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

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KLF5 (Krüppel-like factor 5) is a zinc-finger transcription factor that plays a critical role in the regulation of cellular signalling involved in cell proliferation, differentiation and oncogenesis. In the present study, we showed that KLF5 acts as a key regulator controlling the expression of *FASN* (fatty acid synthase) through an interaction with SREBP-1 (sterol-regulatory-element-binding protein-1) in the androgen-dependent LNCaP prostate cancer cell line. The mRNA level of *KLF5* increased when cells were treated with a synthetic androgen, R1881. Furthermore, KLF5 bound to SREBP-1 and enhanced the SREBP-1-mediated increase in *FASN* promoter activity. The results also demonstrated that the expression of *KLF5* in LNCaP prostate cancer cells enhanced *FASN* expression, whereas silencing of *KLF5* by small interfering RNA down-regulated *FASN* expression. The proximal promoter region and the first intron of the *FASN* gene contain multiple CACCC elements that mediate the transcriptional regulation of the gene by KLF5. However, other lipogenic and cholesterogenic genes, such as those encoding acetyl-CoA carboxylase, ATP-citrate lyase, the LDL (low-density lipoprotein) receptor, HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthase and HMG-CoA reductase are irresponsive to *KLF5* expression, owing to the absence of CACCC elements in their promoter regions. Taken together, these results suggest that the *FASN* gene is activated by the synergistic action of KLF5 and SREBP-1, which was induced by androgen in androgen-dependent prostate cancer cells.

Key words: androgen receptor (AR), fatty acid synthase (FASN), Krüppel-like factor (KLF) family, sterol-regulatory-element-binding protein-1 (SREBP-1), synergism.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among males in the United States [1]. Androgens have been reported to play a critical role in the development and maintenance of the prostate, as well as in the progression of prostate cancer. Therefore androgen ablation therapy has become the standard modality of treating the advanced forms of prostate cancer. The AR (androgen receptor), a member of the nuclear receptor superfamily, is the key player in this action of androgens [2,3]. Androgens bind and activate the AR, which acts as a ligand-inducible transcriptional regulator, to exert their actions on prostate cancer cells.

Several reports have indicated that lipid metabolism is a major target of androgen action in prostate cancer cells. When LNCaP cells are exposed to androgens, they induce a massive accumulation of neutral lipids, which are storage products of fatty acid and cholesterol [4]. Increased synthesis of fatty acids and cholesterol by androgens is mediated by stimulation of the expression of whole sets of lipogenic enzymes, covering the entire pathway of fatty acid and cholesterol synthesis [5]. Up-regulation of *FASN* (fatty acid synthase) mRNA and FASN protein is one of the earliest and most common events in the development of prostate cancer, and a strong association between FASN expression and tumour progression has been shown [5,6]. Accordingly, the level of FASN is much higher in tumours with significantly high Gleason scores than demonstrated for benign prostate hyperplasia [7].

FASN, a 250–270 kDa cytosolic protein, is an enzyme that catalyses *de novo* fatty acid synthesis in the cell. In normal human tissues, endogenous fatty acid synthesis is maintained at a minimum, since the exogenous influx of fatty acids meets most of the cellular requirements for fatty acids. However, FASN is overexpressed in various human malignancies, including prostate, breast, ovarian, endometrial, colorectal, lung, stomach and skin cancers [5,8–15]. Treatment of tumour cells with pharmacological inhibitors of FASN leads to apoptosis and cell-cycle arrest [16]. These observations support the hypothesis that FASN overexpression confers a selective advantage to tumour cells by inhibiting apoptosis and promoting cell-cycle progression. Therefore FASN has become an important target for the potential diagnosis and treatment of cancers. However, relatively little is known about the mechanism controlling the expression of the *FASN* gene in human tumours.

The genes encoding the enzymes involved in fatty acid metabolism are SREBPs (sterol-regulatory-element-binding proteins) [17,18]. SREBPs are a family of basic helix-loop-helix leucine-zipper transcription factors that are synthesized as inactive precursor proteins anchored to the ER (endoplasmic reticulum) membrane [18]. There they interact with an SCAP (SREBP cleavage-activating protein), which is retained in the ER by Insig retention proteins [19]. The SCAP–SREBP–Insig complex is stabilized by cholesterol. When sterol levels are low, the SCAP–SREBP complex is released from Insigs and moves to the Golgi, where the N-terminus of SREBP is released by a two-step proteolysis reaction resulting in its translocation to the nucleus.

Abbreviations used: ACACA, acetyl-CoA carboxylase α; ACLY, ATP citrate lyase; AR, androgen receptor; DTT, dithiothreitol; ER, endoplasmic reticulum; FASN, fatty acid synthase; FBS, fetal bovine serum; CT-FBS, dextran-coated charcoal-treated FBS; HA, haemagglutinin; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGCS1, HMG-CoA synthase 1; HRP, horseradish peroxidase; KLF, Krüppel-like factor; LDL, low-density lipoprotein; LDLR, LDL receptor; PI, promoter I; PII, promoter II; RT-PCR, real-time PCR; siRNA, small interfering RNA; SRE, sterol-response element; SREBP, sterol-regulatory-element-binding protein; SCAP, SREBP cleavage-activating protein; Sp, stimulating protein; Sp/KLF, Sp1-like protein and KLF.

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This transcriptionally active fragment activates the transcription of multiple genes involved in the synthesis, binding, metabolism and uptake of fatty acids and cholesterol by binding to the SRE (sterol-response element) [18].

SREBP-dependent transcriptional regulation of several promoters is dependent on the presence of additional binding sites for ubiquitous transcription factors. In the promoter regions of *ACACA* (acetyl-CoA carboxylase α), *LDLR* [LDL (low-density lipoprotein) receptor] and *FASN* genes, which are targets of SREBP, the binding site for the transcription factor Sp (stimulating protein) 1 plays a critical role in SREBP-mediated transcriptional regulation [20].

KLFs (Krüppel-like factors) have been reported previously to be important components of the eukaryotic transcriptional machinery [21]. The Sp/KLF (Sp1-like protein and KLF) family is composed of at least two highly related zinc-finger proteins, of which the DNA-binding domains are conserved at the C-termini with three tandem Cys_2His_2 zinc-finger motifs [22]. These conserved motifs can bind to DNA as well as functioning in protein–protein interactions [23,24]. Sp/KLF proteins can function as either activators or repressors, depending on the promoters that they bind [23,25,26]. Since many Sp1/KLF proteins regulate cell growth in a variety of ways in different 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 [27] and plays an oncogenic role in human bladder cancer cells through the regulation of a subset of genes [28].

In the present study, we demonstrate that KLF5 can activate the transcription of the *FASN* gene through its synergistic action with SREBP-1 in androgen-dependent LNCaP prostate cancer cells. The expression of KLF5 was consistently associated with high levels of FASN expression in these cells. Analyses of the *FASN* promoter showed that KLF5 functions as a stimulator of *FASN* transcription in co-ordination with SREBP-1. Our findings suggest that KLF5 may be a novel target for anticancer therapy by modulating the expression of FASN with resulting changes in lipid metabolism in cancer cells.

