Sterol-regulatory-element-binding protein 2 and nuclear factor Y control human farnesyl diphosphate synthase expression and affect cell proliferation in hepatoblastoma cells

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INTRODUCTION

FDPS (farnesyl diphosphate synthase) catalyses the formation of farnesyl diphosphate, a key intermediate in the synthesis of cholesterol and isoprenylated cellular metabolites. FDPS is also the molecular target of nitrogen-containing bisphosphonates, which are used as bone-antiresorptive drugs in various disorders. In the present study, we characterized the sterol-response element and NF-Y (nuclear factor Y) binding site in the human FDPS promoter. Using a luciferase assay, electrophoretic mobility-shift assay and chromatin immunoprecipitation assay, we demonstrated that these elements are responsible for the transcription of the FDPS gene, and that their transcriptional activation is mediated by SREBP-2 (sterol-regulatory-element-binding protein 2) and NF-Y. We also investigated whether sterol-mediated FDPS expression is involved in the cell proliferation induced by zoledronic acid, an FDPS inhibitor. We show that the SREBP-2 and NF-Y-mediated regulation of FDPS gene transcription modulates cell proliferation. These results suggest that SREBP-2 and NF-Y are required to trigger cell proliferation through the induction of FDPS expression and that the pharmacological action of zoledronic acid is involved in this pathway.

Key words: cell proliferation, farnesyl diphosphate synthase (FDPS), nuclear factor Y (NF-Y), promoter, sterol-regulatory-element-binding protein (SREBP), zoledronic acid.
sterol-mediated promoters include either an NF-Y-binding site or an Sp1-binding site in addition to SREs.

It has been demonstrated that the promoter of the rat Fdp5 gene contains three cis-acting elements [24]. These elements, an SRE and two NF-Y-binding sites, interact with SREBP-1a and NF-Y respectively. Although FDPS has recently attracted attention as a pharmacological target, the regulation of the human FDPS promoter is unknown. In the present study, we show that the human FDPS promoter is regulated by sterols via a mechanism involving SREBP-2 and NF-Y. The human FDPS promoter is shown to have a functional SRE and one NF-Y-binding site. We also examined the relationship between sterol-regulated FDPS and the effect of zoleodronic acid on cell proliferation, a nitrogen-containing bisphosphonate. We show that FDPS expression induced by SREBP-2 and NF-Y modulates cell proliferation in human hepatoblastoma cells. These results suggest that SREBP-2 and NF-Y are required to trigger cell proliferation through the induction of FDPS expression and that the pharmacological action of zoleodronic acid is involved in this pathway.

EXPERIMENTAL

Cell culture

Huh7 and HepG2 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with heat-inactivated FBS (fetal bovine serum) (5% for Huh7 cells and 10% for HepG2 cells), 100 IU/ml penicillin and 100 μg/ml streptomycin. For the sterol-depletion experiments, the cells were cultured in DMEM supplemented with LPDS (lipoprotein-deficient serum). In some experiments, the cells were cultured in DMEM supplemented with either 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol or 50 μM pitavstatin [an HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitor] plus 50 μM sodium deoxomalone.

SL2 (Schneider line 2) cells, a continuous Drosophila cell line, were maintained in Schneider’s medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin supplemented with 10% (v/v) FBS and were grown at 23°C.

Plasmid constructs

The mature human SREBP-2 expression vector (pcDNA3-nSREBP2, amino acids 1–468) was obtained by subcloning pCMV-nSREBP-2 into the pcDNA3 vector (Invitrogen) [25]. The mature human SREBP-1a expression vector (pcDNA3-nSREBP1a, amino acids 1–460) was constructed as described previously [26]. The human FDPS expression vector (pcDNA3-hFDPS) was created by cloning the appropriate cDNA from HepG2-tet-off-hLXRα cells into the pcDNA3 vector [27]. The murine NF-YA29-expressing plasmid (pcDNA3-NF-YA29) has also been described previously [25]. pPac, a Drosophila actin 5C promoter-driven expression vector, pPac-β-gal, pPAC-nSREBP1a, pPacNF-YA, pPacNF-YB and pPacNF-YC have been described previously [25]. To generate human FDPS promoter-reporter plasmids, the FDPS promoter containing nucleotides −1539 to +27 (FDPS-1539) was isolated by PCR from human genomic DNA extracted from HepG2 cells. The promoter was cloned into the PGV-B vector (Toyo Ink). A deletion construct was made and site-directed mutations were introduced into the FDPS promoter by PCR. The nucleotide sequences of these plasmids were confirmed by sequencing on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

