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Reduced Expression of FASN Through SREBP-1 Down-Regulation Is Responsible for Hypoxic Cell Death in HepG2 Cells

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ABSTRACT

Cells under hypoxic stress either activate an adaptive response or undergo cell death. Although some mechanisms have been reported, the exact mechanism behind hypoxic cell death remains unclear. Recently, increased expression of fatty acid synthase (FASN) has been observed in various human cancers. In highly proliferating cells, tumor-associated FASN is considered necessary for both membrane lipids production and post-translational protein modification, but the exact mechanisms are not fully understood. Further, FASN overexpression is associated with aggressive and malignant cancer diseases and FASN inhibition induces apoptosis in cancer cells. For this reason, FASN is emerging as a key target for the potential diagnosis and treatment of various cancers. Here, we observed decreased FASN expression under hypoxic cell death conditions in HepG2 cells. Thus, we examined the effect of decreased FASN expression on hypoxia-induced cell death in HepG2 cells and also investigated the mechanism responsible for reduction of FASN expression under hypoxic cell death conditions. As a result, reduction of FASN expression resulted in hypoxic cell death via malonyl-CoA accumulation. In addition, SREBP-1 restored FASN reduction and hypoxia-induced apoptosis. Taken together, we suggest that hypoxic cell death is promoted by the reduced expression of FASN through SREBP-1 down-regulation. J. Cell. Biochem. 113: 3730–3739, 2012.

KEY WORDS: FASN; SREBP-1; HYPOXIA; APOPTOSIS

Rapidly growing cancer cells are sometimes exposed to hypoxic conditions [Greijer and van der Wall, 2004], as oxygen cannot diffuse more than 100–200 μm from vessels [Dang and Semenza, 1999; Gatenby and Gillies, 2004]. Cells under hypoxic stress either activate an adaptive response such as glycolysis and angiogenesis or undergo cell death such as apoptosis, necrosis, or autophagy [Lee et al., 2007; Vaupel and Mayer, 2007; Wilson and Hay, 2011]. Although some mechanisms related to the activation of pro-apoptotic genes and acidosis have been reported, the exact mechanism behind hypoxic cell death remains unclear [Kubasiak et al., 2002; Almaas et al., 2003].

Recently, increased expression of fatty acid synthase (FASN), a key enzyme necessary for de novo fatty acid synthesis, has been observed in various human cancers, including prostate, colon, breast, endometrium, parathyroid, kidney, and lung cancers, which is in contrast to low FASN expression in normal cells [Rashid et al., 1997; Pizer et al., 1998; Alo et al., 1999, 2001; Kuhajda, 2000; Swinnen et al., 2002; Horiguchi et al., 2008; Orita et al., 2008]. Importantly, high levels of FASN expression are associated with aggressive cancer diseases [Kuhajda et al., 1994; Semenkovich, 1997; Menendez et al., 2005b].

Previous reports have shown that inhibition of FASN by cerulenin, a natural antibiotic product of the fungus *Cephalosporium cerulens*, leads to growth arrest and apoptosis [Menendez et al., 2004]. In addition, C75, a specific noncompetitive inhibitor of the β-ketoacyl synthase activity of FASN, along with RNA interference-mediated silencing of the FASN gene were previously shown to induce apoptosis [Kuhajda, 2000; Pizer et al., 2000; De Schrijver et al., 2003; Zhou et al., 2003; Menendez et al., 2004; Hu et al., 2005; Bandyopadhyay et al., 2006; Orita et al., 2008]. Although several

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studies have shown that FASN inhibition induces apoptosis in cancer cells, the exact mechanism responsible remains an area of active investigation. For this reason, tumor-associated FASN has become a focus for the potential diagnosis and treatment of cancer [Kuhajda, 2006; Menendez and Lupu, 2007].

Here, we observed decreased FASN expression under hypoxic cell death conditions in HepG2 cells. Thus, we examined the effect of decreased FASN expression on hypoxia-induced apoptosis in HepG2 cells and also investigated the mechanism responsible for reduction of FASN expression under hypoxic cell death conditions.

MATERIALS AND METHODS

CELL CULTURE AND MEASUREMENT OF EXTRACELLULAR pH

HepG2 cells were seeded at low density (2.2×10^6 cells/100 mm dish [SPL, Korea]; 40,000 cells/cm^2) and at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2) in MEM supplemented with heat-inactivated 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (JBI, Korea) in a 37°C incubator containing a humidified atmosphere of 5% CO_2. Before exposure to hypoxia, seeded cells were incubated for 24 h in an incubator. For hypoxic treatment, the cells were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH) containing a humidified atmosphere of 2% O_2 and 5% CO_2 balanced with N_2 and incubated at 37°C for 12 h. For measurement of extracellular pH, culture media of the incubated cells was transferred to a 50 ml tube, after which the pH was immediately measured using a pH electrode (Mettler Toledo, Switzerland).

TWO-DIMENSIONAL ELECTROPHORESIS

The cells in the culture were washed twice with ice-cold Tris-buffered sucrose (10 mM Tris, 250 mM sucrose, pH 7.0), harvested, and then transferred to a 1.5 ml tube. After removing washing buffer, the pellet was dissolved in 2-D lysis buffer (9 M urea, 4% CHAPS, 1% DTT, 1% IPG buffer, 0.002% BPB, 1× Protease inhibitor Mix, 1× Nuclease Mix). Protein extract was then sonicated, followed by incubation for 1 h at room temperature. After centrifugation at 15,000 rpm for 60 min at 20°C, the supernatant was transferred to a fresh tube. Protein concentration was determined by using a BIO-RAD Protein assay kit (Hercules CA).

For the first dimension, 200 μg of total protein was diluted in rehydration solution (8 M urea, 2% w/v CHAPS, 20 mM DTT, 1% v/v IPG buffer pH3-10, 0.002% Bromophenol blue), and a 250 μl total volume was loaded onto a 13 cm Immobiline Dry Strip pH 3-10 linear (Amersham Biosciences, Piscataway, NJ) according to the in-gel rehydration method. IPG strips were then rehydrated for 12 h at 20°C, after which a low voltage (Step-n-Hold mode, 50 V) was supplied for 5 h using the IPGphore system (Amersham Biosciences). Focusing was carried out at Step-n-Hold mode 500 V for 1 h, Step-n-Hold mode 1,000 V for 1 h, and Step-n-Hold mode 8,000 V for up to a total of 17,750 Vhrs. Focused strips were then drained to remove excess oil and stored at −80°C or equilibrated immediately.

