KLF5 enhances SREBP-1 action in androgen-dependent induction of fatty acid synthase in prostate cancer cell line

Min-Young Lee

Department of Medical Science

The Graduate School, Yonsei University

KLF5 enhances SREBP-1 action in androgen-dependent induction of fatty acid synthase in prostate cancer cell line

Directed by Professor Kyung-Sup Kim

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Min-Young Lee

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This certifies that the Doctoral Dissertation

of Min-Young Lee is approved.

Thesis Supervisor : Kyung-Sup Kim

Yong-Ho Ahn

Sung-Kil Lim

Soon Won Hong

Joohun Ha

The Graduate School
Yonsei University

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Min-Young Lee

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ABSTRACT

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Min-Young Lee

Department of Medical Science

The Graduate School, Yonsei University

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The Kruppel-like factor 5 (KLF5) is a zinc-finger transcription factor that plays important roles in regulating all aspects of cellular signaling involved in cell proliferation, differentiation and oncogenesis. In the present study, results explain that KLF5 interacts with sterol regulatory element-binding protein-1 (SREBP-1) in regulating the expression of lipogenic protein called fatty acid synthase (FASN), which plays an important role in androgen-dependent

prostate cancer cells. Genes involved in lipid metabolism, including *FASN*, are reported to be up-regulated by androgens in prostate cancer cells through a coordinated cascade controlled by the activation of SREBP-1 rather than a direct action of androgen. The promoter and intron 1 region of *FASN* gene contains multiple GC-boxes that might show response to KLF5. Deletion and mutation analyses indicated that KLF5 binds to the *FASN* gene and strongly induces its activity. Furthermore, results demonstrated that KLF5 protein is able to bind to SREBP-1 and enhance the SREBP-1 regulated activation of FASN. Transfection of KLF5 into prostate cancer cell lines enhanced *FASN* promoter activity, and conversely, silencing of KLF5 by small interfering RNA in prostate cancer cells down-regulated FASN expression. The multiple GC motifs are scattered throughout the upstream sequence and first intron of *FASN* gene whereas these are scarce in the promoters of other lipogenic and cholesterogenic genes such as acetyl-CoA carboxylase (*ACAC*), ATP-citrate lyase (*ACLY*), LDL receptor (*LDLR*), HMG-CoA synthase (*HMGCS1*) and HMG-CoA reductase (*HMGCR*), which makes *FASN* gene very unique among them. Taken together, *FASN* gene could be activated by the synergistic action of KLF5 and SREBP-1 which is increased by androgen treatment.

Key words: KLF5, SREBP-1, FASN, androgen, prostate

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I. INTRODUCTION

Prostate cancer is the most commonly diagnosed non-cutaneous neoplasm among males and the second leading cause of cancer-related death in western countries[1]. Androgen plays an important role in the development, maintenance, management and progression of prostate cancer[1]. As a consequence, androgen

ablation therapy has become the standard treatment of advanced diseases. Unfortunately, most patients relapse because of the outgrowth of androgen-independent cells[2].

An important key player in prostate cancer development and progression is the androgen receptor (AR)[3]. Androgens exert most of their effects on prostate cancer cells by binding to and activating the AR. The AR is a member of the nuclear receptor super-family which acts as a ligand-dependent transcriptional regulator[4]. It consists of an activation domain (AD), a DNA-binding domain (DBD) and a ligand-binding domain (LBD). Circulating androgens in the plasma enter the cell and bind to AR becoming dimerized and phosphorylated. This complex enters the nucleus and binds on the androgen-response element (ARE) of the target promoter to activate its activity.

The AR promotes the growth and regulation of the normal prostate and remains present in nearly all prostate tumors, even in recurrent androgen-independent tumors[1]. In prostate cancer cells, several alterations in the AR have been found that render the AR more active or that broaden its ligand-specificity, this way helping to the escape from androgen ablation therapy[1]. Mounting evidence demonstrates that altered expression of co-activators and crosstalk between the AR and other signaling pathways play a central role in

the dys-regulation of AR function in prostate cancer resulting in abnormal and sustained expression profiles of AR-regulated genes even after the escape from androgen ablation therapy[2].

Numerous reports have indicated that lipid metabolism is a major target of androgen action in prostate cancer cells[5]. When LNCaP cells were exposed to androgen, it led to a massive accumulation of neutral lipids, which are storage products of fatty acid and cholesterol[5]. Up-regulation of FASN mRNA and protein is one of the earliest and most common events in the development of prostate carcinoma, and a strong association between FASN expression and tumor initiation has been shown[6, 7]. Accordingly, FASN expression was much stronger in tumors than in BPH and this expression correlates with high Gleason scores[8]. Increased synthesis of fatty acid and cholesterol is governed by androgens through stimulation of the expression of whole sets of lipogenic enzymes, covering the entire pathways of fatty acid and cholesterol synthesis[6].

FASN, a 250-270 kDa cytosolic protein, is a sole enzyme which catalyzes *de novo* fatty acid synthesis. In normal human tissues, the endogenous fatty acid synthesis is minimal since exogenous influx supplies most of the fatty acids required. On the contrary, FASN is over-expressed in various human malignancies including the prostate, breast, ovarian, endometrial, colorectal,

lung, stomach and skin[6, 9-18]. Although the exact role of the FASN gene in tumorigenesis is not yet clearly elucidated, treatment of tumor cells with pharmacological inhibitors of FASN leads to apoptosis and cell cycle arrest[19]. These observations illustrate that FASN over-expression confers a selective advantage to tumor cells by inhibiting apoptosis and promoting cell cycle progression. As FASN is expressed at low or undetectable level in most normal human tissues and is over-expressed in a variety of tumors, FASN has become an important target for the potential diagnosis and treatment of cancer. However, relatively not much is known about the mechanisms controlling *FASN* gene expression in human tumors, and understanding these mechanisms is of great interest to design the FASN enzymatic pathway.

The key players involved in fatty acid metabolism are the SREBPs [20, 21]. SREBPs are a family of basic helix-loop-helix leucine zipper transcription factors that are synthesized as inactive precursor proteins anchored to the endoplasmic reticulum (ER) membranes[21]. There they interact with an SREBP cleavage-activating protein (SCAP), which is retained in the ER by Insig retention proteins (Insig-1 and Insig-2)[22]. The SCAP/SREBP/Insig complex is stabilized by cholesterol. When sterol levels are low, SCAP/SREBP complex is released from Insigs and move to golgi, where N-terminus of SREBP is

released by a two-step proteolysis resulting in translocation to the nucleus. This transcriptionally active fragment activates the transcription of multiple genes involved in the synthesis, binding, metabolism and uptake of fatty acids and cholesterol[21].

SREBP-dependent transcriptional activation from several promoters is dependent on the presence of an additional binding site for a ubiquitous transcription factor. In the *LDLR*, *ACACA* and *FASN* promoters, which are all regulated by SREBP, the transcription factor is Sp1[23].

Sp1-like proteins and Kruppel-like factors contain at least 21 members with highly related zinc finger proteins which are important components of the eukaryotic cellular transcriptional machinery[24]. Among the family members, the DNA-binding domain is highly conserved at the carboxyl terminus that has three tandem Cys_2His_2 zinc-finger motifs[25] which binds not only to DNA, but also functions in protein-protein interactions[26, 27]. Individual members of the Sp/KLF family have preferences for binding different DNA sequences in the target gene promoter. Sp/KLF proteins can function as either activators or repressors depending on the environment, such as in which promoter they bind and with which co-regulators they interact[26, 28, 29]. Since many Sp1/KLF proteins regulate cell growth in a variety of cell types, it is not surprising that

some members of the family also appear to participate in carcinogenesis. KLF5 has been reported to encode a delayed early response gene product that positively regulates cellular proliferation[30] and plays an oncogenic role in the human bladder cancer cell line through the regulation of a subset of genes[31].

In the present study, it has been observed that KLF5, another member of the Sp/KLF family, is capable in activating the transcription of *FASN* gene through the synergistic action with SREBP-1 in androgen-dependent LNCaP prostate cancer cell. Moreover, KLF5 expression is consistently associated with high levels of FASN expression in prostate cancer cells. By analyzing the *FASN* promoter activity, KLF5 is a stimulator for FASN expression which shows its activity in concert with SREBP-1. These findings suggest that KLF5 may be a novel target for therapy of prostate cancers.