RNA extraction and real-time RT (reverse transcription)–PCR analysis

Total RNA extraction and cDNA synthesis were performed as described previously [26]. PCRs were performed with a QuantiTect® SYBR Green PCR kit (Qiagen). Amplification specificity was verified by visualizing the PCR products on an ethidium-bromide-stained 2% agarose gel. Cyclophilin A or 36B4 mRNA was used to normalize each expression datum. The primers used were: 5′-AAAGCAGGATTTCGTTCAGCA-3′, and 5′-GGAATGGCTACTACACCCTCA-3′ for FDPS; 5′-GAGTCCCGTACTGGAGCCAATC-3′ and 5′-ATGGTCCCCATAGTGAGATT-3′ for FDPS; 5′-GCCGTTCTTTTGAGCTGTTT-3′ and 5′-TCACACCCTCGACACATAACC-3′ for cyclophilin A; and 5′-TGTTTCTCATTGTGGAGCGACA-3′ and 5′-TGAGGCAGCAGTTTCTCAG-3′ for 36B4.

Immunoblot analysis

To prepare whole-cell extracts, Huh7 cells were lysed in 25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40 (Nonidet P40), 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma). Each cell extract was resolved by SDS/PAGE (10% gels) and electroblotted on to nitrocellulose membrane. Western blot analysis was performed using anti-FDPS antibody (Abgent), anti-NF-YA antibody (Santa Cruz Biotechnology), anti-actin antibody (Santa Cruz Biotechnology) or anti-lamin B2 antibody (Abcam). The signals were visualized with the SuperSignal West Dura Extended Duration chemiluminescent substrate (Pierce).

Transfections and luciferase assay

DNA transfection of HepG2 cells was performed with Lipofectamine™ 2000 (Invitrogen), as described previously [26]. At 1 day before transfection, the cells were seeded at a density of 1.5 × 10^4 cells/well on 24-well plates. The cells were transfected with 200 ng of the FDPS reporter plasmid, 250 ng of pSV-β-galactosidase (Promega) and 100 ng of expression vector. At 4 h after transfection, the cells were cultured in DMEM supplemented with 10% (v/v) FBS. After 20 h, assays for both luciferase and β-galactosidase activities were performed. The reporter activities were expressed as the relative luciferase activity/β-galactosidase activity.

DNA transfection of Huh7 cells was carried out with Lipofectamine™ 2000, as described by the manufacturer. The cells were seeded at a density of 1.2 × 10^5 cells/well on 24-well plates, and transfected with 300 ng of luciferase reporter plasmid, 400 ng of pSV-β-galactosidase (Promega) and 100 ng of expression vector. The cells were harvested 24 h after transfection, and luciferase assays were performed as described above.

In the experiment using the dominant-negative NF-Y mutant (pcDNA3-NF-YA29), HepG2 cells were co-transfected with 200 ng of the FDPS reporter plasmid, 250 ng of pSV-β-galactosidase, 1 ng of pcDNA3-nSREBP1a, and 5, 25 or 100 ng of the NF-YA29 expression vector. The total amount of DNA was adjusted to 550 ng by the addition of the pcDNA3 vector. DNA transfection of SL2 cells was carried out with Lipofectamine™ 2000, as described by the manufacturer. The cells were seeded at a density of 5 × 10^5 cells/well on 24-well plates, and transfected with 100 ng of luciferase reporter plasmid, 20 ng of pPac-β-galactosidase and the indicated amount of pPac-derived expression vector. The total amount of DNA was equilibrated by adding the appropriate amount of pPac vector. The
cells were harvested 24 h after transfection, and luciferase assays were performed as described above.

EMSA (electrophoretic mobility-shift assay)
Nuclear extracts were prepared from Huh7 cells that had been cultured in DMEM supplemented with LPDS for 16 h. EMSAs were performed as described previously [27,28]. In the competition studies, a 10- or 100-fold molar excess of unlabelled DNA was added to the reaction mixture. Supershift assays were performed using anti-human SREBP-2 (Perseus Proteomics), anti-NF-YA antibody (Santa Cruz Biotechnology) or anti-PDI (control) antibody. Double-stranded DNAs of the following sequences were used for the binding and competition assays: wild-type human FDPS SRE, 5′-GCCACGTTCAC-A CGACGGCCAG-3′; mutant FDPS SRE, 5′-GCCATGC-TacaACGAGGGCCAG-3′; wild-type FDPS NF-Y-binding site, 5′-GCCAGACGACACATCGCGGCT-3′; and mutant FDPS NF-Y-binding site, 5′-GCCAGACGACACTACGCGGCT-3′ (mutated bases are shown in lower-case letters).

ChIP (chromatin immunoprecipitation) assay
ChIP assays were performed as described previously [27,28]. Huh7 cells were cultured in DMEM supplemented with LPDS for 16 h. Antibody directed against SREBP-2 (Perseus Proteomics), NF-YA (Santa Cruz Biotechnology), mouse IgG (Santa Cruz Biotechnology) or rabbit IgG (Santa Cruz Biotechnology) was used. The precipitated DNAs were analysed by PCR using the primers 5′-CTAGACCTTCAGGCTTGCATG-3′ and 5′-GTGTCGGGTATCTCTCTAAAG-3′, which flank the SRE and NF-Y-binding site of the FDPS promoter.

