP-2, completely inhibited cell growth in inducible Chinese hamster ovary (CHO) cell lines. Growth inhibition occurred through G₁ cell-cycle arrest, which is observed in various cell types with transient expression of nuclear SREBP-1a. SREBP-1a caused the accumulation of cyclin-dependent kinase (cdk) inhibitors such as p27, p21, and p16, leading to reduced cdk2 and cdk4 activities and hypophosphorylation of Rb protein. In contrast to transactivation of p21, SREBP-1a activated p27 by enhancing stabilization of the protein through inhibition of SKP2 and KPC1. *In vivo*, SREBP-1a-expressing livers of transgenic mice exhibited impaired regeneration after partial hepatectomy. SREBP-1-null mouse embryonic fibroblasts had a higher cell proliferation rate than wild-type cells. The unexpected cell growth-inhibitory role of SREBP-1a provides a new paradigm to link lipid synthesis and cell growth.

Sterol regulatory element-binding protein (SREBP) family members have been established as transcription factors regulating the transcription of genes involved in cholesterol and fatty acid synthesis [1,2]. SREBP proteins are initially bound to the rough endoplasmic reticulum membrane and form a complex with SREBP cleavage-activating protein (SCAP), a sterol-sensing molecule, and insulin-induced gene 1 (Insig-1) [3]. On sterol deprivation, SREBP is cleaved to liberate the N-terminal portion containing a basic helix–loop–helix leucine zipper domain, and enters the nucleus where it can bind to specific sterol response elements (SRE) in the promoters of target genes and activate their transcription [1]. Three isoforms of SREBP are known:

SREBP-1a, -1c, and -2. Whereas SREBP-2 plays a crucial role in the regulation of cholesterol synthesis, SREBP-1c controls the gene expression of enzymes involved in the synthesis of fatty acids and triglycerides in lipogenic organs [4,5]. Meanwhile, SREBP-1a is highly expressed in cells that are actively growing [6], and has strong transcriptional activity in a wide range of genes involved in the synthesis of cholesterol, fatty acids, and phospholipids. All mammalian cells require these lipids for the duplication of membranes in cell division. Depending on the cellular nutritional state and the availability of exogenous lipids, nuclear SREBP-1a is induced in growing cells. Therefore, the functional relevance of this potent lipid-synthesis regulator in cell proliferation has been considered a biological adaptation to meet the demand for cellular lipids. It has never been intensively explored whether this regulatory system for the synthesis of cellular lipids could inversely control cell growth. Recently, we reported that *p21*, a cyclin-dependent kinase (cdk) inhibitor, is a direct SREBP target gene, suggesting that the SREBP family may regulate the cell cycle [7].

In this study, we investigated the potential effects of SREBP-1a on cell growth when its active form was induced.

## Results

### SREBP-1a inhibits cell growth at G$_1$ in cultured cells

To assess the effects of SREBP-1a on cell growth, we examined the growth rates of a stable Chinese hamster ovary (CHO) cell line, in which the mature form of human SREBP-1a (CHO-BP1a) was inducibly expressed by addition of isopropyl thio-β-D-galactoside (IPTG) to the medium, by way of a coexpressed Lac repressor [8]. CHO cells expressing only the Lac repressor (CHO-Lac) were used as a negative control, while another inducible cell line for nuclear SREBP-2 (CHO-BP2) was established for comparison [9]. Overexpression of SREBP-1a completely suppressed cell proliferation 24 h after IPTG induction and the effect was sustained for up to 72 h (Fig. 1A). This observa-
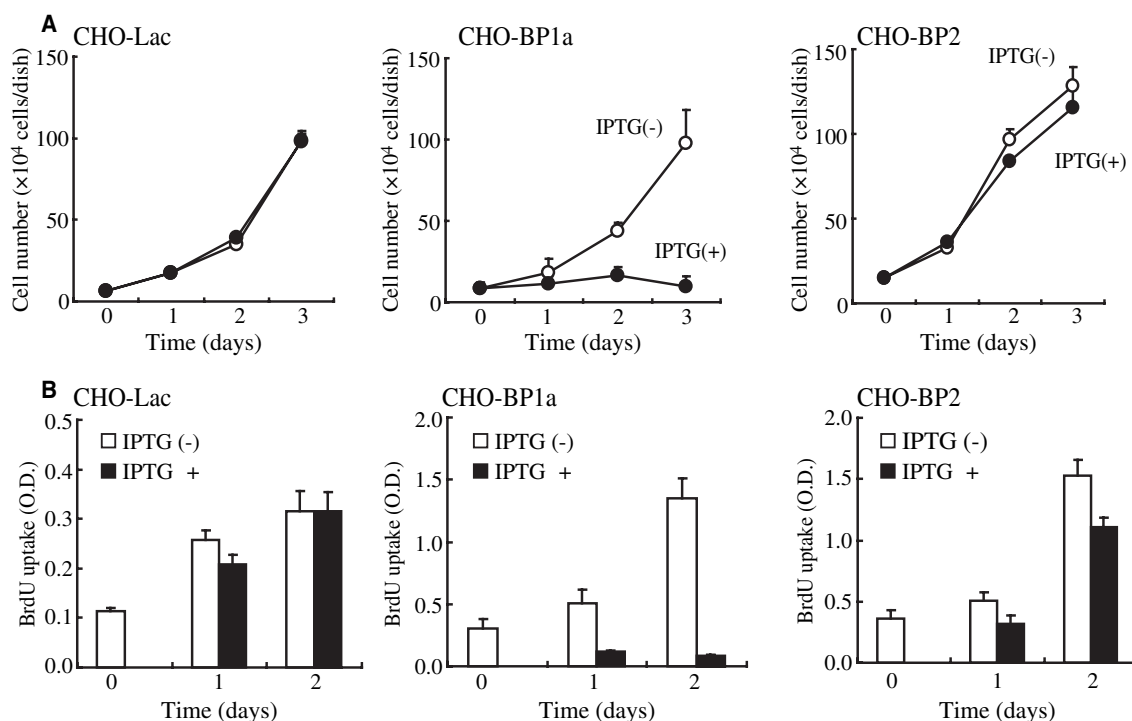


**Fig. 1.** Inhibition of cell proliferation by nuclear SREBP-1a. (A) Time courses of cell proliferation in CHO stable cell lines inducibly expressing nuclear SREBP-1a (CHO-BP1a) or SREBP-2 (CHO-BP2) under the control of an IPTG-regulated promoter, or only Lac repressor as a control (CHO-Lac). CHO stable cell lines were incubated in the absence (white circles) or the presence (black circles) of 0.1mM IPTG to induce expression of nuclear SREBPs. At the indicated days, the number of viable cells was measured using a hemocytometer. (B) BrdU uptake as index of DNA synthesis in CHO stable cell lines that inducibly express nuclear SREBPs. The cells with (black columns) or without (white columns) IPTG induction received a 2 h pulse of BrdU and the incorporation of BrdU into DNA was determined. Data represent mean ± SD in triplicate.