Focused strips were equilibrated for 15 min under gentle agitation in equilibration solution (6 M urea, 30% v/v glycerol, 2%, w/v SDS, 50 mM Tris–HCl pH 8.8, 1% w/v DTT, 0.002% Bromophenol blue). The strips were placed on top of a 12.5% SDS-polyacrylamide gel prepared according to the 2-D electrophoresis Handbook (2-D Electrophoresis: using immobilized pH gradients—Principle and Methods), which can be found at the Amersham Bioscience homepage (http://www.amershambiosciences.com), and sealed with 0.5% w/v agarose in SDS electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS). Invitrogen BenchMark™ Prestained Protein Ladder (Carlsbad, CA) was used as a molecular weight marker. Separation in the second dimension (SDS–PAGE) was carried out at a constant voltage (200 V) by using Hoefer SE 600 vertical chambers until the Bromophenol blue front migrated to the end of the gel.

For visualization of the spots, 2-DE gels were stained by colloidal Coomassie blue G250 (Sigma, St. Louis, MO). Briefly, the gels were incubated overnight in fixing solution (45% methanol, 5% phosphoric acid) with mild shaking at room temperature. The fixed gels were stained with colloidal Coomassie blue solution (0.1% w/v Coomassie blue G250, 17% w/v ammonium sulfate, 3.6% phosphoric acid, 34% methanol) under gentle agitation at room temperature for 36 h. After staining, excessive dye on the gel surface was washed two times with washing solution (1% acetic acid, 15% methanol) at room temperature for 1 h, followed by washing with water at room temperature for 1 h. The stained gels were then digitalized by scanning at 300 dpi resolution on an ImageMaster Scanner (Amersham Bioscience), followed by storage in an airtight container at 4°C.

The 2-DE gel images were analyzed with ImageMaster 2D Elite software version 3.1 (Amersham Bioscience). Spot detecting parameters were set automatically according to the manufacturer's instructions, as follows: sensitivity "8,295," operator size '37', noise factor "5," and background factor "1." To verify the auto-detected result, all spots were automatically filtered, and filtering parameters were set as follows: peak height (≥40), volume (≥1,700), after which all filtered spots were manually edited. After spot detection, background subtraction was performed in "average on boundary" mode, after which spot matching was performed automatically and manually. Next, total spot volume was normalized by using the "multiply by total area" option, followed by analysis of hypoxia-treated HepG2 total protein expression in comparison with the normoxic sample. Difference analysis was performed by using the "volume difference" option and the cut-off value was a 100% increase (twofold increase) or 30% decrease (1.43-fold decrease).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from HepG2 cells was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. First-stranded cDNA was synthesized by using M-MLV reverse transcriptase (Promega, Madison, WI) with 2 μg of total RNA sample and oligo-dTTP according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 20 μl reaction volume containing 1× PCR buffer, dNTPs, Taq DNA polymerase (Bioneer, Korea), and each specific primer. Oligonucleotide primers for PCR were as follows: FASN, 5′-CAC CCC GCA GGA CAG CCC CAT CT-3′ (forward) and 5′-CGC CGC CCG AGC CCG-3′ (reverse) [Kumar-Sinha et al., 2003]; SREBP-1c, 5′-GCC ATG GAT TGC ACT TTC G-3′ (forward) and 5′-CAC AGT GGT CGT TAC AGG GG-3′ (reverse); β-actin, 5′-GTG GGG CGC CCC AGG CAC

CAG GGC-3′ (forward) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (reverse). PCR was carried out for an initial denaturation at 95°C for 5 min, followed by 27 cycles for FASN and SREBP-1c as well as 25 cycles for β-actin of denaturation (95°C, 1 min), annealing (67°C, 1 min for FASN; 59°C, 1 min for SREBP-1c; 57°C, 1 min for β-actin), and extension (72°C, 1 min). This was followed by a final extension step at 72°C for 5 min. Amplification products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining under ultraviolet transillumination. The band intensity was analyzed using Image J software (NIH, USA), and data represent an induction-fold of FASN mRNA expression compared to each control after normalization with the β-actin band.

WESTERN BLOT ANALYSIS

Cells in culture were rinsed two times with ice-cold PBS. Total protein was extracted by RIPA lysis buffer (150 mM NaCl, 1% Nonidet P40, 0.5% DOC, 0.1% SDS, 50 mM Tris [pH 8.0], freshly supplemented with protease inhibitor cocktail) for 30 min on ice. Lysate was sonicated and centrifuged at 13,000 rpm for 30 min at 4°C. Protein concentration of lysis supernatant was determined by using a BCA protein assay kit (Sigma). Equal amounts of cell lysate (50 μg of protein) were separated by SDS–PAGE (6% for FASN, PARP and α-tubulin detection, 8% for SREBP-1, and 12% for caspase-3 and TRAIL) and transferred to PVDF membranes (Amersham Biosciences). The membranes were then blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 30 min at room temperature. The membranes were probed with mouse anti-human FASN (1/2,000 dilution, BD Biosciences, Palo Alto, CA), rabbit anti-PARP (1/2,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-caspase-3 (1/2,000 dilution, Cell Signaling, Danvers, MA), mouse anti-human TRAIL (1/1,000 dilution, BD Biosciences), mouse anti-human SREBP-1 (1/500, Santa Cruz), and mouse anti-human α-tubulin (1/5,000 dilution, BioGenex; San Ramon, CA) for 2 h, followed by washing and incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized using the ECL system (Intron Biotechnology, Korea). Band intensity was analyzed using Image J software, and data represent an induction-fold of FASN protein expression compared to each control after normalization with the α-tubulin band.