MATERIALS AND METHODS

Cell culture

The human prostatic adenocarcinoma cell line LNCaP was obtained from the A.T.C.C., and the PC3 cell line was provided by Dr Joohun Ha (Department of Biochemistry and Molecular Biology, Kyung Hee University School of Medicine, Seoul, Republic of Korea). Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air in RPMI 1640 medium (Invitrogen) supplemented with a reduced level of sodium bicarbonate (1.5 g/l), 10 % (v/v) FBS (fetal bovine serum), 100 μg/ml streptomycin and 100 units/ml penicillin. When appropriate, CT-FBS (dextran-coated charcoal-treated FBS) [29] was used instead of FBS to minimize the concentration of steroids in the culture medium. The synthetic androgen R1881 (DuPont/New England Nuclear) was prepared in absolute ethanol and added to the culture medium to a final concentration of less than 0.1 %.

Promoter reporter constructs and transient transfection assay

The promoters for *ACACA* [PI (promoter I) and PII (promoter II)], *ACLY* (ATP citrate lyase) and *FASN* were amplified by PCR using genomic DNA prepared from LNCaP cells. PCR was performed using the following primers: *ACACA-PI* sense, 5′-CACGAGAATCGCTCGAGCCTGGGAG-3′ and antisense, 5′-TCCTCCCAGTCCTCGAGCACGGGGA; *ACACA-PII* sense, 5′-TGGAACGCTGTGGCAACTATTTTGC-3′ and antisense, 5′-AGTTTCTCCAGGTCCCCGGTCACAG-3′; *ACLY* sense, 5′-GGGTACTCCAGGTCCCAAAGCTGCG-3′ and antisense, 5′-GAACCCCGCAAAATCCGGAGCACCC-3′; and *FASN* sense, 5′-ACACGAACACAATGCTCTGC-3′ and antisense, 5′-TGAGGTTGTCCCAGAACTCC-3′. For *ACACA-PI*, XhoI and BglII restriction sites were introduced using the sense and antisense primers respectively. The amplified PCR fragment of *ACACA-PI* was digested with XhoI and BglII and then inserted into the same restriction sites in 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 by bluntend ligation. PCR was performed using LA Taq polymerase in 2× GC buffer II (TaKaRa) following the manufacturer's instructions. The *HMGCS1* [HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthase 1] promoter construct [17] was provided by Dr Johannes V. Swinnen (Department of Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium).

For androgen-dependent transcriptional activation assays of the promoter constructs, LNCaP cells were cultured at a density of 4×10^6 cells/100-mm-diameter dish in RPMI 1640 medium containing 5 % (v/v) CT-FBS. After 2 days, cells were transfected with the indicated luciferase reporter plasmids (3 μg) using Lipofectamine™ and Plus Reagent (Invitrogen) following the manufacturer's instructions. After 6 h of incubation, the cell medium was exchanged for fresh RPMI 1640 medium containing 5 % (v/v) CT-FBS. To ensure the same transfection efficiency for each treatment group, cells were trypsinized and equal numbers of transfected cells were seeded in six-well dishes 18 h after transfection in the presence or absence of 10 nM R1881. After 72 h of incubation, cells were washed with PBS and harvested in 200 μl of reporter lysis buffer (Promega). The cells were mixed vigorously for 15 s and centrifuged at 12000 ***g*** for 10 min at 4 °C. The supernatants were transferred into a fresh tube, and 5 μl aliquots of the cleared whole-cell lysate were assayed for luciferase activity using a Luciferase Reporter Assay kit (Promega). Each transfection experiment was performed in triplicate, and the luciferase activity was normalized to the amount of protein present in the lysate.

The eukaryotic expression plasmids encoding full-length KLF4, KLF5 or SREBP1c were constructed by PCR using cDNAs synthesized from the total RNA from LNCaP cells. The amplified PCR fragments were cloned into the pSG5-HA (haemagglutinin)-tagged expression vector by blunt-end ligation.

Western blot analysis

Cells were lysed in a buffer containing 25 mM Hepes (pH 7.6), 1.1 M urea, 0.33 M NaCl, 1 % Nonidet P40, 1 mM PMSF, 1 mM DTT (dithiothreitol) and 1 mM sodium orthovanadate. Lysates were briefly vortexed and cleared by centrifugation at 12000 ***g*** for 10 min at 4 °C. The supernatants were collected and protein concentrations were determined using a Bradford assay kit (Bio-Rad). Aliquots of protein (20 μg) were subjected to SDS/PAGE [5 % (for ACACA, ACLY and FASN) or 8 % gels (for all other proteins)] and transferred on to nitrocellulose membranes (Schleicher and Schuell). Western blot analyses were performed using the following antibodies: rabbit polyclonal antibodies against ACACA (1:5000 dilution), ACLY (1:5000 dilution), FASN (1:5000 dilution), SREBP-1 (1:2500 dilution), SREBP-2 (1:2500 dilution), HMGCS1 (1:2000 dilution) and SCAP

(1:2500 dilution) (Genepia), mouse monoclonal anti-HA antibody (1:2000 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-FLAG antibody (1:5000 dilution; Sigma), and mouse monoclonal anti-α-tubulin antibody (1:2500 dilution; Calbiochem). The immunoreactive signals were visualized using an HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) antibody (1:5000 dilution) or HRP-conjugated goat anti-(mouse IgG) antibody (1:5000 dilution) (Pierce) using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce) following the manufacturer's instructions.

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

Total RNA was isolated from cultured cells using TRIzol® (Invitrogen) according to the manufacturer's instructions. For quantitative RT-PCR, cDNAs were synthesized from 5 μg of total RNA using random hexamer primers and SuperScript reverse transcriptase II (Invitrogen) following the manufacturer's instructions. An aliquot (1/50) of the reaction was used for quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers, and this was then subjected to RT-PCR quantification using the ABI PRISM 7300 RT-PCR System (Applied Biosystems). The sequences of the primers used for RT-PCR are listed in Supplementary Table S1 (http://www.BiochemJ.org/bj/417/bj4170313add.htm). All reactions were performed in triplicate. The relative amounts of the mRNAs were calculated using the comparative cycle-time method [Applied Biosystems User Bulletin number 2 (2001); Applied Biosystems]. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) mRNA was also measured as an invariant control.