tion was specific to SREBP-1a and was not seen with SREBP-2, as the growth rates of CHO-Lac cells and the SREBP-2-expressing cell line (CHO-BP2) were almost identical and not affected by IPTG treatment (Fig. 1A). During the growth arrest of CHO-BP1a, cell detachment indicative of cell death was minimal (data not shown). However, DNA synthesis was essentially blocked in these cells, as evidenced by the lack of bromodeoxyuridine (BrdU) incorporation (Fig. 1B), whereas control CHO-Lac and CHO-BP2 cells did not show significant changes. The level of induction of nuclear SREBPs in these cell lines was reported to be physiological, as the amounts of the transgene products were comparable with the levels of endogenous SREBPs in control cells cultured in lipoprotein-deficient medium, which is a standard manipulation for the induction of nuclear SREBPs [8,9]. As shown in

Fig. 2A,B, the level of endogenous human SREBP-1 nuclear protein induced in HeLa cells by incubation with delipidated serum (DLS) was comparable with that induced in CHO-BP1a cells by IPTG at 5 μM, which had already exhibited inhibition of growth. Addition of geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP) restored the growth inhibition caused by a high dose of simvastatin, an HMG-CoA reductase inhibitor, but did not do so in CHO-BP1a (Fig. 2C). Thus, it is unlikely that the cell-growth inhibition observed in CHO-BP1a cells was attributable to altered prenylation, as observed with statins. Simvastatin and cerulenin were added to CHO-BP1a as inhibitors of the biosynthesis of cholesterol and fatty acids, respectively. Neither attenuated the effect of SREBP-1a (Fig. 2C), excluding the possibility that the antiproliferation effect was attributable to
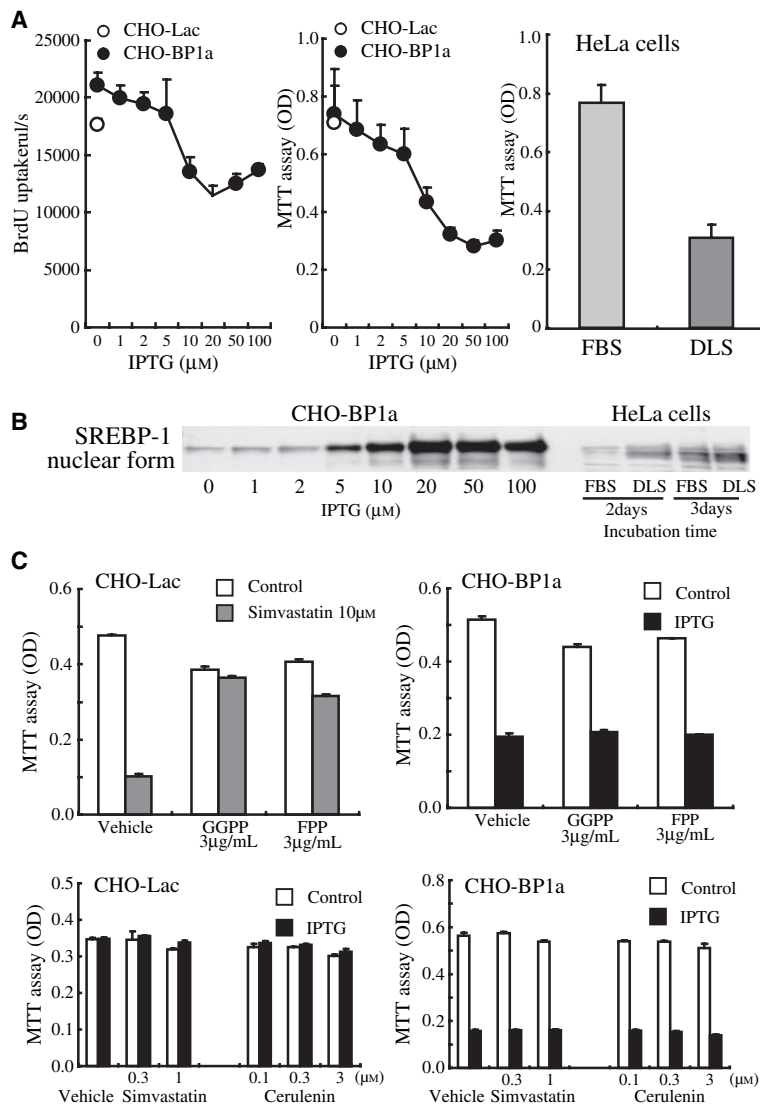


**Fig. 2.** (A) Dose-dependent inhibition of cell proliferation by nuclear SREBP-1a protein in (B) CHO-BP1a with a comparison with endogenous SREBP-1a induced by lipid-deprived condition in HeLa cells. CHO-BP1a cells and CHO-Lac were treated with the indicated dose of IPTG. After 2 days of incubation, MTT assay and BrdU uptake were estimated as described in Fig. 1. In the same procedure, nuclear SREBP-1a protein level in CHO-BP1a induced by IPTG was analyzed by immunoblotting. After HeLa cells had been grown in medium containing delipidated serum for 2 and 3 days, MTT assay for live cell number and estimation of nuclear SREBP-1a by immunoblotting analysis were performed. (C) The antiproliferative action of SREBP-1a was not due to sterol and prenyl synthesis inhibition and lipid accumulation. Stable cell line CHO cells were cultured with the indicated concentration of liposome containing GGPP or FPP, non-sterol metabolites of mevalonate, and with simvastatin or cerulenin to inhibit cholesterol and fatty acid synthesis, under IPTG 0.1 mM for 2 days. Live cell number was estimated by MTT assay. Values are mean ± SD in triplicate.

increased accumulation of cellular lipids. Flow cytometry revealed that the cessation of growth of CHO-BP1a occurred through G$_1$ cell-cycle arrest (Table 1). SREBP-1a and not SREBP-2 evoked a marked decrease in the number of cells in the S phase with a concomitant increase in the G$_1$ population. In transient transfection studies with an SREBP-1a expression plasmid and SREBP-inducible enhanced green fluorescent protein (EGFP) reporter, similar changes in the cell cycle were observed in various cell lines such as HEK293 cells, mouse fibroblast Swiss-3T3 cells, and human osteoblastoma Saos-2, a p53-deficient cell line (Table 2) [10]. These data show that the G$_1$ arrest induced by SREBP-1a is a universal phenomenon and is not mediated through p53, a well-known tumor suppressor that activates the transcription of p21, a cdk

**Table 1.** Cell-cycle profile of CHO-BP1a and CHO-BP2 cells inducibly expressing nuclear SREBP-1a and SREBP-2, respectively, with CHO-Lac cells as control. The three types of CHO stable cell line, after 24 h of culture with 0.1 mM IPTG, were trypsinized, collected, and stained with propidium iodide and analyzed by flow cytometry. Each value is mean ± SD. G$_2$/M, total of G$_2$ and mitotic S phase populations.