Gene silencing experiments
Pre-designed siRNAs (small interfering RNAs) targeting SREBP-1, SREBP-2, NF-YA and FDPS mRNAs were provided by Qiagen. Huh7 cells were transfected with 25 nM siRNA using X-tremeGENE siRNA transfection reagent (Roche), as described by the manufacturer. A control, we used the AllStars negative control siRNA (Qiagen), the sequence of which has no significant similarity to any mammalian gene. For the siRNA experiments, total RNA was extracted using the QuickGene RNA cultured cell HC kit S (Fujifilm).

Assays for cellular proliferation
We obtained zoledronic acid from Sequoia Research Products and dissolved it in PBS. The effects of zoledronic acid on Huh7 cells were measured by the WST-8 (water-soluble tetrazolium salt 8) method (Cell Counting Kit-8; Wako Chemical) based on the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Briefly, 4 × 10^4 cells/well on 96-well plates were incubated for 24 h in LPDS- or sterol-containing medium. At 24 h after seeding, 0.1, 1, 5, 10 or 50 μM zoledronic acid was added to the cells and they were incubated for 48 h. Then, 10 μl of Cell Counting Kit-8 solution was added, and the cells were incubated for another 1 h. The number of surviving cells was measured using a microplate reader (Bio-Rad Laboratories).

For the siRNA experiments, Huh7 cells were seeded at a density of 9 × 10^3 cells/well on 96-well plates and transfected with control, SREBP-1 or SREBP-2 siRNA for 24 h in DMEM supplemented with 5% (v/v) LPDS. After 24 h, PBS or 50 μM zoledronic acid was added to the cells and they were incubated for 48 h.

In the experiment using the NF-YA siRNA, Huh7 cells were seeded at a density of 3 × 10^3 cells/well on 96-well plates and transfected with control or NF-YA siRNA for 48 h in DMEM supplemented with 5% (v/v) FBS. After 48 h, PBS or 50 μM zoledronic acid was added to the cells and they were incubated for 48 h.

Statistical analysis
All data are presented as means ± S.E.M. Statistical analyses were performed with the unpaired Student’s t test or the Dunn–Bonferroni test using Statcel version 2.0 (OMS Publishing).

RESULTS
Sterol depletion mediates FDPS expression in human hepatoblastoma cells
Previous studies have demonstrated that the FDPS gene is regulated by cellular sterols [1]. To confirm this regulation, we examined FDPS expression under various cellular sterol conditions. Human Huh7 hepatoblastoma cells were cultured for 24 h in one of three media supplemented as follows: with sterols (DMEM with LPDS, cholesterol and 25-hydroxycholesterol), LPDS (DMEM with LPDS) or statin (DMEM with LPDS, pitavastatin and sodium mevalonate). These three media contain sterols, LPDS or statin, and represent a decreasing order of intracellular sterols. We prepared total RNA from the cells and quantified the FDPS mRNA levels using real-time RT-PCR. As shown in Figure 1(A), mRNA from the FDPS gene was significantly induced (3.0-fold) in LPDS-containing medium relative to that in sterol-containing medium. FDPS mRNA levels were also increased 3.8-fold when the cells were cultured in statin-containing medium. We examined further the sterol responsiveness of the FDPS gene at the protein level using Western blot analysis. Sterol depletion markedly increased FDPS protein levels, similar to the effect observed for mRNA (Figure 1B). These results indicate that the expression of FDPS is regulated by sterol depletion in human hepatoblastoma cells.

Transcriptional regulation of the human FDPS promoter by SREBPs and NF-Y
We next examined whether the expression of a human FDPS promoter–reporter gene was regulated in response to changes in cellular sterol conditions. We isolated the 5′-flanking region (−1539 to +27 bp) of the FDPS gene by PCR of human genomic DNA. The construct was transiently transfected into HepG2 cells and the cells were cultured with sterols, LPDS or statin for 20 h. Figure 2(A) shows that the activity of the FDPS-1539 promoter was dramatically increased in cells cultured with LPDS or statin compared with that in cells cultured with sterols. Thus the human FDPS promoter activity was regulated by cellular sterol levels.