RNA interference

Two siRNAs (small interfering RNAs) were designed to target *KLF4* and *KLF5* mRNA with the target sequences 5′-GACCG-AGGAGTTCAACGATUU-3′ and 5′-AAAGTATAGACGAGAC-AGTGCUU-3′ respectively. A scrambled control siRNA (siCONTROL; Dharmacon) was used as a transfection control. For siRNA transfection, cells were seeded in 100-mm-diameter dishes or in 12-well plates at a density of 3×10^6 cells/dish in 5% (v/v) CT-FBS-supplemented RPMI 1640 medium without antibiotics and cultured for 48 h. The transfection was performed three times over 3 days with 200 nM chemically synthesized siRNAs using Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's protocol. Briefly, the medium was changed to RPMI 1640 medium containing 1% CT-FBS in the presence of 10 nM R1881. The siRNA solution was then mixed with Lipofectamine™ RNAiMAX in OptiMEM I medium for 20 min before being added to the cultured cells. Total RNA or protein extracts were prepared from the cells 72 h after the first transfection, and RT-PCR and immunoblot analyses were performed.

Immunoprecipitation

Cells were lysed in a buffer composed of 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.3% Nonidet P40, 0.2% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT and protease inhibitors (Sigma) and, after centrifugation, 500 μg of the clarified cell lysate was pre-cleared by incubating for 2 h at 4 °C with 50 μl of a 50% (v/v) Protein A/G Plus–agarose slurry (Santa Cruz Biotechnology). An anti-FLAG antibody (2 μg) was incubated with the pre-cleared cell lysate overnight at 4 °C. After incubation, 50 μl of Protein A/G Plus–agarose was added and incubated for 2 h at 4 °C. The agarose was centrifuged, washed three times with ice-cold PBS, resuspended in SDS/PAGE sample buffer and boiled for 3 min. The immunoprecipitate was then analysed by SDS/PAGE and Western blotting as described above.

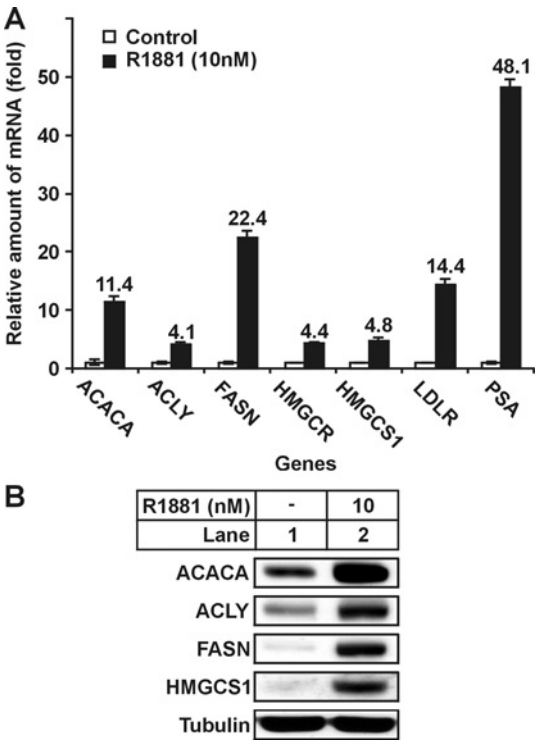

Figure 1 Androgen-dependent regulation of lipogenic gene expression in LNCaP prostate cancer cells

LNCaP cells were incubated for 3 days in the absence (Control) or presence of 10 nM R1881 in 1% CT-FBS medium, which was used to optimize the androgen effect. (**A**) Total RNA was isolated and subjected to RT-PCR with primers for lipogenic and cholesterogenic enzymes as described in Supplementary Table S1 (http://www.BiochemJ.org/bj/417/bj4170313add.htm). The values indicates the fold change between samples of control and R1881-treated mRNAs. Results are means $\pm$ S.D. ($n = 3$) with each experiment performed in triplicate. (**B**) Whole-cell lysate (20 μg) was resolved by SDS/PAGE and immunoblotted for each of the proteins indicated, with α-tubulin (Tubulin) detected as an internal loading control. HMGCR, HMG-CoA reductase; PSA, prostate-specific antigen.

Microarray analysis

Total RNA from LNCaP cells was prepared using TRIzol® (Invitrogen), which were grown in RMPI 1640 medium supplemented with 1% CT-FBS in the absence or presence of 10 nM R1881 for 3 days. The microarray hybridization was carried out by GenoCheck using a human whole 35000 oligonucleotide chip provided by Operon.

RESULTS

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

To determine whether androgen modulates the expression of lipogenic genes in prostate cancer cell lines, LNCaP cells were cultured for 3 days in the absence or presence of 10 nM R1881. After incubation, the expression levels of lipogenic or cholesterogenic enzymes were analysed by immunoblot analyses and RT-PCR. As shown in Figure 1(A), the amount of mRNAs of the genes encoding these enzymes was up-regulated by androgen, and the increase in the level of *FASN* mRNA was the most

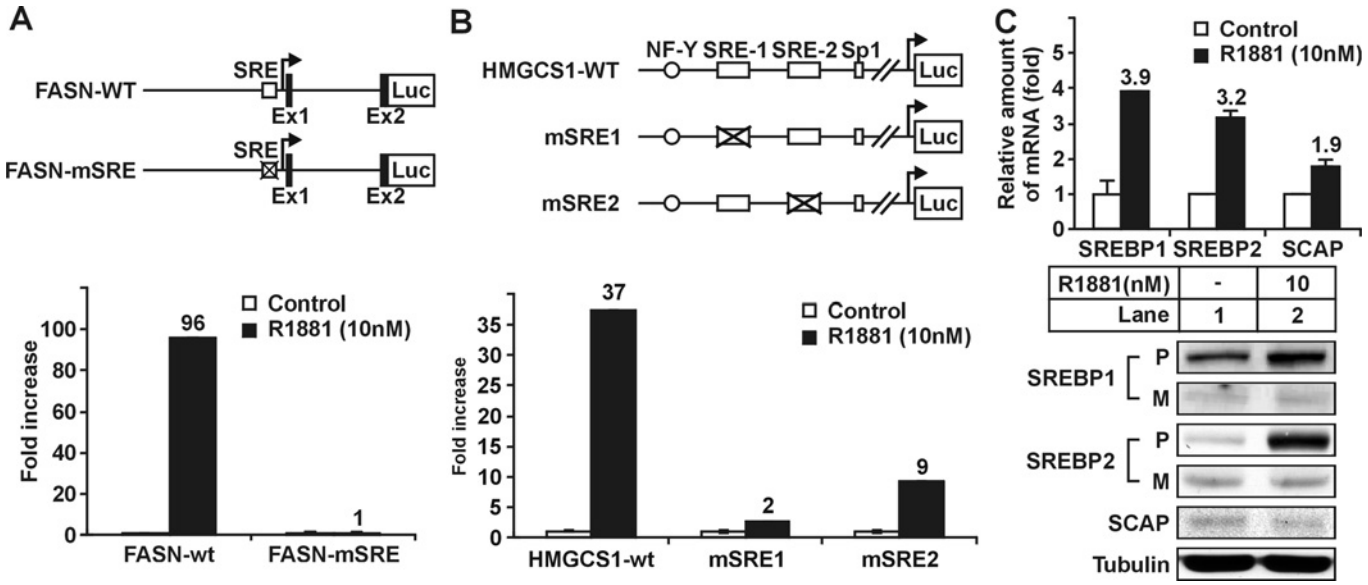

Figure 2 SREBPs are involved in androgen-dependent regulation of lipogenic gene expression