| Cell | IPTG | G$_0$/G$_1$ | S | G$_2$/M |
|---|---|---|---|---|
| CHO-Lac | − | 40.7 ± 1.3 | 37.5 ± 1.6 | 21.8 ± 2.0 |
| | + | 40.4 ± 3.1 | 38.8 ± 1.9 | 20.8 ± 3.2 |
| CHO-BP1a | − | 49.4 ± 1.5 | 22.7 ± 3.8 | 27.9 ± 5.3 |
| | + | 73.7 ± 0.6** | 6.6 ± 1.0** | 19.6 ± 0.9 |
| CHO-BP2 | − | 34.7 ± 1.4 | 43.8 ± 4.0 | 21.6 ± 2.5 |
| | + | 33.2 ± 1.5 | 41.5 ± 0.6 | 25.3 ± 1.5 |

**$P < 0.01$ compared with IPTG non-treated group by Student's *t*-test.

**Table 2.** REBP-1a induces G$_1$ arrest in the three types of cell lines – HEK293, mouse fibroblast Swiss-3T3 cells, and human osteoblastoma Saos-2 cells. Cells were transiently transfected with the indicated expression vectors and the SRE-EGFP vector. Twenty-four hours later, cells were fixed in paraformaldehyde and permeabilized with ethanol followed by staining with propidium iodide. Cell-cycle profiles were estimated within the gate of EGFP-positive cell population. Each value is mean ± SD.

| Cell strain | Group | G$_0$/G$_1$ | S | G$_2$/M |
|---|---|---|---|---|
| HEK293 | pcDNA3.1(+) | 38.5 ± 2.9 | 21.2 ± 3.3 | 40.2 ± 2.4 |
| | SREBP-1a | 50.4 ± 2.0** | 13.8 ± 0.1** | 35.7 ± 2.1 |
| | p21 | 55.6 ± 0.4** | 22.3 ± 1.5 | 22.1 ± 1.2** |
| | p27 | 81.9 ± 1.5** | 5.8 ± 0.5** | 12.2 ± 1.8 |
| Swiss-3T3 | pcDNA3.1(+) | 49.7 ± 1.1 | 18.7 ± 0.6 | 32.0 ± 1.3 |
| | SREBP-1a | 59.5 ± 1.6** | 2.0 ± 1.1** | 28.5 ± 2.6 |
| Saos-2 | pcDNA3.1(+) | 45.4 ± 2.0 | 15.1 ± 2.8 | 39.6 ± 3.3 |
| | SREBP-1a | 53.2 ± 5.0* | 10.3 ± 2.2* | 36.5 ± 4.7 |

*$P < 0.05$, **$P < 0.01$ compared with pcDNA3.1(+) group by Dunnett's multiple comparison test.

**Table 3.** Mutated SREBP-1a does not induce G$_1$ arrest in HEK293 cells. ΔTA–SREBP-1a lacks the N-terminal *trans*-activation domain. YR–SREBP-1a loses the capability of binding to sterol response element of the target gene promoter. Each value is mean ± SD.

| Cell strain | Group | G$_0$/G$_1$ | S | G$_2$/M |
|---|---|---|---|---|
| HEK293 | pcDNA3.1(+) | 40.8 ± 2.9 | 20.4 ± 2.4 | 38.8 ± 3.6 |
| | ΔTA–SREBP-1a | 37.2 ± 2.6 | 23.0 ± 2.6 | 39.8 ± 1.2 |
| | YR–SREBP-1a | 40.8 ± 6.2 | 19.6 ± 3.5 | 39.7 ± 8.2 |
| | SREBP-1a | 51.7 ± 2.6** | 15.0 ± 3.7 | 33.3 ± 2.7 |

inhibitor [11]. To elucidate the functional domains of SREBP-1a involved in this growth-arrest effect, mutational analysis was performed (Table 3). When the N-terminal transactivation domain was deleted (ΔTA–SREBP-1a) [12], SREBP-1a-induced G$_1$ arrest was abolished. Its action was also cancelled by the introduction of a point mutation (YR–SREBP-1a) through which SREBP-1 loses its ability to bind to an SRE, which is generally found in promoters of known SREBP target genes, but still binds to an E-box as a consensus *cis*-element for bHLH proteins [13,14] (Table 3). Therefore, the effect of SREBP-1a on the cell cycle may be mediated through the transactivation of some SREBP target gene(s).

## Involvement of cdk inhibitors in the antiproliferaive action of SREBP-1a

It is highly plausible that cdk inhibitors and cell-cycle-related genes could be involved in the G$_1$ arrest caused by SREBP-1a [15]. We have recently identified *p21* as a direct target of SREBP-1 in the screening of upregulated genes in the liver of SREBP-1a transgenic mice using a DNA microarray [7]. Northern blot analysis showed that gene expression of *p27* and *p16/p19*, in addition to *p21*, was highly elevated only in CHO-BP1a cells, along with key enzymes in the biosynthetic pathways for cholesterol, fatty acids, and phosphatidylcholine (HMG-CoA synthase, FPP synthase, fatty acid synthase, and CTP : phosphocholine cytidylyltransferase α) (Fig. 3A), all of which are well-established SREBP-1a target genes. Luciferase reporter assays in HEK293 cells revealed that SREBP-1a activated mouse *p16* and *p21* promoters, though only marginally compared with an authentic SRE reporter, consistent with the increased mRNA levels in SREBP-inducible cells; however, it did not activate the promoters of *p19* and *p27* (Fig. 3B). Although a precise mechanism for the accumulation of p27 with SREBP-1a has yet to be clarified, p27 is known to be regulated mainly at the post-transcriptional level. Recent reports indicate that p27 protein is regulated through a
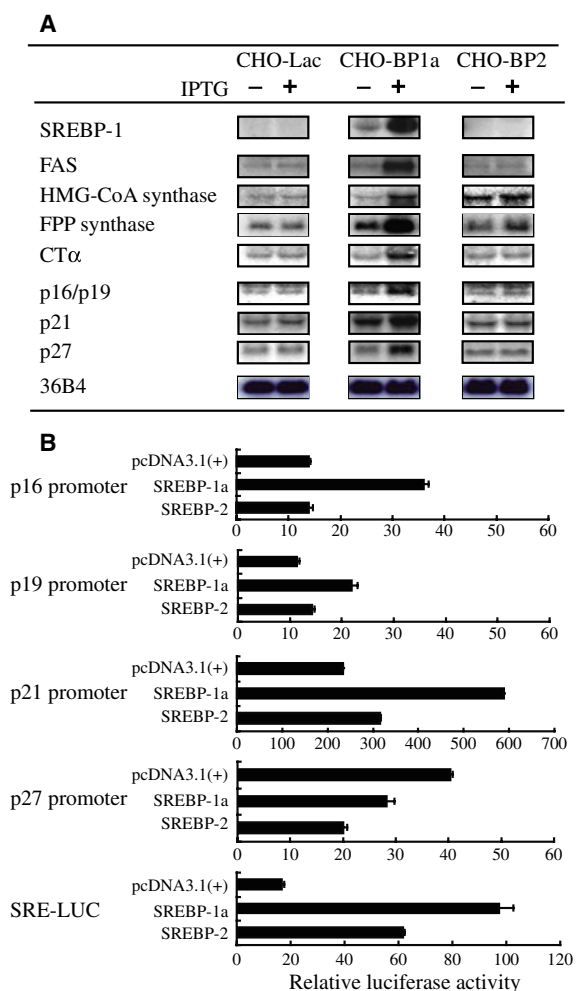
**A**