PLASMID CONSTRUCTION

Full-length human FASN cDNA expression vector was obtained from Dr. Massimo Loda (DANA-FARBER cancer institute, Boston, MA). FASN full sequence of the obtained vector was amplified by using the following primer set: 5′-TGC CAT GGA GGA GGT GGT GAT TGC-3′ (forward) and 5′-GCC CTC CCG CAC GCA CA-3′ (reverse). The PCR product was cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen). The generated plasmid was designated as pcDNA3.1/FASN-V5-His. Human recombinant mature SREBP-1c (rmSREBP-1c) cDNA expression vector (pcDNA3.1/rmSREBP-1c-V5-His, aa. 1-447) was constructed by using the pcDNA3.1/V5-His TOPO vector system. The following primers were used [Kim et al., 2007]: SREBP-1c, 5′-GCG GAA TTC GGA GCC ATG GAT TGC ACT TTC GA-3′ (forward) and 5′-GCC TCG AGC TAT GGC TTT GCC TTG CTG TCC TC-3′ (reverse). For cloning of the FASN promoter, genomic DNA was prepared from HepG2 cells by using an i-genomic CTB DNA Extraction Mini Kit (iNtRON, Korea). The human FASN promoter region, extending from positions −1,108 to +120, was amplified from purified human genomic DNA with the aid of upstream and downstream primers incorporating restriction sites for *Kpn*I and *Hind*III (New England Biolabs, Beverly, MA), respectively. The sequence of the forward primer was 5′-CGG GGT ACC CGC CCG CCC CGG-3′ and that of the reverse primer was 5′-CTC ACC AGG TAC GAG CAG AAG CTT GGG-3′. After enzyme restriction, the PCR product was ligated into pGL4.14-luciferase reporter plasmid (Promega) and named as pGL4/FASNp.

CELL TRANSFECTION AND REPORTER ASSAY

For the promoter assay, HepG2 cells were seeded (7×10^5 cells/well; 200,000 cells/cm^2) onto 12-well plates and transiently transfected using Carrigene transfection reagent (Kinovate; Oceanside, CA) according to the manufacturer's instructions. The pGL4/FASNp was transfected with pCMV/*lac*Z to control for transfection efficiency. To detect FASN promoter activity by SREBP-1 expression, pcDNA3.1/rmSREBP-1-V5-His was co-transfected with pGL4/FASNp and pCMV/*lac*Z. After transfection, hypoxic treatment was performed for 12 h. At the end of the treatment, cells were harvested in Passive Lysis Buffer (Luciferase Assay System, Promega). Luciferase activity was determined using a microplate reader (Wallac Victor, Perkin-Elmer; Boston, MA) following the manufacturer's protocol. Relative luciferase activity was normalized against the activity of β-gal. Each value represents the mean of at least four wells, and similar results were obtained in three different experiments. Statistical comparisons between the groups were performed using Student's *t*-test. Data were considered statistically significant at $P < 0.05$.

For the viability assay, HepG2 cells were seeded (1.7×10^6 cells/well 80,000 cells/cm^2) onto a 60 mm dish (SPL), and pcDNA3.1/FASN-V5-His or pcDNA3.1/rmSREBP-1-V5-His was stably transfected by using Lipofectamine and PLUS reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were subcultured into a 100 mm dish and supplied 640 μg/ml of G418-containing media. Visible individual colonies were transferred onto multi-well plates, after which the stably expressed cells were selected by using RT-PCR and Western blot analysis.

MTT ASSAY

HepG2 wild-type cells or stable cells were seeded at low density (1.4×10^5 cells/well; 40,000 cells/cm^2) and at high density (7.0×10^5 cells/well; 200,000 cells/cm^2) in a 12-well plate (NUNC). Seeded cells were incubated for 24 h in an incubator containing a humidified atmosphere of 5% CO_2. For the viability assay, glucose (25 mM), cerulenin (10 μg/ml), palmitic acid (300 μM), or TOFA (20 μg/ml) was used as a treatment, followed by immediate exposure to hypoxia or normoxia. For hypoxic treatment, the cells were transferred to an anaerobic chamber containing a humidified atmosphere of 2% O_2 and 5% CO_2 balanced with N_2 and then incubated at 37°C for 12 h. A final concentration of 0.6 mg/ml of MTT reagent was added directly to each well. After 4 h, the medium was removed, the formazan crystals in HepG2 cells were dissolved in dimethyl sulfoxide (DMSO), and the absorbance of the formazan

solution was measured by using an ELISA reader (Molecular Devices, Sunnyvale, CA) equipped with a 540 nm filter. Each sample was assayed in triplicate, and the experiment was repeated three times.

ANALYSIS OF DNA FRAGMENTATION

HepG2 cells were seeded at a density of 1.1×10^7 HepG2 cells/100 mm dish (200,000 cells/cm^2) and incubated for 24 h in an incubator. After hypoxic treatment, genomic DNA was extracted [Malhotra and Brosius, 1999] by using lysis buffer (10 mM Tris–HCl [pH 8.0], 10 mM NaCl, 10 mM EDTA [pH 8.0], 0.5% SDS, and 100 μg/ml of proteinase K). The cell lysate was incubated at 50°C for 2 h and centrifuged at 3,000 rpm at 4°C for 30 min. The genomic DNA was then extracted once with phenol, twice with phenol/chloroform, and once with chloroform. Precipitation of DNA was carried out in ethanol and a 1/10 volume of 3 M NaAc, followed by incubation at −20°C overnight. Following this, the DNA was washed with 70% (v/v) ethanol. After drying, the DNA pellet was dissolved in 30 μl of DW containing 200 μg/ml of RNase A and incubated at 37°C for 30 min. The DNA (4 μg) samples were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining under ultraviolet transillumination.