LNCaP cells were incubated in 5 % (v/v) CT-FBS-containing medium for 48 h and then transfected with wild-type (WT) or mutant SRE (mSRE) reporter constructs (0.5 μg/μl) containing *FASN* (**A**) and *HMGCS1* (**B**) promoters. Constructs were incubated for 72 h in the absence (Control) or presence of 10 nM R1881 in 1 % CT-FBS-containing medium, and luciferase activities were measured. The relative luciferase activity was converted into the fold increase in activity (values shown above bars). Results are means $\pm$ S.D. ($n = 3$) with each experiment performed in triplicate. (**C**) Total RNA and protein were isolated from LNCaP cells 3 days after incubation in 1 % CT-FBS medium in the absence (Control) or presence of 10 nM R1881 and were subjected to RT-PCR and immunoblotting to determine the levels of *SREBP-1*, *SREBP-2* and *SCAP* mRNA and protein respectively. Tubulin was used as an internal loading control. Values shown above bars indicate the fold increase in relative mRNA levels. Results are means $\pm$ S.D. ($n = 3$) with each experiment performed in triplicate. Ex, exon; Luc, luciferase; M, mature; NF-Y, nuclear factor-Y; P, premature.

prominent. The mRNA level of the *KLK3* gene [the gene encoding PSA (prostate-specific antigen)] which is known to be a direct target of the AR, was markedly elevated by androgen. Increases in the amounts of these proteins by androgen were verified by immunoblot analyses with available antibodies (Figure 1B).

Since SREBPs are well known principal transcriptional regulators of the expression of lipogenic or cholesterogenic enzymes, we determined the changes in the expression of SREBPs and SCAP in LNCaP cells after androgen treatment. In LNCaP cells, androgen is known to induce the expression of *SCAP*, resulting in an increase in the processing of SREBP precursors into mature forms [17]. To confirm whether SREBPs play an important role in androgen-dependent activation of lipogenic and cholesterogenic genes, we performed transient transfection assays using the reporter constructs harbouring promoters for *FASN* and *HMGCS1* (Figures 2A and 2B). The critical SREs for SREBP-mediated transactivation have been characterized precisely in the promoters of *FASN* [31] and *HMGCS1* [32]. These promoters were drastically activated by androgen treatment, and mutation of the SREs almost completely abolished androgen-dependent activation. These results indicate that the activity of SREBPs plays a critical role in androgen-dependent increases in lipogenic and cholesterogenic gene expression. The transcripts for *SCAP*, *SREBP-1* and *SREBP-2* were minimally increased. The increase in the level of the mature forms of SREBP and SCAP proteins as a result of androgen was not drastic, although the amount of the premature forms of SREBPs detected was up-regulated (Figure 2C).

To evaluate the actions of androgen, free steroids in the serum were removed by the addition of CT-FBS, and 1 % CT-FBS was used instead of 10 % FBS in culture medium. The charcoal treatment does not remove cholesterol in lipoproteins, but depletion of the serum in the medium might significantly reduce cholesterol levels, resulting in a possible induction of processing of SREBPs. To rule out this possibility, the expression levels of SREBPs were verified in cells incubated in medium containing 10 % (v/v) FBS or 1 % CT-FBS. The low levels of cholesterol in the 1 % CT-FBS-containing medium were probably not low enough to change the processing of SREBPs, because no changes in the levels of the premature and mature forms of SREBPs were detected in 1 % CT-FBS medium compared with SREBPs incubated in 10 % (v/v) FBS (results not shown). These observations excluded the possibility that enhanced expression of SREBPs by serum depletion could mask the effects of androgen on SREBP activation. In summary, it suggests the possibility that the existence of other transcription factors affected by androgen treatment might regulate the transactivation activities of SREBPs.

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

In many promoters, the transactivation functions of SREBPs work efficiently in synergy with different ubiquitous transcription factors, such as Sp1 and NF-Y (nuclear factor-Y) [20,33–36]. In previous studies, Sp1 was reported to be required for SREBP-mediated activation of the *FASN*, *ACACA*, *ACACB* (acetyl-CoA carboxylase β) and *LDLR* genes [20,34]. However, the DNA-binding activity and protein levels of Sp1 were not changed by androgen (results not shown). Microarray analysis provided the information that KLF4 and KLF5, which belong to the Sp/KLF family, were induced by androgen. RT-PCR showed that the transcripts of *KLF4* and *KLF5* were significantly increased by androgen up to 5.5- and 3.3-fold respectively (Figure 3).

To confirm further the association between KLF5 and lipogenic enzymes in prostate cancer cells, KLF5 was overexpressed to see whether forced expression of KLF5 could affect the activities of the promoters for various lipogenic genes. Only the *FASN* promoter showed significantly increased activity in

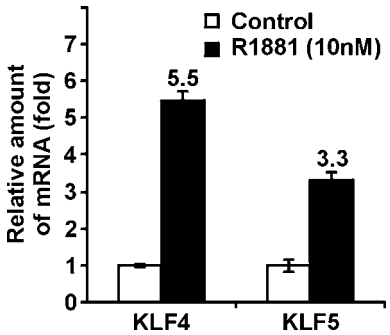

Figure 3 mRNA levels of *KLF4* and *KLF5* are increased by androgen treatment in LNCaP prostate cancer cells

LNCaP cells were seeded in an 100-mm-diameter dish and incubated in 5% (v/v) CT-FBS-containing medium. The cells were incubated for 3 days in the absence (Control) or presence of 10 nM R1881 in 1% CT-FBS-containing medium. Total RNA was prepared and subjected to RT-PCR with *KLF4* and *KLF5* primers [detailed in Supplementary Table S1 (http://www.BiochemJ.org/bj/417/bj4170313add.htm)]. The relative amount of mRNA was converted into the fold increase in relative mRNA levels (values shown above bars). Results are means ± S.D. ($n = 3$) with each experiment performed in triplicate.