**B**



**Fig. 3.** Induction of cdk inhibitors by nuclear SREBP-1a. (A) Expression of genes involved in lipid synthesis and cdk inhibitors in relation to cell-cycle progression. Total RNAs (10 µg) were prepared from each CHO stable cell line (CHO-BP1a, CHO-BP-2 and CHO-Lac as control) 24 h after IPTG addition and used for northern blot analysis with the indicated cDNA probes. Fatty acid synthase (FAS), CTP : phosphocholine cytidylyltransferase α (CTα), 36B4 as loading control. (B) Transcriptional activation of SREBP-dependent promoter-reporter of cdk inhibitors. HEK293 cells were transfected with cdk inhibitor promoter–luciferase constructs fused to the 5′-flanking region of *p16*, *p19*, *p21*, *p27* genes and SRE–luciferase reporter as positive control in the absence or presence of nuclear form of SREBP expression plasmids. The cells were subjected to firefly-luciferase reporter assays with Renilla luciferase as reference. Values are means ± SD.

ubiquitin-dependent proteasome system [16]. Two ubiquitin ligase complexes, Skp1–Cullin1–F-box (SCF) and Kip1 ubiquitylation-promoting complex (KPC), are involved in p27 degradation at the G$_2$ and G$_1$ phases, respectively [17,18]. In CHO-BP1a cells, SKP2 and KPC1, which are key components of SCF and KPC, were markedly decreased by SREBP-1a

induction, at the mRNA level in both cases and at the protein level in SKP2, potentially explaining the p27 protein elevation (Fig. 4A,D). The data show that SREBP-1a regulates an assortment of genes involved in the control of cell proliferation.

On induction of exogenous SREBP-1a protein in CHO-BP1a cells, p21 and p27 proteins were markedly induced, as shown by immunoblot analysis (Fig. 4B). In accordance with the induction of these cdk inhibitors, SREBP-1a-expressing cells exhibited inhibition of cdk2 and cdk4 activities without any change in total protein level (Fig. 4C,D); in particular, the activity of cdk2, which plays an essential role in DNA synthesis and transition into the S phase [19], was almost abolished. Cyclins D and E were slightly decreased. Consequently, Rb protein, the major target of the cdk/cyclin complex, was mainly in a phosphorylated form in the growing control CHO cells (Fig. 4D) [20]. SREBP-1a expression caused a shift to the dephosphorylated form of Rb protein 24 h after induction by IPTG. Our data show that SREBP-1a inhibits the ability of cdk/cyclin complexes to phosphorylate Rb protein, resulting in cell-cycle arrest at the G$_1$ phase [16], and that this partly occurs through the induction of p21 and p27.

## Inhibition of cell growth by SREBP-1a *in vivo*

The antiproliferative activity of SREBP-1a observed in cultured cells was also tested *in vivo*. Partial hepatectomy is an established method for the synchronized induction of cell proliferation in a differentiated organ. Partial hepatectomy was conducted in wild-type and transgenic mice that overexpressed nuclear SREBP-1a in the liver [21] (Fig. 5). After 70% resection, wild-type mouse livers recovered to their original size in 10 days. SREBP-1a transgenic mice have huge, fatty livers containing large amounts of triglycerides and cholesteryl esters due to the activation of lipid synthetic genes [21]. In contrast to wild-type mice, SREBP-1a transgenic mice showed marked impairment in liver regeneration, with essentially no growth of the remaining liver, and about half of the mice died 1–2 days after partial hepatectomy. DNA synthesis in the livers was estimated by incorporation of injected BrdU (Fig. 5A). Consistent with the notion that most normal hepatocytes are in a quiescent stage, BrdU incorporation was very low in both wild-type and SREBP-1a transgenic livers prior to partial hepatectomy. At 36 and 48 h after partial hepatectomy, the number of BrdU-positive cells was dramatically increased in wild-type livers, indicating synchronized entry of the hepatocytes into the S phase. In contrast, overexpression of nuclear SREBP-1a completely suppressed BrdU incorporation in hepatocytes
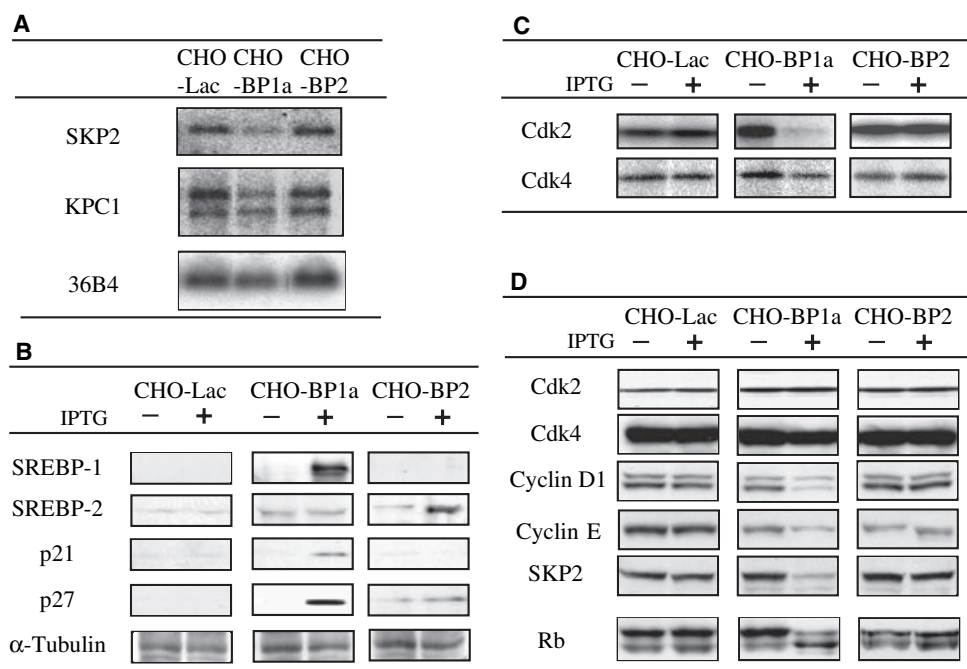
**Fig. 4.** Effects of nuclear SREBP-1a on the cell-cycle regulators, p21(Cip1), p27(KIP1), S-phase kinase-associated protein 2 (SKP2), ubiquitin ligase KPC1, cyclin D1, cyclin E expression, cdk2, cdk4 expression and related kinase activities and Rb protein phosphorylation. (A) Repression of SKP2 and KPC1 which regulate the ubiquitin-dependent degradation of p27 at G$_1$ and G$_2$ phase, respectively, in CHO cells inducibly expressing nuclear SREBP-1a (CHO-BP1a) and -2 (CHO-BP2) and control cells (CHO-Lac) as estimated by northern blot analysis. (B) Nuclear SREBPs, cdk inhibitor proteins cdk2, cdk4, cyclin D1, cyclin E, SKP2 protein levels, and phosphorylation of Rb protein in CHO-BP1a, CHO-BP2, and CHO-Lac after induction by IPTG. Cells were treated with IPTG for 1 day, and nuclear extracts and cell lysates were subjected to immunoblot analysis with antibodies against the indicated proteins. Alpha-tubulin was shown is the loading control. (C) Activities of cdk2 and cdk4 by SREBP-1a. cdk assay was carried out with cdk2 or cdk4 immunoprecipitates from 200 μg of protein of the cell lysates using Rb protein fragment and histone HI as substrate, respectively.