Many studies have reported that the SREBP transcription factors control the gene expression associated with lipid metabolism in response to cellular sterol levels [19]. SREBP-1a and SREBP-2, in particular, activate the cholesterogenesis genes. To determine whether this phenomenon is attributable to the effects of SREBPs, HepG2 cells were co-transfected with FDPS-1539 and the expression construct encoding the active form of SREBP-1a (amino acids 1–460) or SREBP-2 (amino acids 1–468). As under sterol-depleted conditions, the
FDPS expression is regulated by intracellular sterols in Huh7 cells

Huh7 cells were cultured for 24 h in medium containing sterols, LPDS, or statin. (A) FDPS mRNA levels were normalized to those of 36B4 mRNA. Results are means ± S.E.M. (n = 3). **P < 0.01. (B) Cell extracts were prepared and aliquots (100 μg of protein/lane for FDPS or 20 μg of protein/lane for actin) were subjected to SDS/PAGE. Immunoblots were probed with anti-FDPS antibody or anti-actin antibody.

Alignment of the proximal promoters of the human, mouse and rat FDPS genes revealed that the conserved sequence within the region between nucleotides −264 and −151 is an SRE motif (from −234 to −225; Figure 3). Previous work has shown that the SRE motif in the rat Fdps promoter is located in close proximity to the two NF-Y-binding sites, which are apparently essential for the optimal regulation of sterol-sensitive genes [24]. When we searched for the NF-Y-binding sites around the SRE motif of the human FDPS promoter, only one NF-Y-binding site was located between nucleotides −215 and −211, which is a highly conserved region in the three species. Interestingly, this differs from the rat (and mouse) promoter, which has two NF-Y-binding sites. The conservation of these elements suggests that this is likely to be an important regulatory sequence.

To test the hypothesis that the SRE motif and the NF-Y-binding site are responsible for the regulation of FDPS by cellular sterols, we introduced a mutated version of these elements into the human FDPS promoter and performed luciferase assays in HepG2 cells. Luciferase activity expressed from the FDPS-1539 promoter–reporter construct significantly increased in the presence of SREBP-1a or SREBP-2 compared with the luciferase activity in the presence of the empty vector (Figure 2B). We then constructed various deleted versions of the promoter–reporter construct and performed luciferase assays (Figure 2B). Similar regulatory responses were observed for the FDPS-1079, FDPS-577, FDPS-292 and FDPS-264 constructs, whereas there was no increase with the FDPS-151 construct. These results strongly suggest that the region between nucleotides −264 and −151 contains the cis-acting element necessary for the SREBP-mediated transcriptional regulation of FDPS.

Figure 1  FDPS expression is regulated by intracellular sterols in Huh7 cells

Figure 2 The region between nucleotides −264 and −151 of the human FDPS promoter contains sequences that mediate the induction of FDPS expression by sterol depletion

(A) HepG2 cells were transiently transfected with luciferase reporter constructs of the FDPS promoter. The cells were harvested, and the luciferase activity was measured and normalized to the β-galactosidase activity. PGV-B is a control vector that is FDPS promoter-free. Results are means ± S.E.M. (n = 5). **P < 0.01 compared with sterols for FDPS-1539. (B) Reporter constructs were co-transfected into HepG2 cells with pcDNA3 (mock), SREBP-1a (nSREBP1a) or SREBP-2 (nSREBP2) expression vector. At 24 h after transfection, the cells were harvested and the luciferase activity was measured and normalized to the β-galactosidase activity. Results are means ± S.E.M. (n = 3–7). *P < 0.05 or **P < 0.01 compared with mock.

Figure 3 Schematic representation of the human FDPS gene between nucleotides −264 and −151, illustrating the SRE motif and the NF-Y-binding site

An alignment of the human, mouse and rat FDPS promoters is shown. Nucleotides conserved in all three species are indicated by asterisks.
FDPS induced by SREBP-2 and NF-Y modulates cell proliferation

Figure 4  SREBPs and NF-Y are necessary for the activation of FDPS transcription

(A) Scheme representing the luciferase reporter gene construct encoding the FDPS promoter sequence. Wild-type and mutated SRE or NF-Y-binding site sequences are shown. (B) Reporter constructs were co-transfected into HepG2 cells with pcDNA3 (mock), SREBP-1a (nSREBP1a) or SREBP-2 (nSREBP2) expression vector. The cells were harvested, and the luciferase activity was measured and normalized to the β-galactosidase activity. Results are means + S.E.M. (n = 3). **P < 0.01 compared with FDPS-1539 nSREBP1a. ##P < 0.01 compared with FDPS-1539 nSREBP2. (C) Reporter constructs were co-transfected into Huh7 cells with each expression vector. Results are means + S.E.M. (n = 3). (D) HepG2 cells were co-transfected with FDPS-1539, pcDNA3-nSREBP1a, pcDNA3-NF-YA29 and pSV-β-galactosidase. Results are means + S.E.M. (n = 4). **P < 0.01 compared with cells not transfected with NF-YA29. (E) Drosophila SL2 cells were co-transfected with reporter constructs, each expression vector and pSV-β-galactosidase. Results are means + S.E.M. (n = 4). **P < 0.01 compared with FDPS-1539 nSREBP1a/NF-YA/YB/YC.