STATISTICAL ANALYSIS

Data are given as means ± SD. Statistical comparisons between the groups were performed using Student's *t*-test. Data were considered statistically significant at *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

HYPOXIA LEADS TO APOPTOSIS IN HIGH DENSITY CULTURE

Upon exposure to hypoxia, cancer cells secrete angiogenic factors and transform into more aggressive phenotypes [Dang and Semenza, 1999; Gatenby and Gillies, 2004]. On the other hand, cells under severe hypoxic conditions undergo cell death [Schmaltz et al., 1998; Greijer and van der Wall, 2004; Lee et al., 2007]. In this study, we confirmed that severe hypoxic conditions lead to apoptosis. As shown in Figure 1A, the viability of cells at high density was remarkably reduced under hypoxic conditions, whereas that of cells at low density was maintained at a level resembling that under normoxic conditions. These results indicate that both cell density and hypoxia are contributing to cell death. Hypoxia induces several types of cell death such as apoptosis, necrosis, and autophagy [Greijer and van der Wall, 2004; Lee et al., 2007; Vaupel and Mayer, 2007; Wilson and Hay, 2011]. Especially, it was reported that hypoxia induces apoptosis in a high density culture of Myc/Ras-transformed rat embryo fibroblasts [Schmaltz et al., 1998]. Thus, to confirm whether or not hypoxia induces apoptosis in a high density culture of HepG2 cells, DNA fragmentation and Western blot analyses for pro-apoptotic genes were performed. As shown in Figure 1B, DNA fragmentation was induced in the high density cell culture under hypoxic conditions. Further, the cleavage of PARP, cleavage of caspase-3, and the increase in TRAIL were positively changed to apoptotic phenotypes in the high density culture under hypoxic conditions (Fig. 1C).

HYPOXIA-INDUCED APOPTOSIS IS REGULATED BY GLUCOSE LEVEL

Under hypoxic conditions, ATP generation is reduced due to the suppression of oxidative phosphorylation (OXPHOS) [Gatenby and Gillies, 2004; Brahimi-Horn et al., 2011; Wilson and Hay, 2011]. At this point, cancer cells overcome their ATP deficiency through the expression of glycolytic enzymes such as GLUT1, HK2, and LDHA [Dang and Semenza, 1999; Brahimi-Horn et al., 2011; Wilson and Hay, 2011]. Therefore, under hypoxic conditions, cancer cells rapidly consume glucose, which has the effect of decreasing extracellular pH due to the accumulation of glycolysis end-products [Dang and Semenza, 1999; Gatenby and Gillies, 2004; Greijer and van der Wall, 2004]. We observed that while hypoxia did not induce

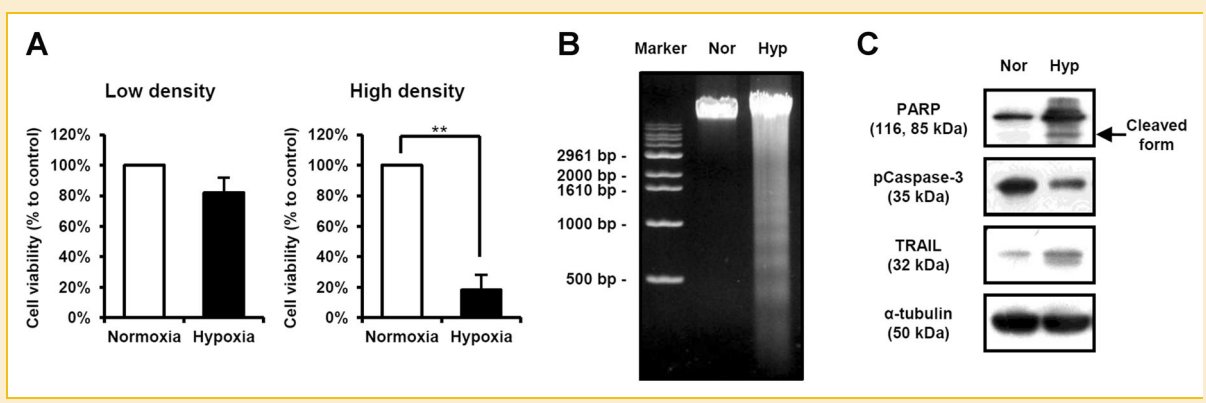

Fig. 1. Hypoxia leads to apoptosis in high density HepG2 cells. A: HepG2 cells seeded at low density (1.4×10^5 cells/well; 40,000 cells/cm^2) and at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, MTT assay was performed to examine cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times. B: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, genomic DNA was isolated and electrophoresed on 1.5% agarose gel, followed by visualization by ethidium bromide staining under ultraviolet transillumination. C: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, Western blot analysis was performed to measure total protein levels of PARP, caspase-3, and TRAIL.

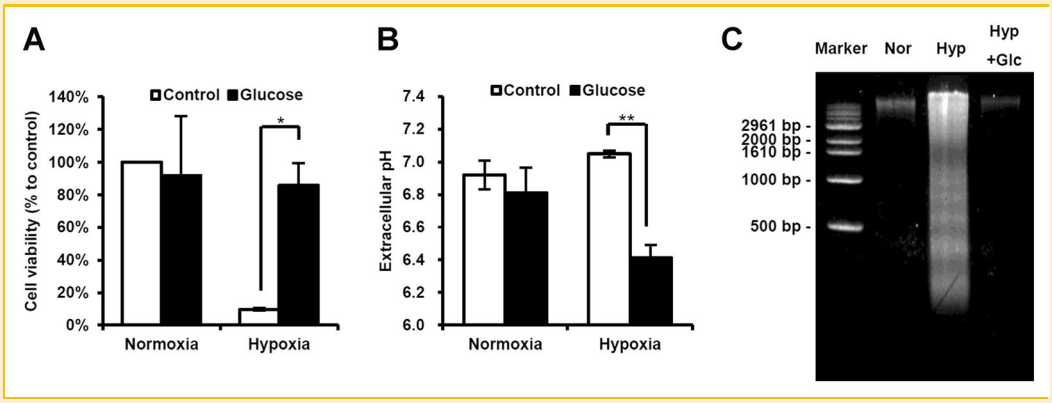

Fig. 2. Hypoxia-induced apoptosis is mediated by glucose deprivation. A: HepG2 cells seeded at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia and 25 mM of glucose for 12 h, MTT assay was performed to examine cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times. B: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia and 25 mM of glucose for 12 h, extracellular pH was determined immediately. Each sample was assayed in triplicate, and the experiment was repeated three times. C: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia and 25 mM of glucose for 12 h, 4 μg of genomic DNA was isolated and electrophoresed on 1.5% agarose gel, followed by visualization by ethidium bromide staining under ultraviolet transillumination.