in transgenic mice, explaining the impaired liver regeneration. It has been established that partial hepatectomy leads to hepatic polyploidy, which reflects an increase in nuclear DNA content [22]. Hepatocytes from SREBP-1a transgenic mice had a higher proportion of 2N cells than did normal hepatocytes (Fig. 5B). SREBP-1a inhibited a change in the polyploidy pattern that was observed in livers from wild-type mice by flow cytometry 10 days after partial hepatectomy. The data provide supporting evidence that SREBP-1a overexpression inhibits cell proliferation *in vivo* as well as in cultured cells, though it is possible that the accumulation of huge amounts of lipids in the transgenic hepatocytes may contribute to the inhibition of cell growth.

### Effects of endogenous SREBP-1 on cell growth

To determine the physiological relevance of the growth-inhibitory action of SREBP-1a, the role of endogenous SREBP-1a in cell proliferation was examined in SREBP-1-null mice. Both cell growth and uptake of BrdU in mouse embryonic fibroblast (MEF) cells prepared from SREBP-1-null mice were significantly elevated compared with wild-type cells (Fig. 5C,D). Uptake of BrdU also tended to increase in hepatocytes from SREBP-1-null mice after partial hepatectomy (Fig. 5E). The data suggest that endogenous SREBP-1a plays a substantial role in the regulation of cell proliferation, though it is possible that SREBP-1c also makes a contribution.

The amounts of nuclear SREBPs, and thus their endogenous activities, in cultured cells are known to be highly induced under lipid-deprived conditions such as culture in DLS or lipoprotein-deficient serum, or with HMG-CoA reductase inhibitors due to activation of the SCAP/Insig system [23]. These lipid-deprivation manipulations induce endogenous nuclear SREBP-1a, as shown by immunoblot analysis of nuclear extracts from HeLa cells (Fig. 6A). The induction of nuclear SREBP-1 accompanied a reduction in cell proliferation and an increase in the population of cells at G$_1$ (Fig. 6A,C). The G$_1$-arrest antiproliferative effect in DLS was cancelled when an unsaturated fatty acid (oleate) was added to the medium in accordance with
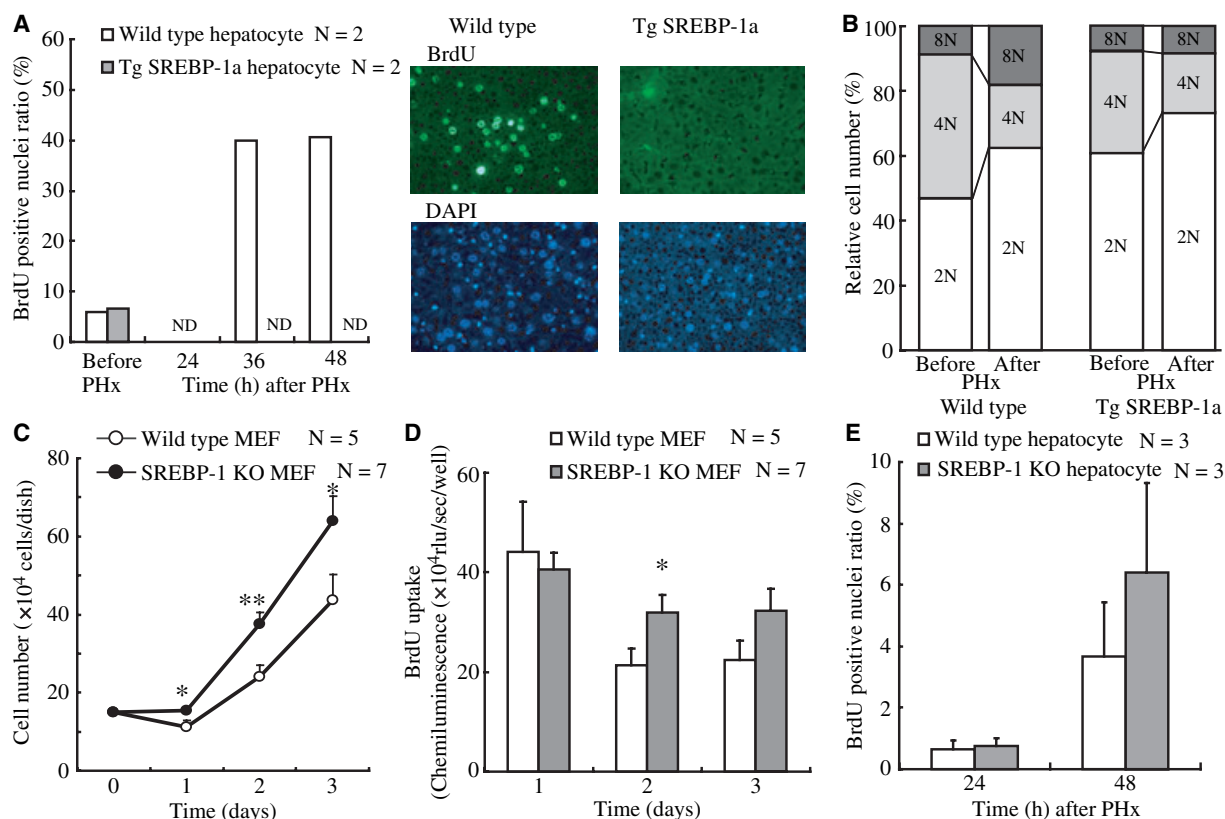
**Fig. 5.** Effects of SREBP-1a on cell growth *in vivo*. Impaired liver regeneration after partial hepatectomy (PHx) in SREBP-1a transgenic mice (A, B) and enhanced cell growth in MEF cells (C, D) and livers from SREBP-1-null mice (E). SREBP-1a transgenic mice overexpressing nuclear human SREBP-1a under the control of rat phosphoenolpyruvate carboxykinase promoter were established as described previously [21]. Non-transgenic littermates (wild-type) were used as controls. Each group of animals was fed a high protein/low carbohydrate diet for 5 days to induce transgene expression. Animals were deprived of food from 6 h before partial hepatectomy. (A) BrdU uptake of hepatocytes at the indicated times (h) after partial hepatectomy from SREBP-1a transgenic mice and wild-types (left graph). BrdU immunostaining and DAPI staining for nuclei were performed as described in Experimental procedures (right panels at 48 h). The incorporation rates of BrdU in livers from SREBP-1a transgenic wild-type mice were represented with the ratio BrdU-positive nuclei to DAPI-stained nuclei. ND, no detection of BrdU-positive nuclei. (B) Analysis of ploidy in hepatocyte cell nuclei by flow cytometry. Nuclei were isolated from resected liver (pre PHx) at the time of partial hepatectomy and from remnant liver 9–10 days after partial hepatectomy (post PHx). Hepatocyte ploidy is shown as 2n, 4n, and 8n. (C) Cell proliferation and (D) BrdU uptake in MEF cells from SREBP-1-null mice and wild-type littermate mice. Results are expressed as the means ± SD of five or seven independent experiments. \*\**P* < 0.01, \**P* < 0.05 compared with littermates by Student's *t*-test. (E) Uptake of BrdU in hepatocytes from SREBP-1-null mice and wild-type littermate mice after partial hepatectomy.

the suppression of nuclear SREBP-1 (Fig. 6A,C). Meanwhile, cholesterol did not suppress nuclear SREBP-1 or restore cell growth. Similar regulation by oleate was observed in Swiss-3T3 fibroblasts (Fig. 6B). Our data indicate that lipid regulation by endogenous SREBP-1a contributes to the cell cycle and growth.