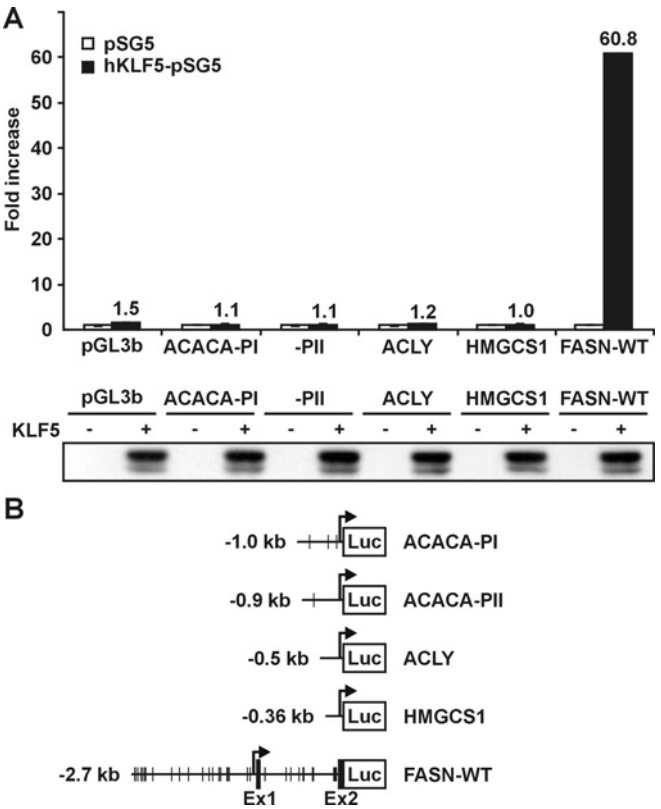

Figure 4 KLF5 regulates wild-type *FASN* promoter activity in LNCaP prostate cancer cells

(**A**) Control and lipogenic promoter constructs (0.1 μg/μl) were co-transfected with control (pSG5) (−) or the KLF5 expression vector (hKLF5-pSG5) (+) (0.9 μg/μl) into LNCaP cells. After 2 days, transfected cells were lysed and their luciferase activities were measured. The relative luciferase activity was converted into the fold increase in activity (values shown above bars) (upper panel). Results are means ± S.D. ($n = 3$) with each experiment performed in triplicate. The samples were then subjected to immunoblotting to confirm whether KLF5 proteins were expressed (lower panel). (**B**) Structures of *ACACA-PI*, *ACACA-PII*, *ACLY*, *HMGCS1* and *FASN*-WT lipogenic promoter constructs used in (**A**), with the lengths of their promoter regions indicated. Black vertical bars represents CACCC elements. Ex, exon; FASN-WT, wild-type FASN; Luc, luciferase.

response to KLF5 overexpression (Figure 4A). Comparisons between the *FASN* promoter and other lipogenic promoters explain that multiple CACCC elements for KLF binding are scattered throughout the upstream sequences and the first intron of the *FASN* gene, whereas these elements are scarce in the *ACACA-PI*, *ACACA-PII*, *ACLY* and *HMGCS1* promoters (Figure 4B).

Since KLF4, which recognizes the same CACCC elements as KLF5, was also increased by androgen, forced expression of KLF4 was performed to test whether similar results were produced. The promoter activity of *FASN* did not show any response to KLF4 overexpression (see Figure 6B). Consequently, KLF5, but not KLF4, increases the promoter activity of *FASN* when overexpressed in LNCaP cells.

KLF5 interacts with SREBP in regulating *FASN* expression

The next step was to determine the role of KLF5 in SREBP-1-mediated activation of the *FASN* promoter. The overexpression plasmids for SREBP-1c and/or KLF5 were transfected in tandem with the *FASN* promoter construct into PC3 prostate cancer cells. The promoter activity was increased by KLF5 and SREBP-1c overexpression up to 14- and 27-fold respectively (Figure 5A). Moreover, the activity was dramatically increased to more than the additive value when SREBP-1c and KLF5 were overexpressed together. On the other hand, the SRE mutant construct almost completely abolished SREBP-1c-mediated activation and the synergy between KLF5 and SREBP-1c without affecting responsiveness to KLF5.

To confirm further the synergistic effect of KLF5 and SREBP-1c, the induction of *FASN* promoter activity was measured when the concentration of SREBP-1c was changed in the absence or presence of KLF5 overexpression. When KLF5 was overexpressed, the *FASN* promoter sensitively and drastically responded to SREBP-1 in a dose-dependent manner, whereas its activation by SREBP-1c alone was low in the absence of KLF5 overexpression (Figure 5B). As a result, SREBP-1-mediated activation of the *FASN* promoter is enhanced by the addition of KLF5.

Next, protein–protein interactions were examined, since KLF5 and SREBP-1 were shown above to act in co-operation. HA-tagged SREBP-1 and/or FLAG-tagged KLF5 constructs were transfected into PC3 cells and incubated for 48 h. Cells were then harvested, the total-cell lysate was immunoprecipitated using an anti-FLAG antibody and then HA-tagged SREBP-1 was detected in the immunoprecipitates. SREBP-1 was markedly increased when FLAG-tagged KLF5 was expressed (Figure 5C, lane 4), indicating that SREBP-1 and KLF5 physically interact with each other (Figure 5C).

To elucidate the correlation between KLF5 and FASN expression in LNCaP cells, the cell line was transfected with the KLF5 expression plasmid and the endogenous level of FASN was measured. When the cells were transfected with empty vector, the endogenous level of FASN was low (Figure 5D). Nonetheless, when the exogenous KLF5 expression vector was introduced by transfection, the endogenous level of FASN protein increased significantly. In the case of other lipogenic enzymes, such as ACACA, ACLY and HMGCS1, the expression levels were not changed at all by KLF5 overexpression.

KLF5 drastically enhances androgen-dependent activation of the *FASN* promoter

The activity of the *FASN* promoter can be activated markedly by a synergistic action between exogenously expressed KLF5 and SREBP-1. However, if SREBP-1 is activated by androgen