I. MATERIALS AND METHODS

1. Cell culture

The human prostatic adenocarcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and PC3 was a kind gift from Prof. Joohun Ha (Biochemistry and Molecular Biology, College of Medicine, Kyunghee University, Seoul, Korea). Cells were maintained at 37 °C in a humidified incubator with a 5% CO_2 in air in RPMI 1640 medium supplemented with reduced level of sodium bicarbonate (1.5 g/L), 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units /ml penicillin. Cell culture medium and supplements were from Invitrogen (Carlsbad, CA). To minimize and reduce the background level of steroids, FBS was pretreated with dextran-coated charcoal (CT-FBS)[32]. The synthetic androgen R1881 was purchased from DuPont/New England Nuclear and dissolved in absolute ethanol (Merck). Control cultures received similar amounts of ethanol only. Final ethanol concentrations did not exceed 0.1%.

2. Promoter-reporter constructs and transient transfection assay

The promoters for ACACA-PI, ACACA-PII, ACLY, HMGCS1 and FASN were constructed to evaluate the promoter activities by KLF5. They were amplified from the genomic DNA isolated from LNCaP cells except for the HMGCS1 promoter[20] which was kindly provided by Dr. Johannes V. Swinnen (Experimental Medicine and Endocrinology, Gasthuisberg, K. U. Leuven, Leuven, Belgium). The primer sequences used for promoter constructions were listed in Table 1. In the case of ACACA-PI promoter construction, XhoI and BglII site were introduced in sense and anti-sense primers, respectively. The amplified PCR fragments for ACACA-PI were digested with XhoI and BglII, individually, and inserted into the XhoI/BglII site of the pGL3-basic vector (Promega). The amplified PCR fragments for ACACA-PII, ACLY and FASN were inserted into the SmaI site of the pGL3-basic vector (Promega) by blunt-end ligation. PCR was performed using LA Taq. polymerase in 2 X GC buffer II (Takara).

For assays of androgen-dependent transcriptional activation, LNCaP cells were seeded in 100-mm dish in RPMI 1640 containing 5 % CT-FBS at a density of 4 X 10^6 cells. Two days later, cells were transfected with the

indicated luciferase reporter plasmids (3 μg) using Lipofectamine and the PLUS reagent according to the manufacturer's protocol (Invitrogen). After 6 h incubation, the cells were changed with fresh medium with 5 % CT-FBS. The next day, cells were trypsinized and transferred to 6-well dishes in the presence or absence of 10^{-8} M R1881. After 72 hours, cells were washed with PBS and harvested in 200 μl reporter lysis buffer (Promega). They were mixed vigorously for 15 seconds and centrifugation was performed at 4 °C, 12,000 X g for 10 minutes. Supernatants were transferred to a fresh tube and aliquots of 5 μl of cleared lysate were assayed for luciferase activity by using a luciferase reporter assay kit (Promega). Each transfection experiment was performed in triplicate and the best result was used among three independent experiments.

The eukaryotic expression constructs containing full-length KLF4, KLF5 and SREBP1c were cloned using cDNAs synthesized from the total RNA of LNCaP cells. PCR was performed with appropriate annealing temperatures ((T_m - 5) °C) by Ex Taq. polymerase (Takara). The amplified PCR fragments were then inserted into the pSG5-HA-tagged expression vector by blunt-end ligation, which was a kind gift from Dr. Ho-Geun Yoon (Yonsei University, College of Medicine, Korea).

Table 1. Oligonucleotide sequences used for

the preparation of promoter-reporter constructs

Name of Oligonucleotide		Oligonucleotide sequence
ACACA-PI	**Sense**	5’ CACGAGAATCGCTCGAGCCTGGGAG 3’
	Anti-sense	5’ TCCTCCCAGTCCT CGAGCACGGGGA 3’
ACACA-PII	**Sense**	5’ TGGAACGCTGTGGCAACTATTT TGC 3’
	Anti-sense	5’ AGTTTCTCCAGGTCCCCGGTCACAG 3’
ACLY	**Sense**	5’ GGGTACTCCAGGTCCCAAAGCTGCG 3’
	Anti-sense	5’ GAACCCCGCAAAATCCGGAGCACCC3’
FASN	**Sense**	5’ ACACGAACA CAATGCTCTGC 3’
	Anti-sense	5’ TGAGGTTGTCCCAGAACTCC 3’

3. Western blot analysis

Cells were lysed in a buffer composed of 1.1 M urea, 0.33 M NaCl, 25mM HEPES (pH 7.6), 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT and 1 mM sodium orthovanadate. Lysates were briefly vortexed and cleared by centrifugation at 12,000 X g for 10 minutes at 4°C. Supernatants were collected and transferred to a fresh tube. Protein concentrations were determined using Bradford assay system (Bio-rad). Equal amounts of proteins were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Shleicher & Schuell). The NC membranes were blocked in phosphate-buffered saline containing 0.05% (v/v) Tween 20 and 5% (w/v) nonfat dried milk, and probed with primary antibodies indicated in the figure legends. The following antibodies were used: polyclonal antibodies against ACACA, ACLY, FASN, SREBP-1, SREBP-2 and SCAP from Genepia (Seoul, Korea), anti-Flag mouse monoclonal antibody (Sigma, F3165), anti-HA mouse monoclonal antibody (Santa Cruz Biotechnology, sc-7392), anti-KLF4 rabbit polyclonal antibody (Chemicon, AB4138), anti-BTEB2 rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-22797), anti-α-tubulin mouse monoclonal antibody (Calbiochem, CP06), anti-rabbit IgG peroxidase

(Pierce, 31462) and anti-mouse IgG horseradish peroxidase (Pierce, 31430). Immunoreactive bands were visualized by secondary antibodies (Pierce) using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce).

4. RNA isolation and real time-PCR (RT-PCR)

Total RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. For quantitative RT-PCR, cDNAs were synthesized from total RNA (5 μg) using random hexamer primer and SuperScript reverse transcriptase II (Invitrogen) according to the manufacturer's protocol. The resulting cDNAs were then diluted with 80 μl of Tris-EDTA (pH 8.0) and 2 μl was subsequently mixed with 2 X SYBR Green PCR Master Mix (Applied Biosystems). Diverse sets of gene-specific primers were mixed and subjected to RT-PCR quantification using the ABI PRISM 7300 Real Time PCR System (Applied Biosystems). The specific sequences of the primers used for RT-PCR are listed in Table 2. All the reactions were performed in triplicate. The relative amounts of the RNAs were calculated by using the comparative C method. As an invariant control, GAPDH mRNA was used.

Table 2. Oligonucleotide sequences used for RT-PCR

Oligonucleotides		Oligonucleotide sequence
ACACA	**Sense**	5’TGGTCTCTTTCCGGACCTTTGAAG 3’
	Anti-sense	5’TCCTCCTCAAACTTATCCCTTGCTCGGA3’
ACLY	**Sense**	5’TGCTCGATTATGCACTGGAAGT3’
	Anti-sense	5’ATGAACCCCATACTCCTTCCCAG3’
FASN	**Sense**	5’TACGTACTGGCCTACACCCAGA3’
	Anti-sense	5’TGAACTGCTGCACGAAGAAGCATAT3’
HMGCS1	**Sense**	5’GACTTGTG CATTCAAACATAGCAA3’
	Anti-sense	5’GCTGTAGCAGGGAGTCTTGGTACT3’
HMGCR	**Sense**	5’CAAGGAGCATGCAAAGATAATCC3’
	Anti-sense	5’GCCATTA CGGTCCCACACA3’
LDLR	**Sense**	5’TGAACTGGTGTGAGAGGACCAC3’
	Anti-sense	5’TGTTCTTAAGCCGCCAGTTGTT3’
SREBP-1	**Sense**	5’AAACTCAAGCAGGAGAACCTAAGTCT3’
	Anti-sense	5’GTCAGTGTGTCC TCCACCTCAGT3’
SREBP-2	**Sense**	5’CAAGCTTCTAAAGGGCATCG3’
	Anti-sense	5’CACAAAGACGCTCAGGACAA3’

SCAP	**Sense**	5’TATCTCGGGCCTTCTA CAACCA3’
	Anti-sense	5’ACACAACTCCTCCAAGCTCCTG3’
KLF4	**Sense**	5’AA GACGATCGTGGCCCCGGA3’
	Anti-sense	5’GGTTTCTCACCTGTGTGGGTTC3’
KLF5	**Sense**	5’TTACCCACCACCCTGCCAGTTAACT3’
	Anti-sense	5’TAAACTTTTGTGCAACCAGGGTAAT3’
PSA	**Sense**	5’ATTTGATGGAGAA ACTGGCTGT3’
	Anti-sense	5’GGATCAGGGTAGGAGACACAAG3’

5. siRNA transfection

Two siRNAs were designed to target KLF4 and KLF5 mRNA. Their target sequences were 5'-GACCGAGGAGTTCAACGATUU-3' (siKLF4) and 5'-AAAGTATAGACGAGACAGTGCUU-3' (siKLF5), respectively. A scrambled siCONTROL (Dharmacon, Chicago, IL) was used as a control. For siRNA transfection, cells were grown in 100-mm plates or 12-well plates for 48 hours in 5% CT-FBS medium without antibiotics. The transfections were performed with 200 nM chemically synthesized siRNAs (Dharmacon, Chicago, IL) using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, three times for three days. Briefly, the medium was change to 1% CT-FBS in the presence of 10^{-8} M R1881. The siRNA solution was then mixed with Lipofectamine RNAiMAX in OPTI-MEM media for 20 min and was added to the cultured cells. Total RNA or protein extracts were collected 3 days after first transfection and RT-PCR or western blot analyses were performed, respectively.