cell death in the low density culture, it did significantly induce apoptosis in the high density culture. Thus, to determine the cause of cell death during high density hypoxia, changes in cytotoxicity and extracellular pH were examined. The cytotoxicity induced by high density hypoxia was restored by sufficient glucose supplementation (Fig. 2A). Further, extracellular pH was measured to verify whether or not hypoxia-induced apoptosis is mediated by acidification. In the result, the acidic environment did not promote apoptosis under this condition. Rather, extracellular pH significantly decreased in response to glucose treatment under hypoxic conditions (Fig. 2B), whereas hypoxia-induced apoptosis did not occur (Fig. 2A). Accordingly, we assumed that additional glucose was rapidly converted to pyruvate and lactate via glycolytic gene expression in response to hypoxia. Next, to confirm whether or not glucose suppresses hypoxia-induced apoptosis, DNA fragmentation assay was performed. As a result, DNA fragmentation was suppressed by glucose addition under hypoxic conditions (Fig. 2C). These results are considered that decreased level of glucose through glycolysis activation induced by hypoxic stress leads to apoptosis.

FASN EXPRESSION IS DECREASED UNDER HYPOXIC CELL DEATH CONDITION

To investigate proteomic alterations under hypoxic cell death conditions, 2-DE proteomic analysis was performed. Among the identified proteins, FASN showed a 6.49-fold decrease in expression upon hypoxia in HepG2 cells (Fig. 3A), which warranted further study. To confirm the results of the proteomic analysis, expression of FASN after exposure to hypoxia was verified. Both the mRNA and protein expression levels of FASN were remarkably reduced under hypoxic cell death conditions, whereas those of FASN in the low density culture were maintained at levels resembling those under normoxic conditions (Fig. 3B,C). Further, RT-PCR and Western blot analysis were performed to determine whether or not glucose affects the expression of FASN under hypoxic cell death conditions. As shown in Figure 3D, the mRNA and protein expression of FASN was restored after sufficient glucose supplementation under hypoxic conditions. These data show that hypoxia reduces FASN expression under hypoxic cell death conditions.

REDUCTION OF FASN INDUCES HYPOXIC CELL DEATH VIA MALONYL-CoA ACCUMULATION

It is known that cells undergo apoptotic cell death when FASN is blocked using a chemical inhibitor or FASN siRNA [De Schrijver et al., 2003; Kridel et al., 2004; Menendez et al., 2004; Little et al., 2007; Zhang et al., 2008]. To verify whether or not FASN is associated with the suppression of apoptosis upon glucose supplementation, MTT assay was performed using cerulenin, a natural FASN inhibitor. As a result, cell viability was reduced by cerulenin treatment under normoxic conditions. Further, cell viability was recovered by glucose addition, but this survival effect disappeared upon treatment with cerulenin under hypoxic conditions (Fig. 4A). These results suggest that reduction in FASN expression is closely associated with hypoxic cell death. Thus, to determine whether or not hypoxic dell death is induced by reduction of FASN expression, cell viability was examined by MTT assay using FASN stable cells. In the MTT assay, cell viability was significantly restored by FASN overexpression under hypoxic conditions (Fig. 4B). In addition, Western blot analysis was performed to measure the expression of apoptotic molecules. As shown in Figure 4C, apoptotic changes under hypoxic conditions disappeared in FASN-overexpressing cells. These results indicate that cell death under hypoxic conditions might be caused by reduced expression of FASN. Although it is unclear how FASN inhibition induces apoptosis in cancer cells, two separate reports provide relevant information. The first report found that fatty acid depletion induces apoptosis in cancer cells. Rapidly growing cancer cells require phospholipids for their membrane synthesis. As such, various human cancer cells exhibit the activated expression of lipogenic enzymes. However, most cancer cells do not store significant amounts of triglycerides. Instead, endogenously synthesized fatty