## Discussion

### SREBP-1a causes G$_1$ arrest through cdk inhibitors

SREBP-1a is highly expressed in actively growing cells and has been considered to be a master transcription factor in lipid synthesis. This study clearly demonstrates that nuclear SREBP-1a can also regulate the cell cycle and growth. Thus, lipid synthesis in proliferating cells is not simply a secondary event under the regulation of cell growth [24], but rather, actively controls cell growth. This unexpected observation explains the difficulty in obtaining cell lines that highly express nuclear SREBP-1a, unlike those that express SREBP-1c and SREBP-2.

Recently, we reported that both SREBP-1a and SREBP-2 directly activate the promoter of the *p21* gene, partially explaining this hypothesis [7]. However, current studies on various cell types show that an
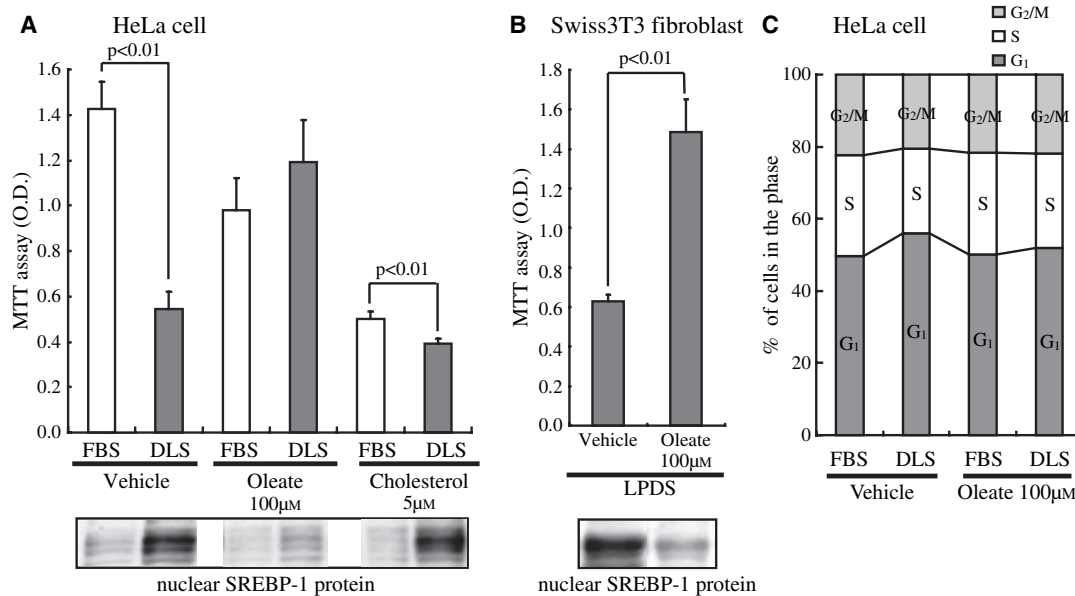
**Fig. 6.** Effects of endogenous SREBP-1a on cell growth and cell cycle in lipid deprivation. (A) Effects of DLS, and rescue effect of oleate (unsaturated fatty acid) and cholesterol on cell proliferation in cultured cells were tested. HeLa cells were plated at $0.5 \times 10^4$ cells per well in 24-well plates. After 1 day's incubation, the medium was switched to DMEM containing 5% DLS, in the presence or absence of 100 μM oleate or 5 μM cholesterol. DMEM containing 10% fetal bovine serum was control. For estimation of cell number, MTT assay (A) and cell-cycle analysis by FACS (C) were performed after 2- and 1-day incubation, respectively. In another set of experiments, nuclear forms of SREBP-1 protein were detected by immunoblot analysis on nuclear extracts from the cells (A, lower). (B) Swiss-3T3 fibroblasts subjected to lipid starvation by lipoprotein deficient medium were incubated with 100 μM oleate. MTT assay and immunoblotting for detection of nuclear SREBP-1 protein (lower) after two and one days, respectively, were performed in the same procedure described previously. $P < 0.01$ compared with fetal bovine serum control group by Student's *t*-test. Values are mean ± SD.

abundance of nuclear SREBP-1a induces various cdk inhibitors, such as *p27* and *p16*, in addition to *p21*, leading to G$_1$ arrest in cell growth. In the current experimental setting, the antiproliferative action was observed only with SREBP-1a; however, SREBP-2 might have a similar, though less efficient, action. The mechanisms for the activation of individual cdk inhibitors are diverse and complex (scheme shown in Fig. 7). Because the ability of SREBP-1a to cause G$_1$ arrest depends on its transcriptional activity (Table 3), some unknown SREBP-1a-regulated genes may also be involved in the mechanisms in addition to direct activation of p21 [7], and repression of SKP2 and KPC1. The relative contributions of factors such as p27, p21, p16, to this new action of SREBP-1a remain unknown, but presumably depend on cell type. Further investigations are needed to clarify the more detailed mechanisms and identify the major upstream mediator(s).

It is well established that the amounts of nuclear SREBPs are regulated by the sterol-regulated cleavage system and primarily depend on cellular demand for sterols. In previous reports, enhanced proliferation on activation of the phosphatidylinositol 3-kinase/Akt pathway, has been linked to activation of SREBP-1a