6. Co-immunoprecipitation

Cells were lysed in a buffer composed of 50 mM Tris, pH 7.4, 150 mM NaCl, 0.3% Nonidet P-40, 0.2% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and protease inhibitors. After centrifugation, 500 μg of the clarified cell lysate was pre-cleared by adding 50 μl of 50 % slurry of G plus / protein A-agarose (Santa Cruz Biotechnology), incubating it for 2 hours. The supernatant was collected and 2 μg of FLAG antibody was added to pre-cleared lysate. After overnight incubation, 50 μl of G plus / protein A-agarose bead was added and the mixture was incubated for 2 hours. The agarose bead was centrifuged, washed three times with ice-cold PBS, suspended in electrophoresis sample buffer, and boiled for 3 min. The immuno-precipitated protein was further analyzed by western blotting as described above.

7. Preparation of fluorescent DNA probe and hybridization

LNCaP cells were seeded in 100 mm dish and incubated for 48 h. Then, cells were treated with androgen at a final concentration of 10^{-8} M. After 72 h incubation, total RNA was extracted from cells with the use of TRIzol reagent (Invitrogen) according to the manufacturer's protocols. Each of the total RNA samples (30 ug) were labeled with Cyanine (Cy) or Cyanine conjugated dCTP (Amersham) by a reverse transcription reaction using reverse transcriptase, SuperScript II (Invitrogen). The labeled cDNA mixture was then concentrated using ethanol precipitation method. The concentrated Cy3 and Cy5 labeled cDNAs were re-suspended in 10 μl of hybridization solution (GenoCheck, Korea). After two labeled cDNAs were mixed, placed on OpArray Mouse genome 35K (OPMMV4, Operon Biotechnologies, GmbH) and OpArray Rat genome 27K (OPRNV3 Operon Biotechnologies, GmbH), and covered by a hybridization chamber. The hybridized slides were washed in 2 X SSC, 0.1 % SDS for 2 min, 1 X SSC for 3 min, and then 0.2 X SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 sec to dry.

8. Micro-array data analysis

Hybridized slides were scanned with the Axon Instruments GenePix 4000B scanner and the scanned images were analyzed with the software program GenePix Pro 5.1 (Axon, CA) and GeneSpring GX 7.3.1 (Sillicongenetics, CA). Spots that were judged as sub-standard by visual examination of each slide were flagged and excluded from further analysis. Spots that had dust artifacts or spatial defects were manually flagged and excluded. To filter out the un-reliable data, signal-to-noise spots (signal - background - background SD) below 10 were not included. Data were normalized by global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes. Data were clustered groups of genes that behave similarly across time course experiments using GeneSpring GX 7.3.1 (Sillicongenetics). An algorithm based on the Pearson correlation was used to separate the gene of similar patterns. The accuracy of micro-array analyses in this study was confirmed by Real-time PCR analysis.

III. RESULTS

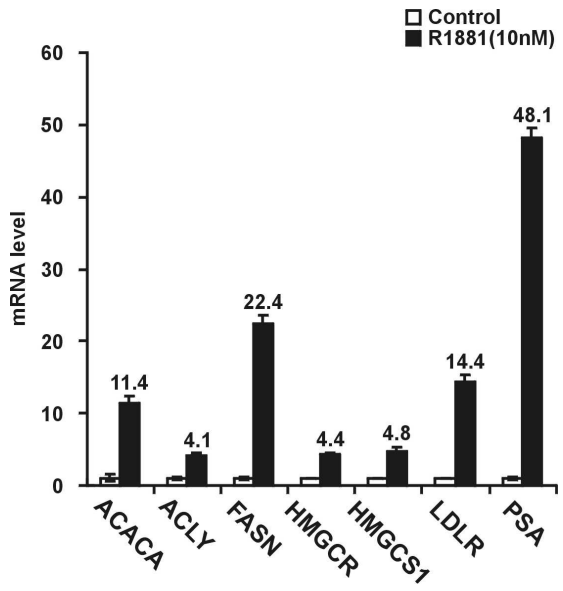
1. Androgen up-regulates the expression of lipogenic enzymes and SREBPs are involved in this regulation.

To investigate that androgen affects the expression of lipogenic genes in prostate cancer cells, LNCaP cells were incubated for 3 days in the absence or presence of 10^{-8} M of the synthetic androgen, R1881. Total RNA was prepared and subjected to RT-PCR with primers for lipogenic (ACACA, ACLY and FASN) and cholesterogenic genes (HMGCR, HMGCS1 and LDLR). The levels of these transcripts were up-regulated by androgen and the increase of FASN gene expression was the most predominant (Figure 1A). The prostate specific antigen (PSA) gene was markedly activated by androgen which is known as a direct target gene of AR. Moreover, the protein levels of lipogenic enzymes were also increased by androgen treatment (Figure 1B).

SREBPs are principle regulators in determining the expression levels of multiple enzymes involved in lipogenesis and cholesterogenesis. In prostate cancer cells, androgen is known to induce the expression of SCAP, resulting in the increase of processing the SREBP precursors to mature forms[20]. To confirm whether SREBPs virtually play an important role in androgen-

dependent activation of lipogenic and cholesterogenic genes, we performed transient transfection assay, using the reporter constructs harboring promoters for FASN and HMGCS1 (Figure 2). The critical elements (SREs) for SREBP-mediated trans-activation have been precisely characterized in promoters for FASN[33] and HMGCS1[34]. These promoters were dramatically activated by androgen treatment and mutations of the SREs almost completely abolished the androgen-dependent activation. This indicates that SREBP activities play a critical role in androgen-dependent increases of lipogenic and cholesterogenic gene expressions. Nevertheless, the increase in the levels of mature form of SREBPs and SCAP protein was not dramatic by androgen treatment although the premature form of SREBPs was up-regulated (Figure 2C). The transcript for SCAP, which is reported as a direct target of AR, was minimally increased as well as those of SREBPs. The above results suggest the possibility that the existence of other transcription factors, controlled by androgen might regulate the trans-activation activities of SREBP.

A



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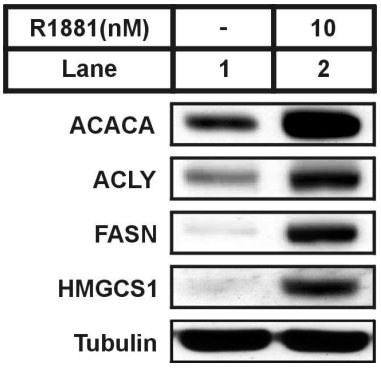
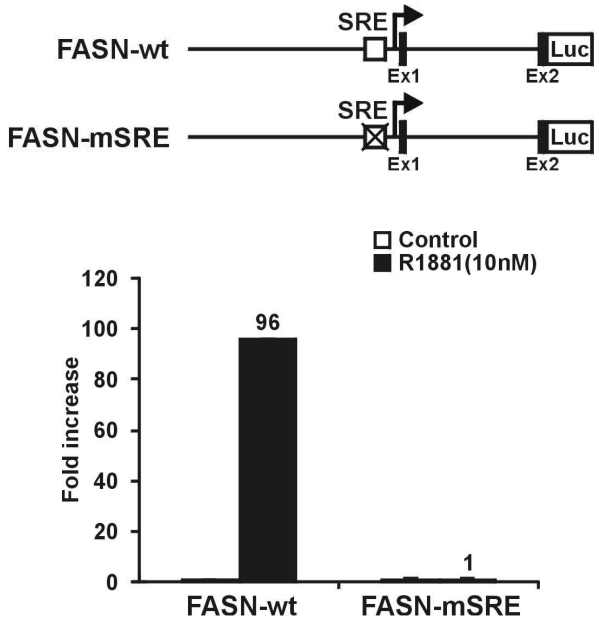


Figure 1. Androgen-dependent regulation of lipogenic gene expressions in LNCaP prostate cancer cells. Cells were incubated for 3 days in the absence or presence of 10^{-8} M R1881 in 1% CT-FBS media. Total RNA was isolated and subjected to RT-PCR with primers for lipogenic and cholesterogenic genes described in materials and methods (A). Cell lysates were prepared and western blot was performed with various antibodies (B).