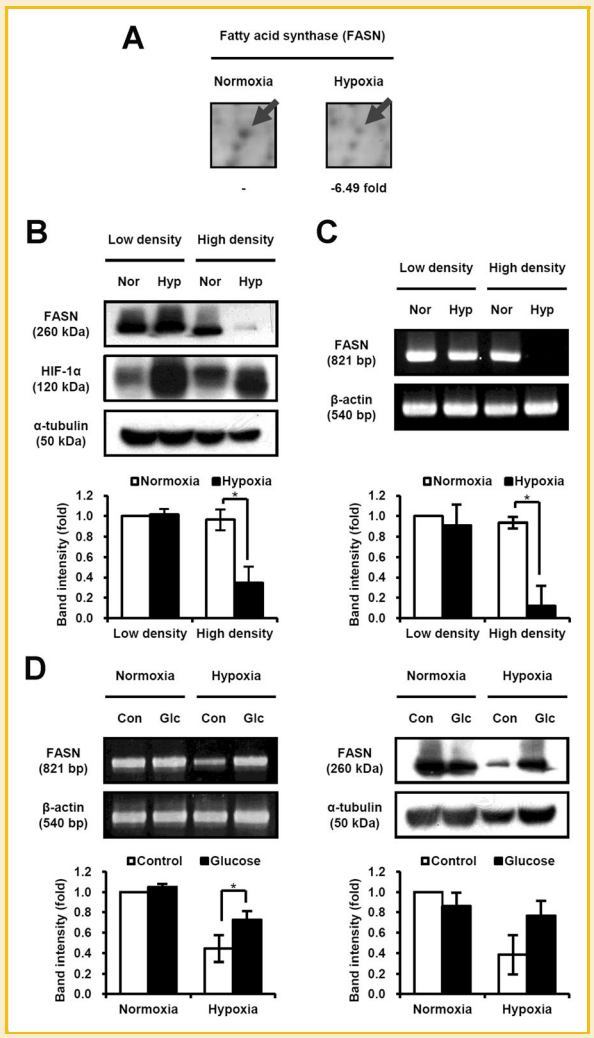

Fig. 3. FASN expression is reduced under hypoxic cell death condition. A: HepG2 cells seeded at 3.0×10^7 cells/150 mm dish, and treated with hypoxia for 12 h. In the first dimension (IEF: 17,750 Vhrs), 200 μg of total protein was loaded onto a 13 cm IPG strip with a linear gradient of pH 3–10. In the second dimension, 12.5% SDS–PAGE gels were used. Proteins were visualized using colloidal Coomassie G250, and the stained gels were digitalized by scanning and analyzed with ImageMaster. Picked spots were determined by mass spectrometry. B: HepG2 cells seeded at low density (2.2×10^6 cells/100 mm dish; 40,000 cells/cm^2) and at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). Cells were treated with hypoxia for 12 h, after which total RNA and total protein were isolated. The protein expression of FASN was examined by Western blot analysis. C: The mRNA expression of FASN was examined by RT-PCR. Band intensity was analyzed using Image J software, and data represents an induction-fold of FASN expression compared to each control after normalization with the α-tubulin and β-actin bands (bottom panel of B,C). D: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia and 25 mM of glucose for 12 h, total RNA and total protein were isolated from HepG2 cells. The mRNA expression of FASN was examined by RT-PCR. Western blot was performed to detect the FASN protein level. Band intensity was analyzed using Image J software, and data represents an induction-fold of FASN expression compared to each control after normalization with the β-actin and α-tubulin bands (bottom panel).

acids in cancer cells are predominantly converted to membrane phospholipids, not triglycerides [Menendez et al., 2005a; Kuhajda, 2006]. Therefore, when FASN is inhibited, cancer cells undergo apoptosis since they cannot synthesize phospholipids. Previous reports have shown that palmitic acid, the end-product of fatty acid synthesis, protects cells from apoptosis induced by chemical FASN blockers [Kuhajda et al., 1994; Chajes et al., 2006]. To confirm whether or not apoptosis by FASN reduction is triggered by palmitic acid depletion, palmitic acid was added to the medium before exposure to hypoxia. As shown in Figure 4D, palmitic acid did not restore cell viability. These results suggest that FASN down-regulation-induced apoptosis is not correlated with palmitic acid depletion under hypoxic conditions. On the other hand, another report found that malonyl-CoA is a potential mediator of cytotoxicity induced by FASN inhibition in human breast cancer cells [Kuhajda, 2000; Bandyopadhyay et al., 2006]. The accumulation of malonyl-CoA, a substrate of FASN, inhibits CPT-1 (carnitine palmitoyltransferase-1) and increases production of ceramide, a membrane sphingolipid. Ceramide increases the permeability of the mitochondrial outer membrane, which ultimately results in apoptosis [Siskind, 2005; Bandyopadhyay et al., 2006]. Therefore, to confirm whether or not malonyl-CoA accumulation induces apoptosis, TOFA (5-(tetraecyloxy)-2-furoic acid) was added to the medium before exposure to hypoxia. TOFA is an allosteric inhibitor of acetyl-CoA carboxylase (ACC) and blocks the carboxylation of acetyl-CoA to malonyl-CoA [Pizer et al., 2000; Ronnett et al., 2005]. As shown in Figure 4E, cell viability was recovered by nearly 50% upon TOFA treatment under hypoxic conditions. These results suggest that FASN down-regulation causes accumulation of malonyl-CoA under hypoxic conditions, followed by hypoxic cell death.

SUPPRESSION OF SREBP-1 LEADS TO FASN REDUCTION AND HYPOXIC CELL DEATH

In this study, the level of FASN mRNA significantly decreased under hypoxic cell death condition. Thus, to confirm whether or not FASN promoter activity is reduced by hypoxia, we performed a promoter activity assay using luciferase reporter vector containing the FASN promoter construct. In the results, FASN promoter activity was dramatically reduced by hypoxia (Fig. 5A). Human FASN gene expression is mainly up-regulated by the transcription factors SREBP and ChREBP [Griffin and Sul, 2004; Dentin et al., 2005; da Silva Xavier et al., 2006; Furuta et al., 2008]. To confirm the expression of these transcription factors under hypoxic conditions, RT-PCR and Western blot analysis were performed. As shown in Figure 5B, expression of SREBP-1 was significantly reduced in hypoxia-exposed cells, whereas expression of ChREBP was not affected (data not shown). These data indicate that expression of SREBP-1 is reduced by hypoxia. Next, to examine whether or not FASN transcription is reduced by decreased SREBP-1 expression, V5-tagged recombinant mature SREBP-1 (rmSREBP-1) expression vector was constructed. Transiently rmSREBP-1 transfected cells were exposed to hypoxia for 12 h, after which luciferase reporter assay was performed. As shown in Figure 5C, decreased FASN promoter activity was restored by rmSREBP-1 overexpression under hypoxic conditions. These data suggest that decreased SREBP-1