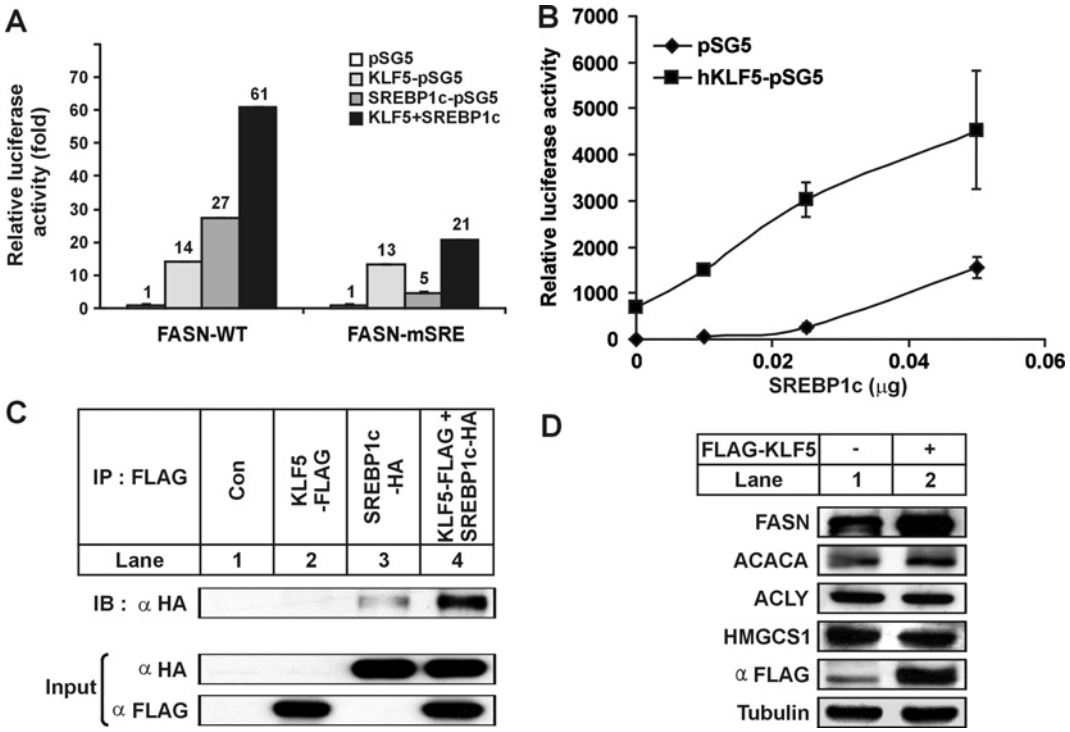

Figure 5 Co-operative action and physical interaction between SREBP and KLF5 in regulating *FASN* transcription

(**A**) The wild-type FASN (WT-FASN) and the mutant SRE FASN (FASN-mSRE) reporter constructs (0.1 μg/μl) were co-transfected with an empty vector (control; pSG5), SREBP1c (SREBP1c-pSG5) (0.45 μg/μl), KLF5 (KLF5-pSG5) (0.45 μg/μl), or both KLF5 and SREBP1c (KLF5 + SREBP1c) in PC3 cells, and the promoter activities were measured. The relative luciferase activity was converted into fold increase in luciferase activity (values indicated above bars). Results are means ± S.D. ($n = 3$) with each experiment performed in triplicate. (**B**) The wild-type FASN (WT-FASN) promoter construct (0.1 μg/μl) was co-transfected with control (◆, pSG5) or KLF5 (■, hKFL5-pSG5) (0.1 μg/μl), and various concentrations of the SREBP-1c expression plasmid in LNCaP cells seeded in six-well plates. The relative luciferase activities were measured after 48 h of incubation and the luciferase activity was normalized to the amounts of protein present in the lysate. Results are means ± S.D. ($n = 2$) with each transfection performed in triplicate. (**C**) PC3 cells were seeded in a 100-mm-diameter dish and transfected with HA-tagged SREBP-1c (3 μg/μl) (SREBP1c-HA), FLAG-tagged KLF5 (3 μg/μl) (KLF5-FLAG) or both (KLF5-FLAG + SREBP1c-HA). After 2 days, cells were lysed and subjected to immunoprecipitation (IP). The pre-cleared cell lysate (500 μg) was incubated with 2 μg of anti-FLAG antibody overnight at 4 °C. The following day, 50 μl of Protein A/G Plus–agarose was added and incubated for 2 h. The agarose was centrifuged, washed and resuspended in SDS/PAGE sample buffer (see the Materials and methods section for details). The immunoprecipitated protein was analysed by immunoblotting (IB) using an anti-HA antibody, and cell lysates (Input) were immunoblotted using anti-HA and anti-FLAG antibodies. (**D**) A control plasmid (−) or KLF5 expression plasmid (+) (FLAG-KLF5) (5 μg/μl) was transfected into LNCaP cells seeded in a 100-mm-diameter dish, and cells were lysed 48 h after transfection. Total protein was then prepared for immunoblotting with the indicated antibodies, with tubulin used as an internal loading control.

in LNCaP cells, exogenous KLF5 could enhance the androgen-dependent activation of the *FASN* promoter. Therefore the androgen-dependent activity of the *FASN* promoter was measured in the absence or presence of KLF5. The promoter activity of *FASN* was increased to 68- and 34-fold by androgen treatment and KLF5 overexpression respectively (Figure 6A). Furthermore, the activity markedly increased to 545-fold when androgen treatment was added in the presence of KLF5 overexpression.

Next, the promoter activity of *FASN* was measured using the KLF4 expression plasmid instead of KLF5. Contrary to the previous result, the increased promoter activity of *FASN* by androgen did not have any synergistic action when KLF4 was overexpressed (Figure 6B). In order to determine whether the synergistic activation between KLF5 and androgen occurs in other SREBP-controlled promoters, we measured the effect of KLF5 on *HMGCS1* promoter activity in androgen-treated LNCaP cells. As shown previously, the *HMGCS1* promoter responded to androgen treatment effectively, but not to KLF5 expression (Figure 6C). KLF5 slightly increased the activity of the *HMGCS1* promoter by androgen, but the increased amount was small when compared with the increase in the *FASN* promoter induced by KLF5. These results demonstrate that KLF5 could specifically reinforce the androgen-dependent activation of the *FASN* promoter, which makes *FASN* unique among lipogenic genes.

KLF5 siRNA suppresses androgen-mediated *FASN* expression

FASN expression is most prominently activated by androgen in LNCaP cells, and SREBP-1 plays a critical role in this induction. However, a dramatic change in the level of the mature form of SREBP-1 after androgen treatment was not detected. The fact that KLF5 is increased, even by a small amount, by androgen and synergistic action with SREBP, suggested the possibility that KLF5 could also play a critical role in FASN induction. On the basis of this hypothesis, knockdown of *KLF5* was performed to test whether *FASN* induction was blocked by androgen in LNCaP cells. A specific *KLF5* siRNA, which was synthesized as described previously [37], decreased the mRNA level of *KLF5* to 80 % compared with treatment with control siRNA, which did not markedly decrease *KLF5* mRNA levels (Figure 7A). However, even this small knockdown of *KLF5* expression significantly increased *KLF4* expression. The increase in the level of *KLF4* by *KLF5* siRNA might be explained by a previous report in which *KLF4* transcription was demonstrated to be strongly suppressed by *KLF5*, and as a result, repression by *KLF5* knockdown is sufficient to induce physiological changes [38]. The mRNA level