A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SRE
Ex1
Ex2
Luc
FASN-mSRE
SRE
Ex1
Ex2
Luc
Control
R1881(10nM)
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100
80
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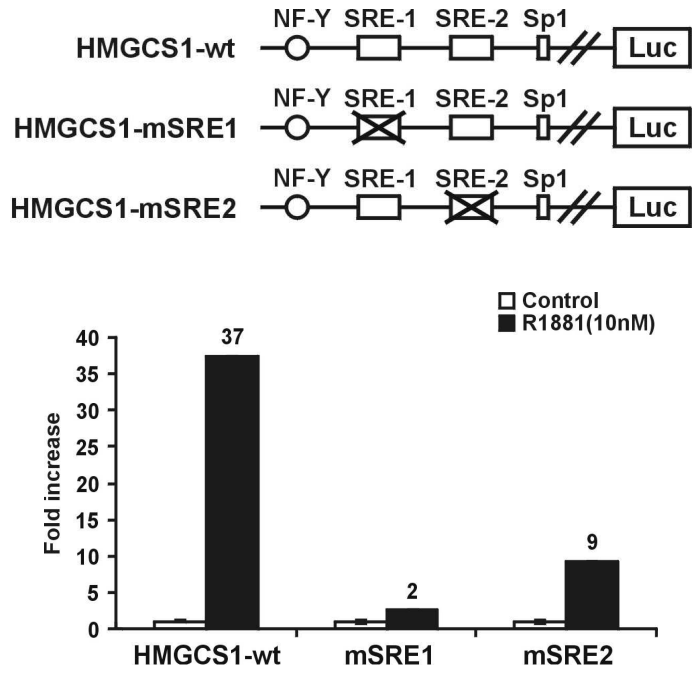
40
20
0
96
1
FASN-wt
FASN-mSRE

B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Luc
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NF-Y SRE-1 SRE-2 Sp1
Luc
HMGCS1-mSRE2
NF-Y SRE-1 SRE-2 Sp1
Luc
Control
R1881(10nM)
Fold increase
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15
10
5
0
37
2
9
HMGCS1-wt
mSRE1
mSRE2

C

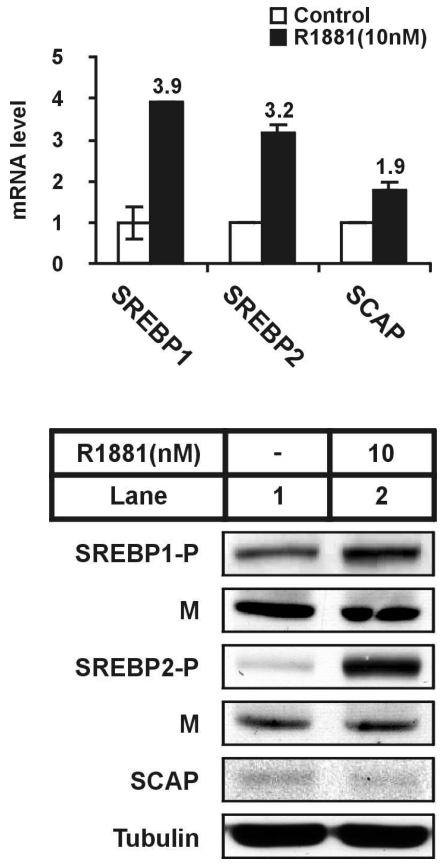


Figure 2. SREBPs are involved in androgen-dependent regulation of lipogenic gene expressions. LNCaP cells were transfected with wild- or SRE-mutant types of reporter constructs containing FASN (A) and HMGCS1 (B) promoters. Constructs were incubated for 72 h in the absence or presence of 10 nM R1881. Total RNA and proteins were isolated from LNCaP cells 3 days after hormone treatment and were subjected to RT-PCR and western blot to determine the levels of SREBP-1, SREBP-2 and SCAP mRNAs and proteins, respectively (C). SRE, sterol response element; mSRE, mutant sterol response element; NF-Y, nuclear factor Y; Sp1, stimulatory protein 1

Table 3. Micro-array analysis data*

Name	Gene Symbol	Intensity (R1881 / Ethanol)
LIPOGENIC ENZYMES		
Fatty acid synthase (FASN) [2.3.1.85]	FASN	9.7
Acetyl-CoA carboxylase (ACCa1) [EC 6.4.1.2]	ACACA	2.4
CHOLESTEROGENIC ENZYMES		
3-hydroxy-3-methyglutaryl-Coenzyme A synthase 1 (soluble) [EC 4.1.3.5]	HMGCS1	6.0
3-hydroxy-3-methyglutaryl-Coenzyme A reductase [EC 1.1.1.88]]	HMGCR	3.2
Low-density lipoprotein receptor precursor (LDL receptor)	LDLR	2.8
SREBP cleavage-activating protein (SCAP)	SCAP	2.6
Farnesyl pyrophosphate synthetase (FPS) [EC 2.5.1.10]	FDPS	3.3
Lanosterol synthase	LSS	3.3
Squalene monooxygenase [EC 1.14.99.7]	SQLE	3.7
Sp / KLF FAMILY		
Kruppel-like factor 4 (Gut-enriched)	KLF4	2.7
Kruppel-like factor 5 (Intestinal-enriched) (Colon kruppel-like factor)	KLF5	2.5

* LNCaP cells were seeded in 100 mm dish and incubated for 48 h. After that, cells were treated with androgen at a final concentration of 10^{-8} M. After 72 h incubation, total RNA was extracted from cells with the use of TRIzol reagent (Invitrogen). Micro-array analysis was performed with human 35 K oligo-chip as described in Materials and Methods.

2. KLF5 level is increased by androgen in LNCaP prostate cancer cells and is associated with the expression of FASN gene.

In many promoters, the trans-activation function of SREBP work efficiently in synergy with different ubiquitous transcription factors, such as Sp1 and NF-Y[23, 35, 36]. In previous studies, Sp1 was reported to be required for SREBP-mediated activation of FASN, ACACA, ACACB and LDLR genes[23, 37]. Contrary to prior reports, the DNA-binding activity and protein level of Sp1 was not changed at all by androgen treatment (data not shown). Nonetheless, micro-array analysis data provided the fact that KLF4 and KLF5, belonging to Sp/KLF family, are induced by androgen (Table 1). As a result of RT-PCR for these factors, KLF4 and KLF5 were significantly increased in their transcripts by androgen treatment up to 5.5 and 3.3 folds, respectively (Figure 3).

To further confirm the association among KLF5 and lipogenic enzymes in prostate cancer, KLF5 was over-expressed to see whether forced expression of KLF5 could affect the activity of the promoters for various lipogenic genes. Only FASN promoter showed significantly increased activity in response to KLF5 over-expression (Figure 4B). Accordingly, comparison among FASN promoters and other lipogenic enzyme promoters explains that multiple GC motifs for KLF5 binding are scattered throughout the upstream sequence and

the first intron of FASN gene whereas GC elements are scarce in ACACA-PI, ACACA-PII, ACLY and HMGCS1 promoters (Figure 4A).

Because KLF4 recognizes the same GC elements as KLF5 and were also increased by androgen, forced expression of KLF4 was done to test whether it could show similar results. However, the promoter activity of FASN did not show any response to KLF4 over-expression (Figure 4C). As a result, KLF5, but not KLF4, increases the promoter activity of FASN when over-expressed.