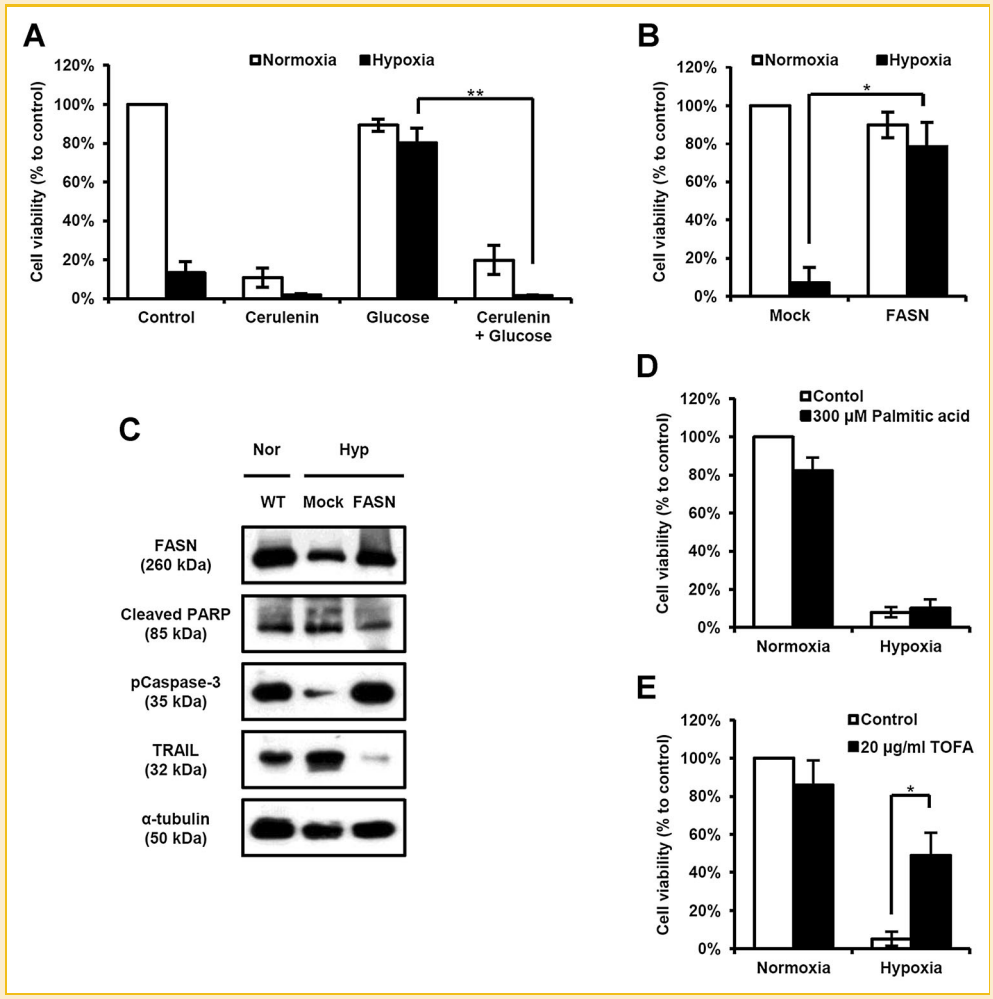

Fig. 4. Reduction of FASN induces hypoxic cell death via malonyl-CoA accumulation. A: HepG2 cells seeded at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia, 25 mM of glucose and 10 μg/ml of cerulenin for 12 h, MTT assay was performed to examine cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times. B: Cells stably expressing FASN expression vector and empty vector seeded at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, MTT assay was performed to examine the cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times. C: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, Western blot analysis was performed to measure total protein levels of PARP, caspase-3, and TRAIL. D: HepG2 cells seeded at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia and palmitate (300 μM) for 12 h, MTT assay was performed to examine cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times. E: HepG2 cells seeded at high density (7×10^5 cells/well; 200,000 cells/cm^2). After exposure to hypoxia and 20 μg/ml of TOFA for 12 h, MTT assay was performed to examine cell viability. Each sample was assayed in triplicate, and the experiment was repeated three times.

expression may cause a reduction in FASN expression under hypoxic conditions. In addition, to confirm whether or not hypoxic cell death is induced by down-regulation of SREBP-1, rmSREBP-1 stably expressing HepG2 cells were exposed to hypoxia for 12 h, after which cell viability was examined. Viability of the SREBP-1-stably expressing cell line under hypoxic conditions was restored to about 62% of that of the normoxic control (Fig. 5D). These data suggest that decreased SREBP-1 expression may be responsible for cell death under hypoxic conditions.

DISCUSSION

In contrast to normal cells, the majority of fatty acids in cancer cells are derived from de novo fatty acid synthesis rather than dietary fats [Menendez and Lupu, 2004; Menendez et al., 2005a]. This increased lipogenesis is reflected in a significantly elevated activity of lipogenic enzymes such as ATP citrate lyase and FASN [Kuhajda, 2006; Menendez and Lupu, 2007]. FASN, the key enzyme in de novo fatty acid biosynthesis, is overexpressed in several human carcinomas and their pre-neoplastic lesions, but it is not expressed in most normal tissues [Menendez and Lupu, 2004]. Especially, FASN has been identified as tumor associated protein oncoantigen-519 (OA-519) in breast cancer [Kuhajda et al., 1994; Semenkovich, 1997]. Further, high levels of FASN expression are associated with an aggressive cancer phenotype [Baron et al., 2004]. In addition, various reports have shown that inhibition of FASN induces apoptosis in FASN-expressing cancer cells [Menendez and Lupu, 2007]. Collectively, these reports indicate that FASN could