cells (Figure 4A). The mutated constructs of either the SRE (SREmut) or NF-Y-binding site (NF-Ymut) resulted in approx. 30% of the wild-type (FDPS-1539) luciferase activity when HepG2 cells were co-transfected with an nSREBP-1a expression plasmid (Figure 4B). A construct with mutations at both the SRE and NF-Y-binding site (SRE/NF-Ymut) showed markedly reduced luciferase activity. We also performed luciferase assays with all of the mutated constructs in the context of nSREBP-2 overexpression (Figure 4B). The luciferase activity showed a similar tendency to the results seen for nSREBP-1a, although the reduction in luciferase activity was weak compared with that for nSREBP-1a. We also performed the luciferase assay with all the mutated constructs in Huh7 cells, and the results were consistent with the results for HepG2 cells described above (Figure 4C).

To investigate further the requirement for NF-Y binding to the FDPS promoter, a dominant-negative NF-YA mutant expression vector, NF-YA29, was co-transfected with the FDPS-1539 promoter construct and nSREBP-1a into HepG2 cells.
NF-Y is a heteromeric transcription factor consisting of at least three subunits (NF-YA, NF-YB and NF-YC) [29,30]. NF-YB and NF-YC form a tight complex, and this complexation is a prerequisite for NF-YA binding to form NF-Y. NF-YA29, in which three amino acids in the DNA-binding domain of NF-YA have been mutated, forms a complex with NF-YB and NF-YC, rendering it functionally inactive as a transcription factor. As shown in Figure 4(D), the luciferase activity in the FDPS promoter was reduced in a dose-dependent manner. These results demonstrate that both the SRE and the NF-Y-binding site are important in the regulation of FDPS transcription.

Having demonstrated that SREBP and NF-Y are required for the sterol responsiveness of FDPS expression, we examined the ability of NF-Y to directly activate the FDPS gene together with SREBP. For this purpose, we took advantage of a modified co-transfection assay system in Drosophila SL2 cells. The cell line does not express functional equivalents of several mammalian transcriptional regulatory proteins, including the active endogenous SREBP-1 and NF-Y [31]. Therefore it is an ideal cell-based assay system for the analysis of transcription factor requirements for promoter activation because it provides a negative background [32]. Figure 4(E) shows the results of co-transfection assays in SL2 cells, where we evaluated the roles of NF-Y and SREBP-1a in the transcriptional activation of the FDPS gene promoter. Co-transfection with the wild-type FDPS promoter construct (FDPS-1539) together with plasmids that express either nSREBP-1a or all three NF-Y subunits resulted in 12- and 102-fold activation respectively. Co-transfection with both the nSREBP-1a and NF-Y subunit expression plasmids synergistically activated the wild-type FDPS promoter approx. 260-fold. In contrast, three mutated constructs (SREmut, NF-Ymut and SRE/NF-Ymut) were not synergistically activated by co-expression with SREBP-1a and the NF-Y subunits. These results are consistent with our mammalian cell experiments and also indicate that NF-Y functions together with SREBP to transactivate the FDPS promoter.

**SREBP-2 and NF-YA gene knockdown affects FDPS expression**

We next examined whether the expression of FDPS was reduced when Huh7 cells were transfected with SREBP-specific siRNA. We have already evaluated the SREBP siRNAs in a previous study, and these siRNAs function effectively and in a subtype-specific manner [26]. Quantitative RT–PCR demonstrated that FDPS mRNA levels were reduced by 40% after cell transfection with SREBP-2 siRNA, whereas FDPS mRNA did not change in cells transfected with SREBP-1 siRNA (Figure 5A). We also examined FDPS protein in a Western blotting experiment. Consistent with the results for mRNA, the level of FDPS protein was reduced in SREBP-2-knockdown cells compared with that in the control cells (Figure 5B). These results indicate that endogenous FDPS expression is regulated by SREBP-2 in Huh7 cells.

We also suppressed the expression of the endogenous NF-YA gene in Huh7 cells using a NF-YA-specific siRNA and examined the expression of FDPS. The mRNAs encoding NF-YA were reduced by approx. 60% in cells transfected with NF-YA siRNA compared with those of the control cells (see Supplementary Figure 1A at http://www.BiochemJ.org/bj/429/bj4290347add.htm). Consistent with the reduction in NF-YA mRNA, the levels of NF-YA protein were reduced in cells transfected with siRNA for NF-YA compared with those of the control cells (Supplementary Figure 1B). We then examined FDPS mRNA levels to analyse whether the knockdown of NF-YA gene reduces FDPS expression. Quantitative RT–PCR demonstrated that FDPS mRNA levels were reduced by approx. 30% after cell transfection with NF-YA siRNA (Figure 5C). The expression of FDPS protein was reduced by transfection of NF-YA siRNA into Huh7 cells compared with control cells (Figure 5D).