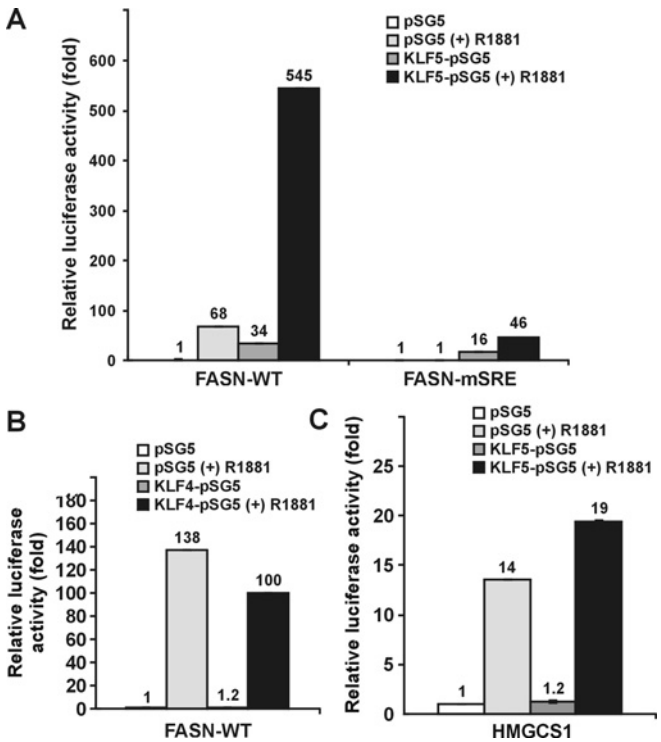

Figure 6 Effect on wild-type *FASN* promoter by SREBP, KLF5 and AR

Wild-type FASN (FASN-WT) or FASN-mSRE (mutant SRE) constructs (0.1 μg/μl) were co-transfected with control plasmid (pSG5), KLF5 plasmid (KLF5-pSG5) (**A**) or KLF4 plasmid (KLF4-pSG5) (**B**) (0.9 μg/μl). The following day, cells were changed to 1 % CT-FBS-containing medium in the absence or presence (+) of R1881, and incubated for a further 3 days. Cells were harvested and the relative luciferase activity was measured and is expressed as the fold increase (values shown above bars). (**C**) The *HMGCS1* promoter (0.1 μg/μl) was co-transfected with control (pSG5) or KLF5 plasmid (0.9 μg/μl) (KLF5-pSG5) and treated as stated in (**A** and **B**). Cells were harvested and the relative luciferase activity was measured and is expressed as the fold increase (values shown above bars). All results are means $\pm$ S.D. ($n = 3$) with each experiment performed in triplicate.

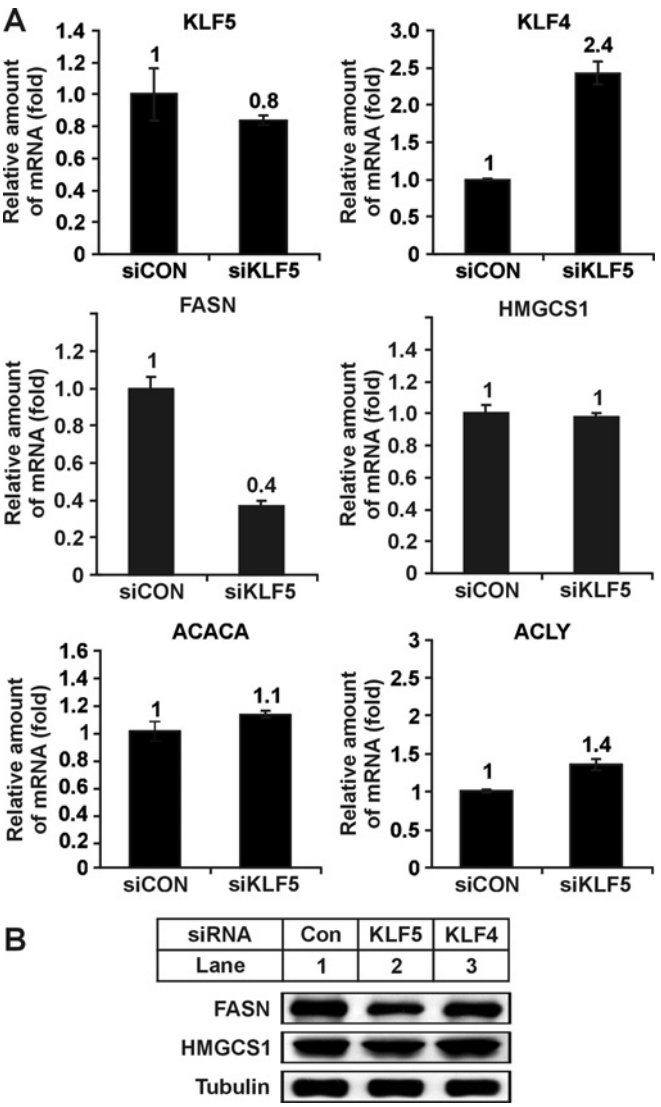

Figure 7 Effect on *KLF5* or *KLF4* siRNA on the expression levels of endogenous *KLF5*, *KLF4* and lipogenic genes

LNCaP cells were seeded in 5 % (v/v) CT-FBS medium without antibiotics and incubated for 48 h. The cells were transfected with 200 nM control siRNA (siCON/Con), *KLF5* siRNA (siKLF5) or *KLF4* siRNA using Lipofectamine™ RNAiMAX three times over 3 days in 1 % CT-FBS medium in the presence of 10 nM R1881. Total RNA (**A**) or protein (**B**) was collected 3 days after the first transfection, and RT-PCR (**A**) and immunoblotting (**B**) was performed as indicated. For RT-PCR, the fold increase in mRNA is shown (values shown above bars) and results are means $\pm$ S.D. ($n = 2$), with each experiment performed in triplicate. For immunoblotting, detection of α-tubulin (Tubulin) was used as an internal loading control.

of *FASN* was effectively suppressed by *KLF5* siRNA to 40 % of that of the control siRNA group, whereas the mRNA levels of *ACACA*, *ACLY* and *HMGCS1* were not significantly affected. Moreover, the protein level of FASN was decreased notably after treatment of cells with *KLF5* siRNA, whereas the protein level of HMGCS1 was not affected (Figure 7B). Since the mRNA level of *KLF4* was increased by androgen and it binds to the same CACCC element as KLF5, the expression level of FASN was also tested in *KLF4* siRNA-treated cells [39]. Transfection of *KLF4* siRNA neither reduced the protein level of FASN nor HMGCS1. These results indicate that KLF5 is involved in androgen-dependent expression of FASN in LNCaP cells, but KLF4 is not involved.

CACCC elements are scattered in the *FASN* promoter and intron 1, which makes KLF5 a positive regulator

The *FASN* promoter, which shows responsiveness to KLF5, contains multiple CACCC elements for KLF5 binding. To discriminate which CACCC elements play a critical role in the responsiveness to KLF5, serial deletion constructs of the *FASN* promoter were generated as shown in Figure 8(A), and a transient transfection assay was performed in PC3 prostate cancer cells overexpressing KLF5 (Figure 8B). When KLF5 was overexpressed, the promoter activity of the pLuc-(2.7 kb) construct increased by 58-fold. Moreover, pLuc-(1.0 kb) and pLuc-(0.4 kb) plasmids were increased 42.1- and 39.7-fold by KLF5 overexpression respectively. To test the contribution of intron sequences for the responsiveness to KLF5, the intron region was deleted from the construct (Δintron constructs). The basal activity was greatly increased by deletion of the intron, but the responsiveness to KLF5 was still present. This result indicates that the first intron strongly suppressed *FASN* promoter activity and suggests the possibility that KLF5 binding to multiple CACCC elements in the first intron might play a significant role in ablating the suppressive activity residing in the first intron. Furthermore, CACCC elements within the pLuc-(0.4 kb) promoter region might play a critical role in activating the *FASN* promoter, since the promoter activity was still present despite complete deletion of the first intron. Consequently, the scattered multiple KLF5-binding