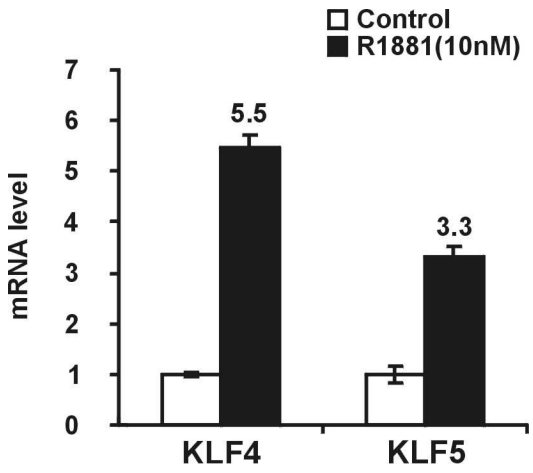
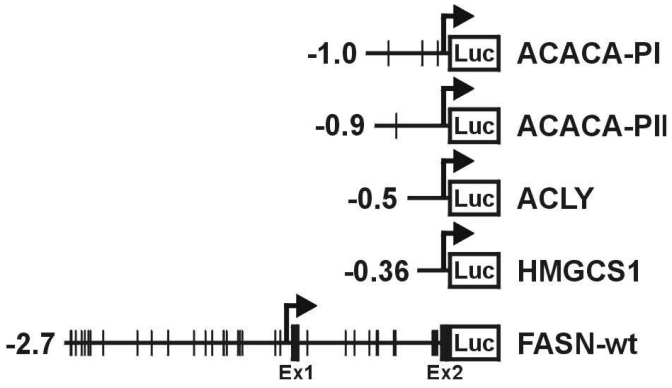
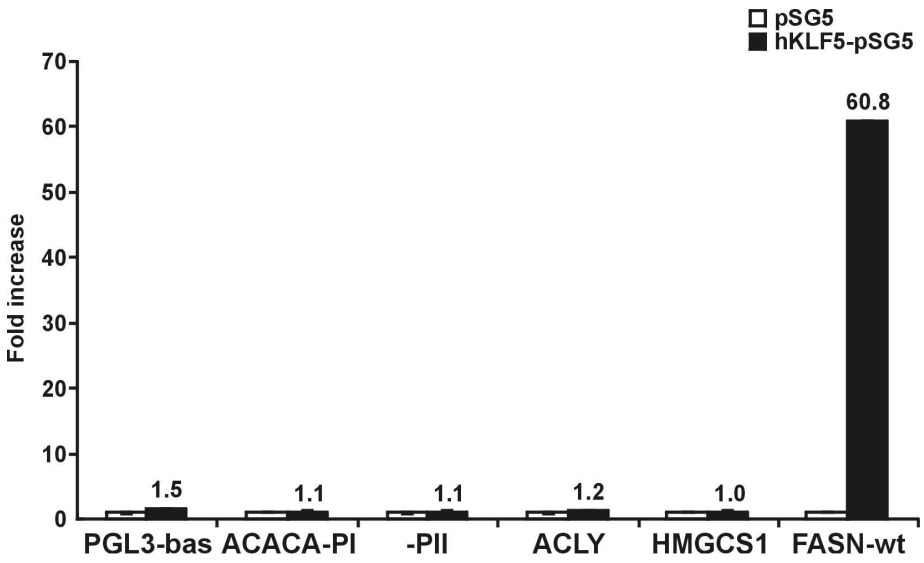


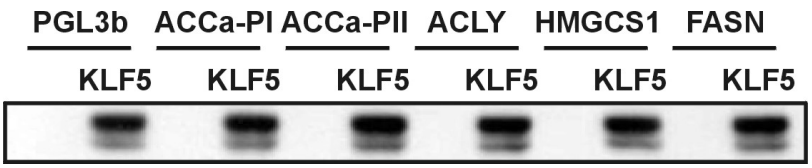
Figure 3. The mRNA level of KLF4 and KLF5 are increased by androgen treatment in LNCaP prostate cancer cells. LNCaP cells were incubated for 3 days in the absence or presence of 10nM R1881. Total RNA was prepared and subjected to RT-PCR with primers described in Materials and Methods.

A


-1.0
Luc
ACACA-PI
-0.9
Luc
ACACA-PII
-0.5
Luc
ACLY
-0.36
Luc
HMGCS1
-2.7
Luc
FASN-wt
Ex1
Ex2

B


pSG5
hKLF5-pSG5
Fold increase
70
60
50
40
30
20
10
0
60.8
1.5
1.1
1.1
1.2
1.0
PGL3-bas
ACACA-PI
-PII
ACLY
HMGCS1
FASN-wt


PGL3b
ACCa-PI
ACCa-PII
ACLY
HMGCS1
FASN
KLF5
KLF5
KLF5
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KLF5

C

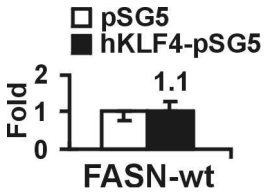


Figure 4. KLF5 regulates the FASN promoter activity in LNCaP prostate cancer cells, but KLF4 is not involved in this regulation. (A) Structures of lipogenic promoter constructs, ACACA-PI, ACACA-PII, ACLY, HMGCS1 and FASN, with GC-boxes indicated with black vertical bars. (B) Promoters of lipogenic enzymes were co-transfected with control or KLF5 expression vector and luciferase activities were measured. (C) FASN reporter construct was co-transfected with control or KLF4 expression vector and the activity of the promoter was measured.

3. KLF5 interacts with SREBPs in regulating FASN expression.

The next step was to determine the role of GC-boxes in SREBP-1-mediated activation of FASN promoter. The over-expression plasmid(s) for SREBP-1c and/or KLF5 were transfected in company with FASN promoter construct into PC3 prostate cancer cells. The promoter activity was increased by KLF5 and SREBP-1c over-expression up to 14 and 27 folds, respectively (Figure 5A). Furthermore, the activity was dramatically increased to more than the additive value when SREBP-1c and KLF5 were over-expressed together. Nevertheless, the SRE mutated FASN plasmid almost completely abolished the SREBP-1c-mediated activation and the synergistic action between KLF5 and SREBP-1c, without affecting KLF5 responsiveness.

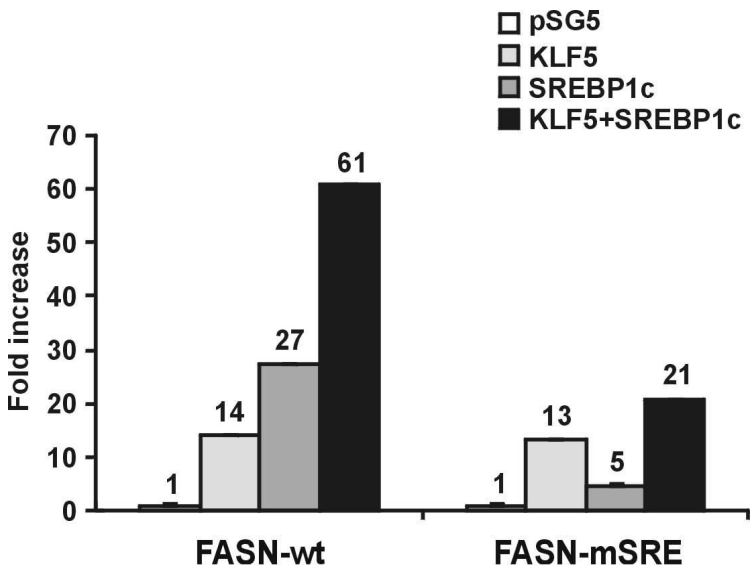
To confirm synergistic effect between KLF5 and SREBP-1c, the induction of FASN promoter activity was measured when the amount of SREBP-1c was changed in the absence or presence of KLF5 over-expression. The promoter activity of FASN was already saturated at only 25 ng of SREBP-1c plasmid alone and remained constant even when more SREBP-1c was added (Figure 5B). On the other hand, when KLF5 was over-expressed, the activity of FASN promoter continuously increased by SREBP-1c in a dose-dependent manner without becoming saturated. As a result, SREBP-1c-mediated activation of

FASN promoter was enhanced by the addition of KLF5.

Next, protein-protein interaction was observed since KLF5 and SREBP-1c showed cooperative action. HA-tagged SREBP-1c and/or Flag-tagged KLF5 were transfected in PC3 cells. After 48 h incubation, the cells were harvested and total lysate was precipitated by anti-Flag antibody. The lysate was then subjected to western blot analysis. This result indicates that SREBP-1c and KLF5 could physically interact with each other (Figure 5C).

To elucidate the correlation between KLF5 and FASN expression in LNCaP cells, KLF5 expression plasmids were transfected and the endogenous level of FASN was measured. When the cells were transfected with the empty vector, the endogenous level of FASN was low. Nonetheless, when exogenous KLF5 expression vector was introduced by transfection, the endogenous level of FASN protein increased dramatically (Figure 5D).