**SREBP-2 and NF-YA bind directly to each cis-acting element in the FDPS promoter**

SREBP-2 affinity for the FDPS SRE sequence was evaluated by EMSA using nuclear extracts prepared from LPDS-treated Huh7 cells (Figure 6A). When the FDPS SRE was used alone, a protein–DNA complex was detected (lane 2). This complex appeared to be specific because the addition of excess unlabelled FDPS SRE probe (FDPS) prevented its formation (lanes 3 and 4), whereas an unlabelled mutated SRE probe (FDPSmut) did not (lanes 5 and 6). The consensus SRE sequence identified in the promoter of the LDLR (low-density lipoprotein receptor) gene (positive control) was used as the control. The addition of excess unlabelled LDLR SRE specifically competed with the protein–DNA complex (lanes 7 and 8). Supershift analysis was then performed to identify the protein bound to the SRE. The protein–DNA complex band was supershifted by the addition of anti-SREBP-2 antibody (lane 9). Conversely, a control antibody had no significant effect (lane 10).
FDPS induced by SREBP-2 and NF-Y modulates cell proliferation

These results demonstrate that SREBP-2 binds to the FDPS SRE at nucleotides $-234$ to $-225$.

To investigate the DNA-binding activity of NF-Y at the NF-Y-binding site of the FDPS promoter, an EMSA was performed using this response element (Figure 6B). A specific protein–DNA complex was detected (lane 12). Excess unlabelled NF-Y DNA probe or the consensus NF-Y sequence competed with the protein–DNA complex formation, whereas a mutated version of the NF-Y-binding site failed to compete for NF-Y binding (lanes 13–18). The complex was supershifted by the addition of anti-NF-YA antibody, confirming that NF-YA binds to the NF-Y-binding site of FDPS (lanes 19 and 20). These results demonstrate that NF-Y binds to the NF-Y-binding site at nucleotides $-215$ to $-211$ of the FDPS promoter.

To determine whether SREBP-2 and NF-Y bind to the FDPS promoter in Huh7 cells, we performed a ChIP assay (Figure 7). The binding of both SREBP and NF-Y to their sites on the FDPS promoter was confirmed by the ChIP assay using specific antibodies against each of these transcription factors. The precipitated cis elements (SRE and the NF-Y-binding site) were detected by PCR amplification of the FDPS promoter fragment (nucleotides $-287$ to $-30$), which included the SRE (nucleotides $-234$ to $-225$) and the NF-Y-binding site (nucleotides $-215$ to $-211$). As shown in Figure 7, SREBP-2 bound to the human FDPS promoter in cells, whereas a negative mouse IgG showed very low non-specific background binding (lanes 2 and 3). Similarly, antibody directed against NF-YA detected that protein bound to the NF-Y-binding site on the FDPS promoter (lane 6). These results indicate that SREBP-2 and NF-YA bind to the cis-acting elements in the FDPS promoter in Huh7 cells.

FDPS induced by SREBP-2 and NF-Y regulates cell proliferation in hepatoblastoma cells

Zoledronic acid, which is one of the bisphosphonates, suppresses cell proliferation by inhibiting FDPS activity [33]. Zoledronic acid is used to prevent skeletal fractures in patients with cancers such as multiple myeloma and prostate cancer [34]. It can also be used to treat the hypercalcaemia of malignancy and can be helpful for treating pain from bone metastases. However, how sterol-mediated FDPS is affected by zoledronic acid in the liver is largely unknown. To address this question, we investigated how cell proliferation is affected by sterol depletion and zoledronic acid.

We first examined whether cell proliferation changed with different cellular sterol contents. Figure 1(B) showed that the expression of FDPS protein in LPDS-containing medium is similar to that in statin-containing medium. Thus we adopted LPDS-containing medium as a sterol-depleted condition. We
examined cell proliferation in LPDS- or sterol-containing medium by the WST-8 method, based on the MTT assay. As shown in Figure 8(A), cell proliferation of Huh7 cells was reduced to 65% in sterol-containing medium relative to that in LPDS-containing medium. These results suggest that cell proliferation is regulated in a sterol-dependent manner. To provide more direct evidence that FDPS affects cell proliferation, we examined cell viability in hepatoblastoma cells with elevated level of FDPS by transfection of FDPS cDNA. Figure 8(B) shows that FDPS overexpression in Huh7 cells significantly increased the cell viability compared with the mock. In addition, we examined whether the cell viability was reduced when Huh7 cells were transfected with FDPS-specific siRNA. To evaluate FDPS mRNA and protein expression after FDPS knockdown, we performed quantitative RT-PCR and Western blotting. When compared with control cells, FDPS significantly reduced FDPS mRNA and protein expression in FDPS-knockdown cells (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/429/bj4290347add.htm). Suppression of the FDPS expression with siRNA resulted in the inhibition of cell proliferation (Figure 8C). These results suggest that the FDPS induced by sterol depletion modulates cell proliferation.