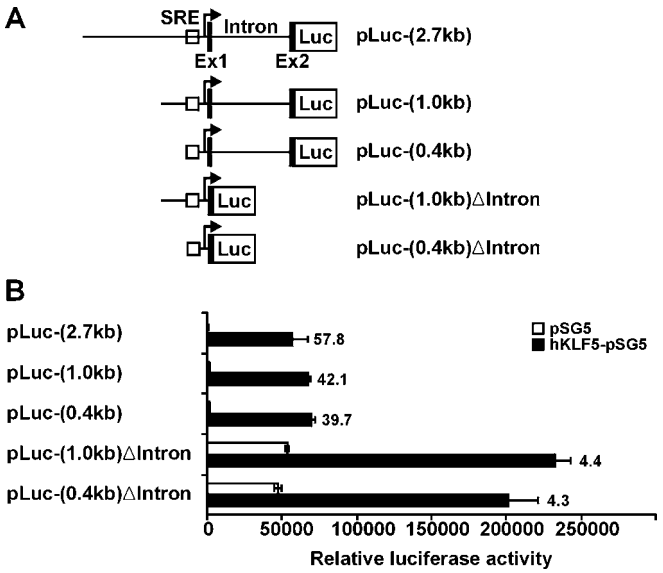

Figure 8 Deletion and mutation analysis of *FASN* promoter activity

(**A**) Structures of deletion and mutation *FASN* promoter constructs. The numbers in parentheses represent the length of each promoter. (**B**) Transient transfection and luciferase assay was performed in PC3 cells. *FASN* promoter constructs [0.1 μg/μl, see (**A**) for details] were co-transfected with either KLF5 (hKLF5-pSG5) or control (pSG5) (0.9 μg/μl) plasmids. At 48 h, cell extracts were analysed for luciferase activity. Fold increase in luciferase activity is also shown (values shown above bars). Results are means $\pm$ S.D. ($n = 3$) with each experiment performed in triplicate. Ex, exon; Luc, luciferase.

sites around the proximal promoter region and the first intron can activate the *FASN* promoter.

DISCUSSION

The present study demonstrates that KLF5 is expressed in LNCaP prostate cancer cells and acts as a positive regulator of the expression of the oncogenic protein FASN. KLF5 can also enhance the activity of SREBP. Both KLF5 and SREBP are increased by androgen treatment and their synergistic action results in much higher induction of *FASN* than other SREBP target genes, such as *ACACA*, *ACLY* and other cholesterogenic genes.

Previous reports have demonstrated that the enzymes engaged in fatty acid synthesis and cholesterol synthesis are up-regulated in prostate cancer cells by androgen [4,40,41] and SREBPs are involved in this regulation [17,18,42]. Lipogenic enzymes are reported to be up-regulated by androgen treatment through an increase in mature SREBP levels [17], and this increased processing of SREBPs is performed by an escort protein called SCAP, which is a direct target of the AR [42].

In the present study, FASN was most prominently increased by androgen treatment in LNCaP prostate cancer cells, and its up-regulation occurred at the transcriptional level (Figure 1A). This is in contrast with HER2 (human epidermal growth factor receptor protein 2)-mediated increases in ACACA and FASN in breast cancer cells, which takes place at the translational level by activation of the mTOR (mammalian target of rapamycin) signalling pathway [43]. However, it was difficult to detect the increase in mature SREBP-1 and SREBP-2 in comparison with the prominent increase in FASN expression. Furthermore, mRNA and protein levels of SCAP, known to be a direct target of the AR [42], were increased minimally by androgen treatment. These results suggest the possibility that other androgen-regulated transcription factor(s) might participate and/or assist in regulating *FASN* levels in concert with SREBPs in prostate cancer cells, and that KLF5 might be the most promising candidate.

The KLF family of proteins are transcription factors controlling cell proliferation, differentiation and development [44,45]. Although most KLF family members have not been characterized for their cellular function, it has been revealed previously that several KLF members are linked to the control of proliferation and apoptosis in cancer cells [27,28,37,46–49]. Among these, 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 CACCC homology or is G/C-rich, previous reports have demonstrated that they have opposing effects on the transcription of target genes [50], such as *KLF4* [38], *TAGLN* [transgelin; also known as *SM22α* (smooth muscle 22α) [51] and *CYP1A1* (cytochrome P450 1A1) [52]. The differences in the biochemical behaviour of KLF4 and KLF5 reflects their biological behaviour. For example, the cellular distribution of the two KLFs is different from each other. KLF4 is mainly expressed in the post-mitotic differentiated villus of epithelial cells of the intestinal tract [53], whereas KLF5 is expressed mostly in proliferating crypt cells [54]. KLF4 is mainly associated with a process of growth arrest [53], whereas KLF5 mainly accompanies cell proliferation [27]. Moreover, forced expression of KLF4 leads to a G_1/S-phase cell-cycle arrest [47], but that of KLF5 causes a transformed phenotype [27].

In the present study, KLF4 and KLF5 were shown to be increased by androgen treatment. Promoters for *ACACA-PI*, *ACACA-PII*, *ACLY* and *HMGCS1* contain either none or very few CACCC consensus sequences for KLF binding, but the *FASN* promoter contains multiple CACCC elements throughout the promoter and intron 1 region. In the transient transfection assay, KLF5, but not KLF4, increased the promoter activity of *FASN*, but the other promoters were not affected by KLF5. When the cells were cultured in 1 % CT-FBS-containing medium, the endogenous levels of KLF5 and FASN were very low. However, when exogenous *KLF5* DNA was introduced by transfection, the endogenous level of FASN protein increased significantly. In contrast, the inhibition of *KLF5* by siRNA was accompanied by the down-regulation of FASN. Furthermore, CACCC elements, scattered throughout the *FASN* proximal promoter and intron 1 region, were proven to act as response elements for KLF5 to induce *FASN* promoter activity. Taken together, these results indicate that among the lipogenic enzymes regulated by androgen through SREBPs, *FASN* is the only gene that is regulated by KLF5.

KLF5 showed synergism with SREBP in the regulation of FASN expression. In the transient transfection assay, increased *FASN* promoter activity by SREBP was strongly up-regulated by the addition of KLF5. Moreover, SREBP and KLF5 physically interacted with each other. The synergism between SREBP and KLF5 implies that small changes in these transcription factors could drastically change the expression of FASN, which acts as an important oncogenic protein in cancer cell growth and proliferation. The molecular target of the KLF5/FASN pathway revealed in the present study requires further investigation for the potential development of diagnostic and/or therapeutic approaches cancer treatment.

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