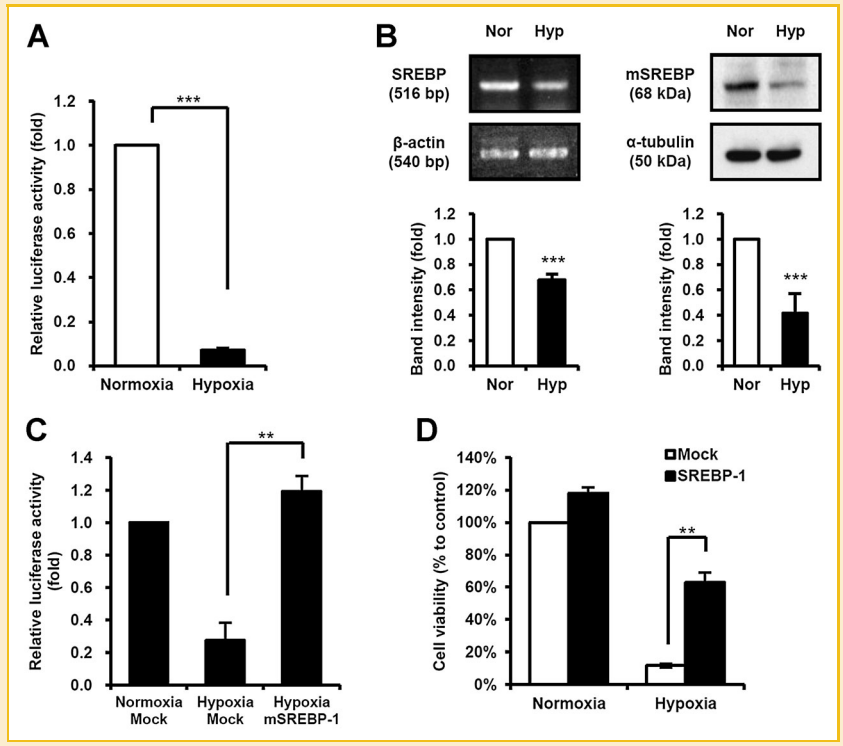

Fig. 5. Cell viability and FASN expression are restored by SREBP-1. A: HepG2 cells seeded at high density (7×10^5 cells/100 mm dish; 200,000 cells/cm^2) and transiently transfected with FASN promoter constructs. After exposure to hypoxia for 12 h, FASN promoter activity was measured by luciferase assay. B: HepG2 cells seeded at high density (1.1×10^7 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, total RNA and total protein were isolated from HepG2 cells. The mRNA expression of SREBP-1 was examined by RT-PCR. Western blot was performed to detect the SREBP-1 protein level. Band intensity was analyzed using Image J software, and data represents an induction-fold of SREBP-1 expression compared to each control after normalization with the β-actin and α-tubulin bands (bottom panel). C: HepG2 cells seeded at high density (7×10^5 cells/100 mm dish; 200,000 cells/cm^2) and transiently co-transfected with FASN promoter constructs and rmSREBP-1 expression constructs. After exposure to hypoxia for 12 h, FASN promoter activity was measured by luciferase assay (rmSREBP-1; recombinant mature SREBP-1). D: HepG2 cells stably expressing rmSREBP-1 seeded at high density (7×10^5 cells/100 mm dish; 200,000 cells/cm^2). After exposure to hypoxia for 12 h, MTT assay was performed to examine cell viability.

ultimately be used for diagnosis or prognosis [Kuhajda, 2000]. In highly proliferating cells, tumor-associated FASN overexpression is considered necessary for both membrane lipids production and post-translational protein modification [Baron et al., 2004; Menendez and Lupu, 2007]. However, the exact mechanisms of tumor-associated FASN overexpression are not fully understood.

Generally, glycolysis increases upon a lack of oxygen in cancer cells away from blood vessels [Malhotra and Brosius, 1999]. Therefore, cancer cells rapidly uptake and consume glucose, which results in the accumulation of pyruvate and lactate. This exhaustion of glucose and accumulation of lactate is the main cause of hypoxia-induced apoptosis [Dang and Semenza, 1999; Almaas et al., 2003; Gatenby and Gillies, 2004; Greijer and van der Wall, 2004]. In our experiments, exhaustion of glucose was associated with hypoxia-induced apoptosis. Glucose level was extremely reduced by hypoxia in the high density culture (data not shown), whereas the acidic environment did not induce apoptosis (Fig. 2). In addition, in the experiment with glucose-free media, apoptosis was not induced under normoxic conditions. On the other hand, under hypoxic conditions, glucose-free media more efficiently induced apoptosis in comparison with normal media containing glucose (data not shown). That is, these results suggest that glucose exhaustion by hypoxic adaptation induces apoptosis.

Recently, it was suggested that hypoxia triggers FASN over-expression via Akt and SREBP-1 activity in breast cancer cells [Furuta et al., 2008]. However, in our experiment, FASN expression did not increase under hypoxic conditions in both the low and high density HepG2 cell culture. It can be assumed that FASN is fully expressed in HepG2 cells. However, 2-DE proteomic analysis found that FASN expression was reduced under hypoxic cell death conditions (Fig. 3A). Further, it was confirmed that FASN promoter activity, mRNA expression, and protein expression were all reduced under hypoxic conditions in the high density HepG2 cell culture (Figs. 5A and 3B,C). These results show that reduced expression of FASN is associated with hypoxia-induced apoptosis. According to various reports, FASN expression is regulated by glucose level [Semenkovich et al., 1993; Towle, 2005]. As shown in Figures 2 and 3D, sufficient glucose treatment restored hypoxia-induced apoptosis as well as recovered FASN expression. Therefore, decreased level of glucose under hypoxic conditions reduces FASN expression, which is associated with cell viability. Additionally, we identified that FASN plays a protective role in hypoxic cell death (Fig. 4).

SREBPs, of which there are two genes, SREBP-1 and SREBP-2, encoding three proteins, SREBP-1a, 1c, and 2, are the main regulators in lipogenesis and the sterol response. SREBPs located at

ER membrane are primarily activated and translocated into the nucleus by post-translational cleavage of a precursor form [Li et al., 2011]. Among these SREBPs, fatty acid synthesis is predominantly regulated by SREBP-1c. However, the effects of FASN and SREBP-1 expression under hypoxic cell death conditions are not yet known. In this study, recovery of SREBP-1 expression restored FASN promoter activity (Fig. 5C). We collectively identified that hypoxia reduces SREBP-1 expression in HepG2 cells, thereby decreasing FASN expression and resulting in cell death. In previous reports, SREBP-1 expression was regulated by glucose through activation of the PTEN/Akt pathway [Hao et al., 2011] and inactivation of the AMPK pathway [Hardie and Pan, 2002; Li et al., 2011]. Therefore, to determine the mechanisms of SREBP-1 down-regulation under hypoxic cell death conditions, the expression and phosphorylation levels of AMPK and PTEN were investigated. However, we obtained no evidence that decreased SREBP-1 is associated with the AMPK or PTEN pathway (data not shown). Therefore, the mechanisms of SREBP-1 down-regulation remain unknown.

Taken together, hypoxic cell death is induced by the reduced expression of FASN through SREBP-1 down-regulation. As high density cell culture is similar to a tumor environment, this study suggests that apoptosis in high density cultured cancer cells under hypoxic conditions represents cancer cell death in regions of limited oxygen and glucose diffusion. In conclusion, our results suggest that FASN expression is important for cancer cell survival under hypoxic conditions and that high FASN expression is a potential target for cancer diagnosis and treatment.

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