Next, Huh7 cells were treated with various concentrations of zolendronic acid (0.1, 1, 5, 10 and 50 μM) in LPDS- or sterol-containing medium, and the effects of zolendronic acid on cells were measured by the WST-8 method (Figure 8D). In LPDS-containing medium, the exposure of Huh7 cells to increasing concentrations of zolendronic acid caused a dose-dependent reduction in cell proliferation. This reduction in cell proliferation was almost identical with that in the sterol-treated cells described above. When cells were cultured with sterols, the reduction induced by zolendronic acid did not occur. These results suggest that the inhibition of cell proliferation by zolendronic acid occurs under sterol-depleted conditions when FDPS expression increases.

Finally, we determined the effects of zolendronic acid on the proliferation of Huh7 cells transfected with control, SREBP1 or SREBP2 siRNA. We found that zolendronic acid treatment significantly reduced the proliferation of cells transfected with either the control or SREBP1 siRNA (Figure 8E). However, when the cells were transfected with SREBP2 siRNA, treatment with zolendronic acid affected cell proliferation negligibly. In addition, we investigated the effects of zolendronic acid on the proliferation of Huh7 cells transfected with NF-YA siRNA. Consistent with the SREBP2 siRNA, treatment with zolendronic acid did not affect cell proliferation when the cells were transfected with NF-YA siRNA (Figure 8F). These results show that FDPS induced by SREBP2 and NF-Y regulates cell proliferation in hepatoblastoma cells.

DISCUSSION

The FDPS gene encodes an enzyme that catalyses the production of geranyl pyrophosphate and farnesyl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate [1]. The resulting product, FPP, is a key intermediate in cholesterol and sterol biosynthesis, a substrate for protein farnesylation and geranylgeranylation, and a ligand or agonist for certain hormone receptors and growth factor receptors [2–7]. The present study confirms that the human FDPS promoter is a target of SREBP-2 and provides several novel insights into the cellular response to sterol depletion in terms of the expression of FDPS. We have shown that sterol depletion induces the mRNA and protein expression of the FDPS gene in human hepatoblastoma cells (Figure 1). The transient overexpression of SREBP-1a or SREBP-2 activates the human FDPS promoter (Figures 2 and 4). Conversely, the suppression of SREBP-1 or SREBP-2 by siRNA indicated that endogenous SREBP-2, but not SREBP-1, regulates the FDPS gene (Figure 5). Interestingly, previous studies have reported that the SRE of the rat Fdps promoter is regulated by SREBP-1a overexpression [24]. SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids and triacylglycerols [35]. However, SREBP-1a is expressed only at low levels in the livers of adult mice, rats, hamsters and humans. Studies of Srebp-1a transgenic mice have demonstrated that SREBP-1a has a relative preference for activating fatty acid synthesis rather than cholesterol synthesis. SREBP-2 preferentially enhances the transcription of genes involved in cholestrogenesis. These observations support the proposition that FDPS, a key enzyme in the cholesterol synthesis pathway, is mainly controlled by endogenous SREBP-2.

The ubiquitous transcription factor NF-Y is also an important regulator of FDPS gene expression, and the transcription of the FDPS gene is regulated by a combination of SREBPs and NF-Y (Figures 4 and 5). NF-Y binds to NF-Y-binding sites in the promoter regions of a variety of genes involved in early mouse development and cell-cycle progression [30,36]. The NF-Y complex comprises three subunits, NF-YA, NF-YB and NF-YC, all required for DNA binding. The SREBP-mediated induction of target gene expression occurs in concert with the additional binding of co-regulatory NF-Y to the promoter, resulting in synergistic levels of transcriptional activation [23]. Several genes, including those encoding squalene synthase, HMG-CoA synthase and lipin 1, are regulated by the same combination of transcription factors [26,37,38]. NF-Y-binding sites are usually found in close proximity to SREs in the promoters of these genes. In the human FDPS gene, the distance between the SRE and the NF-Y-binding
FDPS induced by SREBP-2 and NF-Y modulates cell proliferation

Figure 8 FDPS induced by SREBP-2 and NF-Y regulates cell proliferation in hepatoblastoma cells

(A and D) Huh7 cells were treated with 0, 0.1, 1, 5, 10 or 50 μM zoledronic acid (Zol) for 48 h in LPDS- or sterol-containing medium. Cell numbers were counted in triplicate by the WST-8 method. Cell viability was determined as a percentage of the value for 0 μM Zol in LPDS. Results are means ± S.E.M. (n = 4). **P < 0.01 compared with LPDS-containing medium. #P < 0.05 or ##P < 0.01 compared with the effect of Zol on LPDS-containing medium. (B) Huh7 cells were transiently transfected with pcDNA3 (mock) or FDPS expression vector. Results are means ± S.E.M. (n = 4). **P < 0.01. (C) Huh7 cells were transfected with 25 nM control (cont) or FDPS (S-2) siRNA. Results are means ± S.E.M. (n = 4). **P < 0.01. (E and F) Huh7 cells were transfected with 25 nM control (Cont), SREBP-1 (S-1), SREBP-2 (S-2) or NF-YA siRNA. Huh7 cells were treated with 0 or 50 μM zoledronic acid (Zol) for 48 h. Results are means ± S.E.M. (n = 4). **P < 0.01. N.S., not significant.