[25,26]. More recently, it has been reported that activation of SREBP-1a is crucial for cell growth [27,28]. In contrast, our data imply that the presence of abundant nuclear SREBP-1a, indicating that cells are deficient in lipid stores, not only activates transcription of its target genes involved in lipid synthesis, but also delays cell growth, particularly in case of severe depletion with very strong activation of SREBP-1a, until a time when sufficient lipids are available for membrane synthesis. In this respect, our data apparently contradict previous reports indicating a link between SREBP-1a and cell growth. However, SREBP-1a may have biphasic effects depending on its nuclear amount. In the absence of IPTG, incorporation of BrdU was greater in CHO-BP1a and CHO-BP2 cells than in CHO-Lac cells (Figs 1,2). Because expression of SREBP-1a in CHO-BP1a cells may be leaky (Fig. 3A), one interpretation is that both transcription factors promote proliferation at low expression levels (i.e. in the absence of IPTG), whereas overexpression of SREBP-1a blocks proliferation. In knockout studies, trends of increasing cell growth and uptake of BrdU in SREBP-1-null MEFs or hepatocytes were marginal and may be related to compensated activation of SREBP-2.
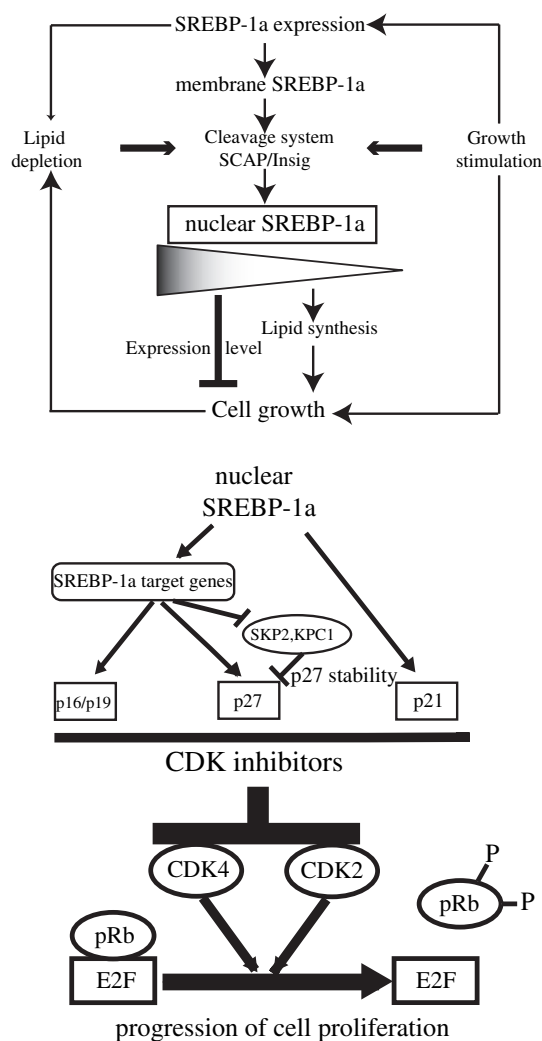
**Fig. 7.** Schematic diagram illustrating the mechanisms by which SREBP-1a causes cell-cycle G$_1$ arrest.

Considering these biphasic actions, the physiological roles of SREBP-1a in the regulation of cell growth may be complex, and should be investigated carefully. Unsaturated fatty acids suppressed the cleavage of SREBP-1, consistent with previous studies [29,30], and cancelled the cell-growth inhibition (Fig. 6). These data suggest that regulation of SREBP-1a may be related to cellular fatty acid metabolism linked to cell growth, although a lack of oleate could affect cell growth independent of SREBP-1.

## Physiological relevance of SREBP-1a activation

Recently, an intriguing study was reported suggesting that SREBP-1a is involved in regulation of the cell cycle. Nuclear SREBP-1a is hyperphosphorylated at

G$_2$/M, which is associated with increased transcriptional activity, explaining the activation of lipid synthetic genes at mitosis [31]. In addition to G$_1$ arrest, our data suggest that nuclear SREBP-1a could potentially modify the cell cycle at the G$_2$/M phase. No marked reduction in the number of cells in the G$_2$/M phase was observed despite a marked decrease in S-phase cells in SREBP-1a overexpression, indicating a concomitant G$_2$/M arrest by SREBP-1a.

The nuclear forms of SREBPs have been speculated to be degraded by the ubiquitin–proteasome pathway, because N-acetyl-leucyl-leucyl-norleucinal, a calpain inhibitor, stabilizes them experimentally [32]. Recently, it was reported that Fbw7, an F-box and a component of an SCF-type ubiquitin ligase complex, is responsible for the degradation of SREBP-1a after phosphorylation by GSK-3 [33]. Fbw7 in SCF also regulates the stability of c-Myc, cyclin E, and c-Jun and the JNK signal, supporting its involvement in cell growth. It can be speculated that the cellular lipid balance regulates SREBP-1a activity through cleavage by the SCAP/Insig system, whereas cell-cycle-associated regulation involves the stability of nuclear SREBP-1a through Fbw7 activity. Thus, both SREBP-1a and p27 are regulated by SCF ubiquitin pathways in a cell-cycle-dependent manner and could thereby regulate the cell cycle and growth. It is important to investigate endogenous Fbw7 activity in relation to the cell cycle and lipid availability.

Our data also suggest a new mechanism for the anti-proliferative activity of statins, which are HMG-CoA reductase inhibitors [34], through the activation of nuclear SREBP-1a, though the main mechanism has been considered to be inhibition of protein prenylation [35]. Further studies of this strong lipid synthetic factor will reveal new aspects of a link between the regulation of lipid synthesis and the cell cycle and growth.

# Experimental procedures

## Cell proliferation and cell-cycle analysis of CHO stable cell lines

CHO cell lines, CHO-BP1a and CHO-BP2, expressing a mature form of human SREBP-1a (amino acids 1–487) and human SREBP-2 (amino acids 1–481), respectively, with a Lacswitch inducible mammalian expression system, and CHO cells constitutively expressing the Lac repressor (CHO-Lac) were constructed as described previously [8,9]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U·mL$^{-1}$ penicillin, and 100 µg·mL$^{-1}$ streptomycin and incubated at 37°C in a humidified 5% CO$_2$ atmosphere. For induction

of SREBP, IPTG was added to the medium at 0.1 mM. For cell-proliferation analysis, cells were seeded at $1 \times 10^5$ per 10 cm dish. At the indicated time after treatment with 0.1 mM IPTG, the cells were trypsinized, collected, and counted using a hemocytometer. To determine of the BrdU uptake, $1.5 \times 10^3$ cells per well were harvested in a 96-well plate. After 24 h of treatment with 0.1 mM IPTG, the cells were incubated with 10 μM BrdU for 4 h in a CO$_2$ incubator at 37°C, and BrdU uptake was measured with a BrdU Labeling and Detection kit (Roche Diagnostics, Basel, Switzerland) or cell proliferation ELISA, BrdU (chemiluminesence) (Roche Applied Science Inc., Basel, Switzerland). For determination of the cell-cycle profile, the cells were harvested and resuspended with 0.1% Triton X-100 in NaCl/P$_i$ solution containing 0.1 mg·mL$^{-1}$ of RNAse and 25 μg·mL$^{-1}$ of propidium iodide (Sigma Chemical Co., St Louis, MO). The stained cells were examined by flow cytometry (FACScaliber; Becton Dickinson, Franklin Lakes, NJ). For all experiments, the cells were harvested at pre-confluency, the stage of exponential proliferation.