A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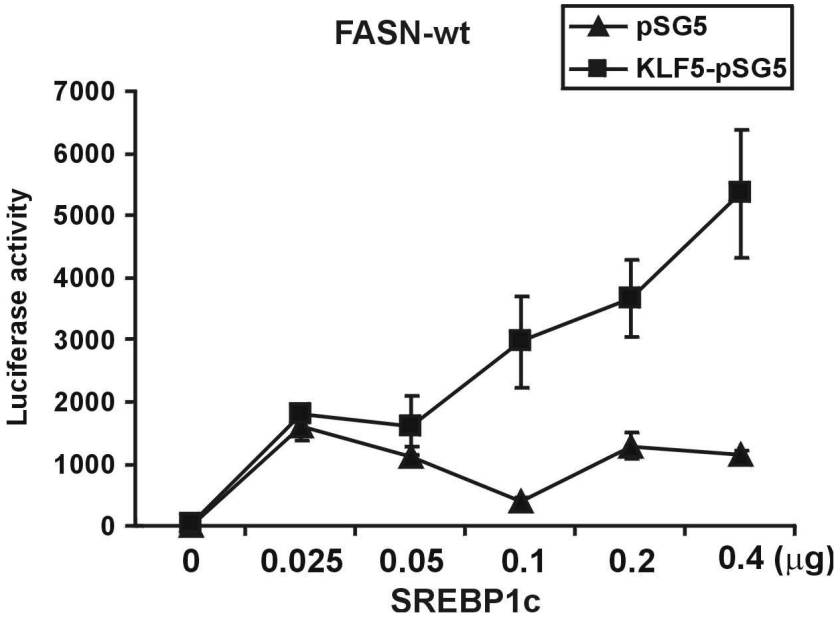
KLF5
SREBP1c
KLF5+SREBP1c
Fold increase
70
60
50
40
30
20
10
0
1
14
27
61
1
13
5
21
FASN-wt
FASN-mSRE

B


FASN-wt
pSG5
KLF5-pSG5
Luciferase activity
7000
6000
5000
4000
3000
2000
1000
0
0
0.025
0.05
0.1
0.2
0.4 (μg)
SREBP1c

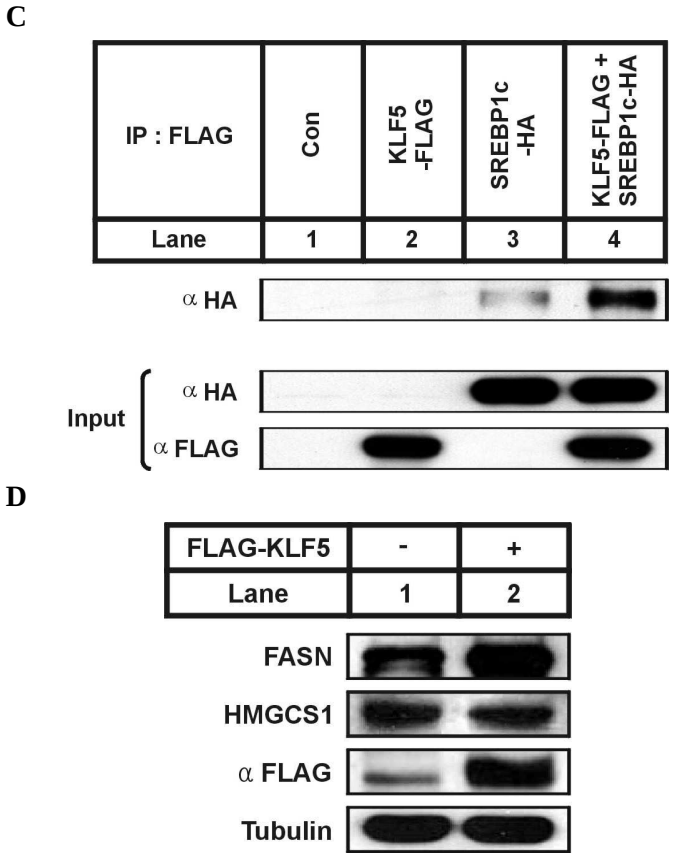


Figure 5. Co-operate action and physical interaction between SREBP and KLF5 in regulating FASN transcription. (A) FASN reporter construct was cotransfected with SREBP1c, KLF5 expression plasmid or both and promoter activities were measured in PC3 cells. (B) FASN promoter construct was co-transfected with control or KLF5 expression plasmids and different doses of SREBP-1c expression plasmid in LNCaP cells and activities were measured after 24 h incubation. (C) Control plasmid or KLF5 expression plasmid was transfected in LNCaP cells and whole cell lysates were prepared for western blot analysis. (D) HA-tagged SREBP-1c, FLAG-tagged KLF5 or both were transfected in PC3 cells and samples were subjected to IP-western blot assays.

4. KLF5 dramatically enhances androgen-dependent activation of FASN promoter.

FASN promoter can be markedly activated by a synergistic action between exogenously introduced KLF5 and SREBP-1 in PC3 cells. If SREBP-1c is activated by androgen treatment in LNCaP cells, the exogenous KLF5 could enhance the androgen-dependent activation of FASN promoter. Consequently, the androgen-dependent activity of FASN promoter construct was measured in the presence or absence of KLF5 over-expression. The promoter activities of FASN by androgen treatment and KLF5 expression increased up to 68 folds and 34 folds, respectively. Furthermore, the activity markedly increased up to 545 folds when androgen was added in the presence of over-expressed KLF5 (Figure 6A).

Next, the promoter activity of FASN was measured using KLF4 expression plasmid instead of KLF5. Contrary to previous results, the increased promoter activity of FASN by androgen treatment did not show any synergistic action when KLF4 was over-expressed (Figure 6B). Besides, the HMGCS1 promoter was affected by androgen but not by KLF5 transfection. Furthermore, the activity remained unchanged when androgen was added in the presence of over-expressed KLF5 indicating that HMGCS1 promoter activity is regulated

only by SREBP-1c (Figure 6C). A possible explanation for these results is that KLF5, but not KLF4 specifically regulates the promoter activity of FASN and this is synergistically activated when androgen is added. In addition, this action is limited to FASN promoter among various lipogenic gene promoters.

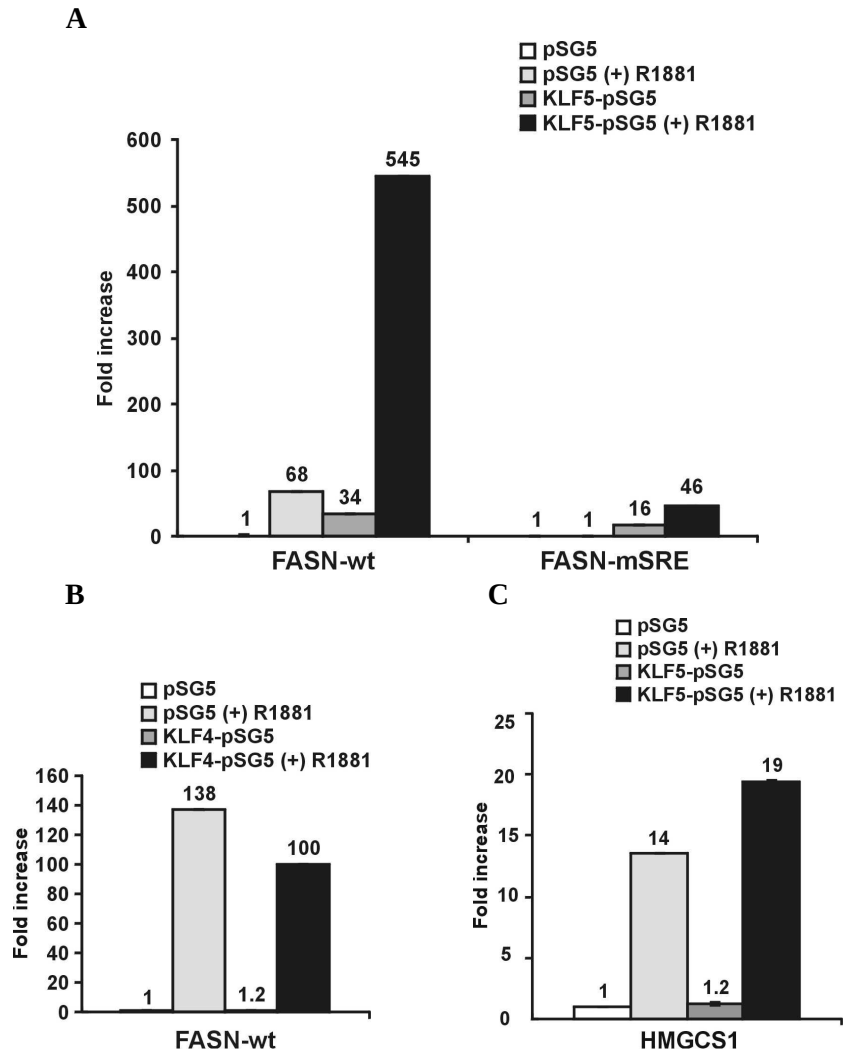
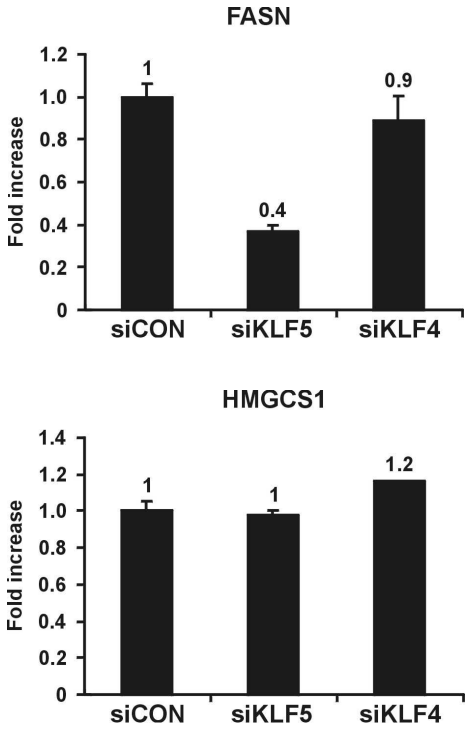


Figure 6. The effect of FASN promoter by SREBP, KLF5 and AR. FASN wt- or mSRE-construct was transfected with control, KLF5 (A) or KLF4 (B) plasmid. After 24 h incubation, media was changed in the presence or absence of 10^{-8} M R1881 and cells were incubated for 3 days. Cells were harvested and luciferase activity was measured. (C) Equal experiment was performed except that HMGCS1 promoter was used instead of FASN-wt promoter.