The distances between the two binding sites for SREBP and NF-Y in the promoter of rat Fdps are in the 21-bp range and this spacing is highly conserved in the mouse Fdps gene (Figure 3). It is likely that the regulation of the human FDPS gene differs from that of the rodent genes. Recently, Romanelli et al. [39] showed that the FDPS gene expression of humans and chimpanzees,
but not of rodents, involves the transcription factors Pax5 and OCT-1 [39]. Martín et al. [40] showed that human FDPS has a novel alternatively spliced isoform, which is expressed in the mitochondria. These observations suggest the different regulation of human and rodent FDPS genes.

It is clear from these results that the effect of zoledronic acid on cell proliferation is critical for the effect of SREBP-2 (Figure 8). Studies in Dictyostelium discoideum have reported that the overexpression of FDPS leads to resistance to the growth-inhibitory effects of nitrogen-containing bisphosphonates [41,42]. Recent studies have also demonstrated that HMG-CoA reductase inhibitors (statins), cholesterol-lowering agents, suppress cell proliferation in various types of tumour cells [43]. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol synthesis pathway, and the growth-inhibitory effect of statins acts with the same mechanism as zoledronic acid. These observations support our results demonstrating that FDPS induced by SREBP-2 regulates cell proliferation. The anti-proliferative effect of zoledronic acid works effectively when SREBP-2 is activated. Interestingly, it has been reported that FDPS is highly expressed in tumour cells. Notarnicola et al. [11] showed that FDPS expression levels are significantly higher in cancer samples than in normal mucosa. Elevated FDPS expression has also been observed in rat prostate tumour cell lines [44]. FDPS probably plays a significant role in various types of tumour cells. Therefore the potential therapeutic applicability of zoledronic acid in various types of tumour cells appears to be relevant and it could be used not only in tumour-associated bone disease therapies, but also in the treatment of liver cancer.

In conclusion, we have demonstrated that intracellular sterols modulate the expression of the human FDPS gene via SREBP-2 and NF-Y. We have also clarified that the expression of FDPS induced by SREBP-2 and NF-Y controls cell proliferation. This regulation of the human FDPS gene may play a role in tumour therapies through the anti-proliferative effects of zoledronic acid.

AUTHOR CONTRIBUTION
Kenji Ishimoto contributed to experimental design, conducted the experiments and wrote the paper. Ikuo Hanano, Hiroki Nakamura and Megumi Kawai carried out the experiments. Keisuke Tachibana, Daisuke Yamasaki, Yasuomi Urano, Toshihisa Tanaka, Takao Hamakubo, Juro Sakai and Tatsuhiko Kodama helped to design the experiments and helped to write the manuscript. Taketumi Doi designed and analysed the experiments, supervised the overall work and contributed to the writing of the paper.

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SUPPLEMENTARY ONLINE DATA

Sterol-regulatory-element-binding protein 2 and nuclear factor Y control human farnesyl diphosphate synthase expression and affect cell proliferation in hepatoblastoma cells

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Figure S1 Knockdown of NF-YA reduces NF-YA expression in Huh7 cells

Huh7 cells were transfected with 25 nM control (Cont) or NF-YA siRNA. (A) Total RNA was extracted for analysis by real-time RT–PCR 48 h after transfection. Target mRNA levels were normalized to cyclophilin A mRNA levels. Results are means ± S.E.M. (n = 3). *P < 0.05. (B) At 48 h after transfection, cell extracts were prepared, and aliquots (30 μg of protein/lane) were subjected to SDS/PAGE. Immunoblots were probed with anti-NF-YA antibody or anti-lamin B2 antibody.

Figure S2 Knockdown of FDPS reduces FDPS expression in Huh7 cells

Huh7 cells were transfected with 25 nM control (Cont) or FDPS siRNA. (A) Total RNA was extracted for analysis by real-time RT–PCR 48 h after transfection. Target mRNA levels were normalized to cyclophilin A mRNA levels. Results are means ± S.E.M. (n = 3). **P < 0.01. (B) At 48 h after transfection, cell extracts were prepared, and aliquots (30 μg of protein/lane) were subjected to SDS/PAGE. Immunoblots were probed with anti-FDPS antibody or anti-lamin B2 antibody.

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