## Expression plasmids and cell-cycle analysis of transiently transfected cell lines

cDNAs encoding a mature form of human SREBP-1a (amino acids 1–487) and human SREBP-2 (amino acids 1–481), a transactivation domain-deleted form of SREBP-1a, and a YR-mutant of SREBP-1a (substitution of tyrosine at amino acid 335 for arginine) were inserted into a pcDNA3.1(+) expression plasmid [13,14] (Invitrogen, Carlsbad, CA). An SRE–EGFP vector encoding an enhanced green fluorescent protein under control of the SRE was prepared by subcloning a region containing the SRE and Sp1 site derived from the human LDL receptor [36] into pEGFP-1 (Clontech Laboratories Inc., Palo Alto, CA). Transfection studies were conducted with cells plated on 10 cm dishes using Transfection Reagent Fugene 6 (Roche Diagnostics). For suppression of intrinsic SREBP, 25-hydroxycholesterol was added to the medium 4 h after transfection. Twenty-four hours after transfection, the cells were harvested, fixed, permeabilized, and resuspended in NaCl/P$_i$ containing propidium iodide and RNAse. EGFP-positive cell populations expressing transfected nuclear srebps were analyzed by flow cytometry [37].

## Northern blot analysis and immunoblot analysis

Total RNA was isolated from the cells using Trizol reagents (Life Technologies, Rockville, MD) and subjected to northern blot analysis as described previously [38] using the indicated $^{32}$P-labeled cDNA probe. Total cell lysates and nuclear extracts from CHO cells were prepared as described previously [39,40] and subjected to immunoblot analysis using the indicated monoclonal or polycolonal antibodies (IgG). Horseradish peroxidase-linked mouse or rabbit IgG was used as a secondary antibody and the target protein was visualized using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

## Cloning of promoter of cdk inhibitor and transfection and luciferase assay

A *Sac*I–*Xho*I fragment of human p16 INK4A, an *Nhe*I–*Hin*dIII fragment of human p19INK4D, and a *Bgl*II–*Hin*dIII fragment of mouse p27(KIP1) extending from the 5′-UTR to each promoter region were subcloned into a pGL3 basic vector (Promega, Madison, WI). The primers used for PCR were as follows: P16: 3′ primer, 5′-TGCCTGCTCTCCCC CTCTCC-3′, 5′ primer, 5′-GCCACCGCGTCCTGCTCCA AAG-3′; p19: 3′ primer, 5′-ACACTGGCGGCCTGACAA AG-3′, 5′ primer, 5′-AGCTCGTAGTAAGGGCCAATGA ATGTTCT-3′; p27: 3′ primer, 5′-CAAAACCGAACAAA AGCGAAACGCCA-3′, 5′ primer, 5′-CAACCCATCCAA ATCCAGACAAAAT-3′. All constructs were confirmed by sequencing. The *p21* (Waf1/Cip1) promoter luciferase construct has been described previously [7]. For transfection and luciferase assay, HEK293 cells were cultured in DMEM containing 25 mM glucose, 100 unit·mL$^{-1}$ penicillin, and 100 μg·mL$^{-1}$ streptomycin sulfate supplemented with 10% fetal bovine serum. On day 0, cells were plated on a 24-well plate at $2.5 \times 10^4$ per well. On day 1, each luciferase reporter plasmid (0.25 μg) and pRL-SV40 reference plasmid (0.02 μg) (Promega) were transfected into cells using the transfection reagent Fugene 6 (Roche Diagnostics) according to the manufacturer's protocol. Expression plasmid (pcDNA3.1(+)–SREBP-1a, -1c, or -2) (0.25 μg) or basic plasmid pcDNA3.1(+) as a negative control were also cotransfected. Four hours after transfection, cells were exchanged into fresh medium, followed by culture for 1 day before harvesting. The luciferase activity was measured and normalized to the activity of co-transfected pRL-SV40 Renila luciferase reporter.

## Immunoprecipitation kinase assay of cdk2 and cdk4

Cdk2 and cdk4 were immunoprecipitated with mouse monoclonal anti-cdk2 and anti-cdk4 sera (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The immunocomplexes were then subjected to an *in vitro* kinase assay with cdk2 substrate histone HI protein (Santa Cruz Biochemistry) and the cdk4 substrate, Rb protein fragment (Santa Cruz Biochemistry), as described previously [41].

## Partial hepatectomy of SREBP-1a transgenic mice and SREBP-1 knockout mice

All animal studies were approved by the Animal Care Committee of the University of Tsukuba. The mice were

housed in colony cages, maintained on a 12 h light/12 h dark cycle, and given free access to water and a standard chow diet (MF, Oriental yeast).

Transgenic mice expressing a mature form of human SREBP-1a [21], SREBP-1-null mice, and littermates (wild-type) were subjected to partial hepatectomy as described previously [42]. Approximately 70% of each liver was resected. For *in vivo* BrdU incorporation experiments, mice were given an intravenous injection of BrdU (60 mg·kg) 2 h before sacrifice. Liver tissue was immediately fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned. Brdu immunohisochemistry was performed using the Amersham cell proliferation kit. The number of BrdU-positive hepatocytes was counted. For cell-cycle profile analysis of hepatocytes, resected and remnant livers were minced and filtered through a filter mesh (BD Falcon cell strainer), and examined by flow cytometry.

## Growth rate of embryonic fibroblasts from SREBP-1 knockout mice

Primary MEFs were prepared from post-implantation embryos (day 13–15) derived from the mating of hemizygote SREBP-1-null mice. The genotype for SREBP-1+/+ and –/– was determined by Southern blot analysis [43]. MEFs of both genotypes were grown in DMEM supplemented with 10% fetal bovine serum. For cell-growth assays, MEFs were seeded at a density of $0.5 \times 10^5$ cells per 10 cm dish on day 0. Cell numbers were counted daily using the hemocytometer. Data were collected from triplicate cell counts from five to seven independent experiments. For DNA synthesis measurements, MEFs were plated at a density of $0.5 \times 10^4$ cells per well in a 24-well plate. On the indicated day, a BrdU uptake assay was performed according to the manufacturer's protocol.

## Response of cell proliferation and SREBP-1 protein to lipid starvation

For lipid starvation, DMEM containing DLS, which was prepared from fetal bovine serum as described previously [30], and lipoprotein-deficient serum (Sigma) was used. HeLa cells and Swiss-3T3 fibroblasts were plated at a density of $0.5 \times 10^4$ cells per well in 24-well plates and grown for 1 day in DMEM containing 10% fetal bovine serum and antibiotics. The medium was switched to standard medium (DMEM +10% fetal bovine serum), delipidated medium or lipoprotein-deficient medium. After 2 days of incubation, a cell viability test was performed using the MTT assay to estimate quantitatively the number of cells. For cell-cycle analysis, the cells were harvested after 2 days of lipid starvation and samples for FACS were prepared by the method described previously. To rescue lipid-deprived cells, a fatty acid and cholesterol addition experiment was performed. HeLa cells were treated with 100 μM oleate or 5 μM cholesterol together with the lipid-deprived medium, and Swiss-3T3 fibroblasts were treated with 100 μM oleate, followed by the MTT assay and cell-cycle analysis. For estimation of the nuclear form of SREBP-1 protein, nuclear extracts from HeLa cells and Swiss-3T3 fibroblasts were prepared and subjected to immunoblot analysis as described previously.

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