5. KLF5 siRNA suppresses androgen-mediated FASN expression by inhibiting the cooperative action of KLF5 with SREBP-1c.

FASN expression is most prominently activated by R1881 in LNCaP cells and SREBP-1 plays a critical role in this induction. However, dramatic change in the levels of SREBP-1 mature form after R1881 treatment was not detected. The fact that KLF5 is activated by R1881, even if not much, and has the properties of the synergistic action with SREBP, suggested the possibility that KLF5 also plays a critical role in FASN induction by androgen as well as SREBP-1. Based on this hypothesis, knock-down of KLF5 was performed to test whether FASN induction is blocked by R1881 treatment in LNCaP cells. A specific KLF5 siRNA, which was synthesized as reported previously[14, 38], effectively suppressed FASN mRNA level up to 40% of that of control siRNA group (Figure 7A) and also decreased the protein level of FASN (Figure 7B). Since the mRNA level of KLF4 was increased by androgen and it binds to the same GC-box as KLF5, it was tested whether KLF4 siRNA also repressed the expression of FASN in prostate cancer cells. Transfection of KLF4 siRNA did not reduce the level of FASN mRNA (Figure 7A) or protein level (Figure 7B). These results indicate that KLF5 is directly involved in androgen-dependent expression of FASN in prostate cancer cells, but not KLF4.

A



B

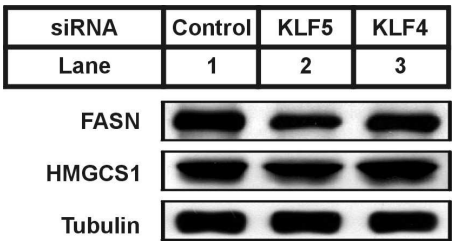


Figure 7. The effects of KLF5 siRNA on expression of endogenous KLF5 and FASN mRNA and protein level. LNCaP cells were transfected with 200nM of control, KLF4 or KLF5 siRNAs three times for three days. Total RNA or protein extracts were collected 3 days after first transfection and RT-PCR (A) and western blot (B) were performed.

6. GC-boxes are scattered on the FASN promoter and intron 1 region which makes KLF5 a positive regulator.

The promoter of FASN, which shows the responsiveness to KLF5, contains multiple GC-boxes for KLF5 binding. To discriminate which GC-boxes play a critical role in KLF5 responsiveness, serial deletion constructs of FASN promoter were generated as shown in Figure 8A and transient transfection assay was performed in prostate cells with over-expressed KLF5 (Figure 8B). When the (-2.7kb) construct was over-expressed with KLF5, the promoter activity increased up to 58 folds. Moreover, (-1.0 kb) and (-0.4 kb) constructs were increased up to 42.1 and 39.7 folds by KLF5, respectively. To narrow down the specific binding sites, the intron region was deleted from the construct. The basal activity of FASN promoter with deleted intron were highly increased even though the KLF5-responsiveness still exists, suggesting that the intron region strongly suppressed the FASN promoter activity. As a result, scattered multiple KLF5 binding sites play a role in this promoter, and these GC-boxes helps the SREBP-mediated activation of FASN promoter.

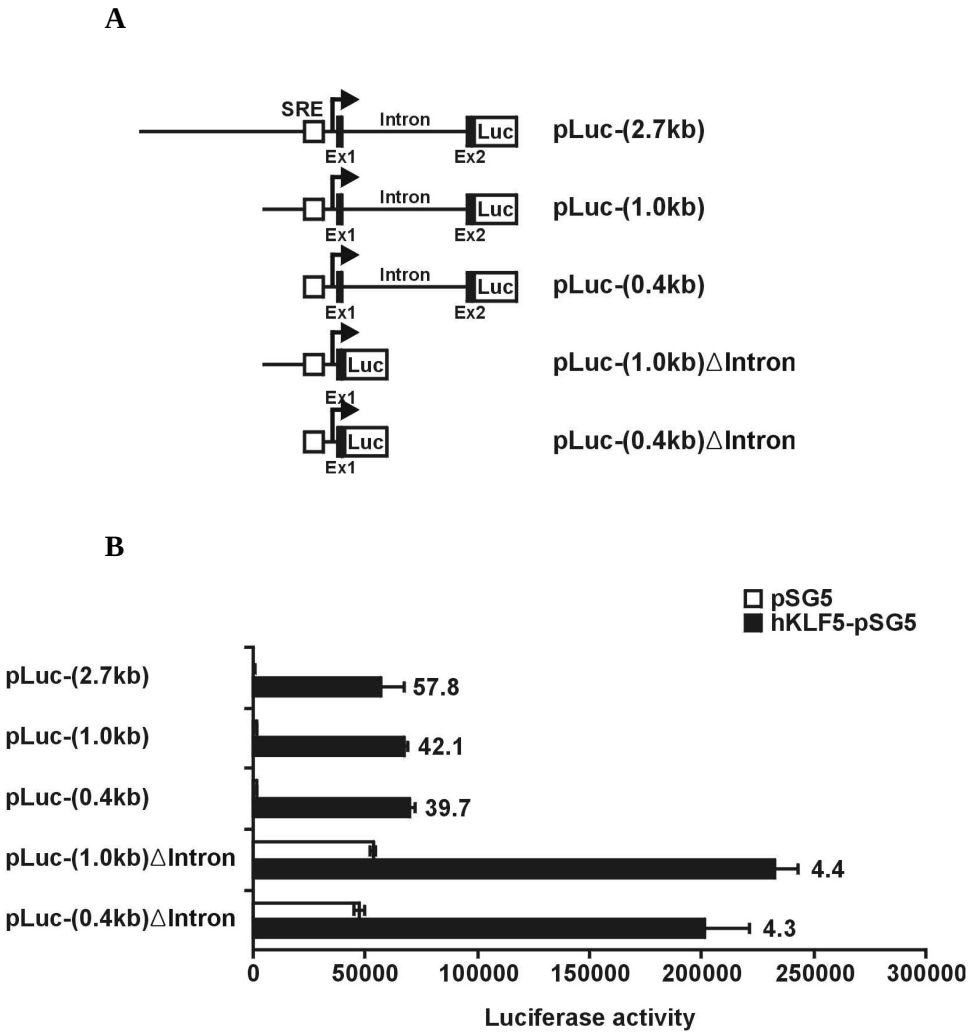


Figure 8. Deletion and mutation analysis of the FASN promoter activity. (A) Structures of deleted and/or mutated FASN promoter constructs. SRE, sterol response element (B) Transient transfection and luciferase assay was performed in PC3 cells with over-expressed KLF5. FASN promoters were co-transfected with either KLF5 or control constructs. At 48 h post-transfection, cell extracts were analyzed for luciferase activity.

IV. DISCUSSION

The present study demonstrates that KLF5 enhances the activity of SREBP, resulting in the activation of FASN expression in androgen-treated LNCaP prostate cancer cells. KLF5 is expressed in LNCaP prostate cancer cells and acts as a positive regulator inducing the expression of the oncogenic protein FASN. KLF5, as well as SREBP, is increased by androgen treatment and this result in up-regulation of FASN expression. Furthermore, silencing of KLF5 by siRNA in LNCaP cells decreased the expression of FASN.

Previous reports have revealed that lipogenic enzymes involved in fatty acid and cholesterol synthesis are up-regulated in prostate cancer cells by androgen[2, 5, 39] and are coordinately regulated by SREBPs[20, 21, 40]. Specifically, lipogenic enzymes are reported to be up-regulated through an increase in mature SREBP level by androgen[20] and this increased processing of SREBP occurs by an escort protein called SCAP which is known as a direct target of AR[40]. Among those lipogenic enzymes, FASN is known to be over-expressed in not only prostate cancer cells but also in different cancer cell lines[6, 7, 9-19, 41] and is thought as an oncogenic protein[8].

In the study presented here, lipogenic enzymes were increased by androgen

treatment in LNCaP cells and these up-regulation occurred at transcriptional level (Figure 1A) in contrary to HER2-mediated increases of ACACA and FASN in breast cancer cells, which take place at translational levels by the activation of mTOR signaling pathway[42]. However, results from this study illustrates that the mRNA (Figure 2A) and protein (Figure 2B) levels of SREBP-1 and SREBP-2 were increased minimally by androgen treatment. Furthermore, SCAP, known as a direct target of AR[40], did not increase dramatically by androgen (Figure 2). Therefore, other androgen-regulated transcription factors might help and/or participate in regulating FASN level by androgen.

KLF family proteins are transcription factors controlling the expression of genes linked to either positive or negative regulation of cell proliferation and growth[24, 43-48]. Consequently, members of this family are associated in the pathological process of cancers. Although most of KLF family members have not been characterized for their cellular function, several KLF members have recently been revealed to be linked to human cancers and induced apoptosis in cancer cells[30, 31, 38, 49-58]. However, the molecular mechanisms of KLF proteins are not clearly defined yet.

Among those transcription factors, KLF4 and KLF5 are two closely related

members of the zinc finger-containing KLFs. Although they bind to a similar DNA sequence that has either a CACCC homology or is rich in GC content, previous reports have been demonstrated that they show opposing effects on transcription of target genes[46]. For example, KLF4 activates the promoter of its own gene which is a negative regulator of cell proliferation, while KLF5 suppresses the KLF4 promoter[57]. Moreover, KLF4 and KLF5 bind to the same sequence in the promoters of two smooth muscle genes, α-smooth muscle actin and SM22α[59]. Here, KLF4 inhibits and KLF5 activates TGFβ-dependent stimulation of the SM22α promoter. In another example, KLF4 and KLF5 antagonize each other in regulating the CYP1A1 promoter activity[14, 43-45].

The difference in the biochemical behavior of KLF4 and KLF5 reflects their biological behavior. For example, the cellular distribution of the two KLFs is different with each other. Usually, KLF4 is mainly expressed in the post-mitotic differentiated villus epithelial cells of the intestinal tract[50], while KLF5 is expressed mostly in the proliferating crypt cells[44]. Moreover, the pattern of expression of the two genes in vitro contrasts each other. KLF4 is mainly associated with a process of growth arrest[50], while KLF5 mainly accompanies cellular proliferation[30]. Moreover, forced expression of KLF4 leads to a G1/S cell cycle arrest[48, 53] but that of KLF5 causes a transformed

phenotype[30].

In this study, KLF4 and KLF5 were revealed to be increased by androgen (Table 3, Figure 3). Promoters for ACACA-PI, ACACA-PII, ACLY and HMGCS1 contain none or very scarce GC-boxes but, on the contrary, FASN promoter contain multiple GC-boxes throughout the promoter and intron 1 (Figure 4A). In the transient transfection assay, KLF5, but not KLF4 (Figure 4C), increased the promoter activity of FASN while the remaining promoters were not affected by KLF5 at all (Figure 4B). This demonstrates that among lipogenic enzymes which are regulated by androgen through SREBPs, FASN is the only gene that is also regulated by KLF5 at the transcriptional level. This makes KLF5 a very important and unique transcription factor which controls the transcriptional level of an oncogenic protein FASN.

Further study was done to elucidate the role of the correlation between KLF5 and FASN expression in LNCaP prostate cancer cell. When the cells were cultured in CT-FBS media, the endogenous level of KLF5 and FASN was very low. However, when exogenous KLF5 DNA was introduced by transfection, the endogenous level of FASN protein increased dramatically (Figure 5D). In contrast, inhibition of KLF5 by siRNA was accompanied by down-regulation of FASN (Figure 7). Furthermore, GC-boxes scattered within

the FASN promoter and intron 1 region act as response elements for promoter activity, and KLF5 strongly induces FASN promoter activity through these GC-boxes (Figure 8).

Another novel founding of the present study is that KLF5 is involved in SREBP-mediated regulation of FASN. In the transient transfection assay, increased FASN promoter activity by SREBP was strongly up-regulated by the addition of KLF5 (Figure 5A-B). Moreover, SREBP and KLF5 physically interact with each other (Figure 5C). Indeed, results indicated that SREBP activates FASN through interaction with KLF5.

The interaction between SREBP and KLF5 in regulating FASN expression has possible clinical significance. KLF5 strongly up-regulates the expression of FASN which acts as an important oncogenic protein in cancer cell growth and proliferation. Targeting the KLF5-FASN pathway using siRNA against either KLF5 or FASN may be a treatment approach for prostate and other cancers with activated SREBPs. Furthermore, it will be a good progress to find the role of KLF5 in other cancer cells such as breast, ovarian and colon since FASN is known to be over-expressed in these cells.

V. CONCLUSION

In androgen-dependent LNCaP prostate cancer cell lines, the expression of lipogenic enzymes, including FASN, is up-regulated by androgen through a coordinate cascade involving transcription factors called SREBPs. Previous reports elucidate that the levels of active mature SREBPs are increased by an escort protein called SCAP which is known as a direct target of AR. On the other hand, results in this study demonstrate that the mRNA and protein levels of SREBPs and SCAP are minimally increased by androgen. Therefore, other transcription factors were considered which might enhance the transactivation activity of SREBP by androgen treatment. Supporting this idea, the level of KLF5, a member of Sp/KLF family, was increased by androgen treatment and was associated with the expression of FASN gene. However, the promoter activity of other lipogenic genes was not affected by KLF5. Furthermore, the FASN promoter activity was not increased by KLF4, which binds to the same region of KLF5. On the promoter and intron 1 region of FASN gene, multiple GC-boxes are scattered which enhance the action of SREBP on FASN gene expression. Over-expression of KLF5 by transfection dramatically enhances the androgen-dependent activation of FASN promoter and silencing of KLF5

by siRNA specifically suppresses the expression. Taken together, FASN gene is activated by the synergistic action of KLF5 and SREBP-1, which is increased by androgen treatment.

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synthase and fatty acid synthetic activity in colorectal neoplasia. *Am J Pathol.* Jan 1997;150(1):201-8.

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ABSTRACT (IN KOREAN)

전립선암 세포주에서 안드로겐에 의한 fatty acid synthase 발현 증가를 매개하는 SREBP-1 작용에 KLF5의 보조 역할

〈지도교수 **김경섭**〉

연세대학교 대학원 의과학과

이민영

Kruppel-like factor 5 (KLF5)는 세포 증식과 발암에 관여하는 세포 신호 조절에 중요한 역할을 하는 전사 인자이다. 본 연구에서는, KLF5가 sterol regulatory element-binding protein-1 (SREBP-1)과 상호작용하여 androgen 의존적인 전립선 암에서 중요한 역할을 하는 지방산 합성 단백질인 fatty acid synthase (FASN)의 발현을 조절한다는 사실을 보고하였다. 현재까지, FASN을 포함한 지방산 대사에 관여하는 유전자들의 발현은 전립선 암에서 androgen에

의해 직접적으로 증가되기보다는 SREBP-1의 활성 증가에 의해 순차적으로 증가된다고 보고되어 있다. FASN 유전자 상의 promoter와 intron 1 위치에는 KLF5에 의해 반응을 보이는 여러 GC-boxes들이 존재한다. Deletion과 mutation 분석을 통해, KLF5가 FASN 유전자에 결합하며 그 활성을 강하게 유도하는 것을 확인할 수 있었다. 더 나아가, KLF5 단백질은 SREBP-1과 결합하여 SREBP-1 조절에 의한 FASN의 활성을 더욱 증가시켜 주는 것을 증명하였다. 전립선 암세포에 KLF5를 transfection시켜 주었을 경우, FASN의 promoter 활성이 증가하였고, 반면에, small interfering RNA를 이용하여 KLF5를 억제하였을 경우, FASN의 발현이 감소하였다. FASN 유전자의 promoter와 첫 번째 intron의 경우에는 GC motif들이 많이 산재해 있는 반면에, acetyl-CoA carboxylase α (ACACA), ATP-citrate lyase (ACLY), HMG-CoA synthase (HMGCS1), HMG-CoA reductase (HMGCR) 그리고 LDL receptor (LDLR)의 경우에는 매우 드물게 존재한다. 이는 FASN 유전자가 지방상 합성에 관여하는 유전자들 중에서도 매우 특이하다는 것을 보여준다. 위 내용을 종합해 보면, FASN 유전자는 androgen에 의해서 증가하는 KLF5와 SREBP-1의 synergistic한 작용에 의해서 활성화될 수 있다.

핵심되는 말: KLF5, SREBP-1, FASN, androgen, prostate