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Squalene Synthase, a Determinant of Raft-associated Cholesterol and Modulator of Cancer Cell Proliferation*

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Koen Brusselmans[‡1], Leen Timmermans[‡2], Tine Van de Sande[‡2], Paul P. Van Veldhoven[§], Guimin Guan[¶], Ishaiahu Shechter[¶], Frank Claessens[‖], Guido Verhoeven[‡], and Johannes V. Swinnen[‡3]

From the [‡]Laboratory for Experimental Medicine and Endocrinology and Department of Molecular Cell Biology, [§]Divisions of Pharmacology and [‖]Biochemistry, Katholieke Universiteit Leuven, Herestraat 49, B-3000 Leuven, Belgium and [¶]Department of Surgery, Uniformed Services University, School of Medicine, Bethesda, Maryland 20814-4799

Several cues for cell proliferation, migration, and survival are transmitted through lipid rafts, membrane microdomains enriched in sphingolipids and cholesterol. Cells obtain cholesterol from the circulation but can also synthesize cholesterol *de novo* through the mevalonate/isoprenoid pathway. This pathway, however, has several branches and also produces non-sterol isoprenoids. Squalene synthase (SQS) is the enzyme that determines the switch toward sterol biosynthesis. Here we demonstrate that in prostate cancer cells SQS expression is enhanced by androgens, channeling intermediates of the mevalonate/isoprenoid pathway toward cholesterol synthesis. Interestingly, the resulting increase in *de novo* synthesis of cholesterol mainly affects the cholesterol content of lipid rafts, while leaving non-raft cholesterol levels unaffected. Conversely, RNA interference-mediated SQS inhibition results in a decrease of raft-associated cholesterol. These data show that SQS activity and *de novo* cholesterol synthesis are determinants of membrane microdomain-associated cholesterol in cancer cells. Remarkably, SQS knock down also attenuates proliferation and induces death of prostate cancer cells. Similar effects are observed when cancer cells are treated with the chemical SQS inhibitor zaragozic acid A. Importantly, although the anti-tumor effect of statins has previously been attributed to inhibition of protein isoprenylation, the present study shows that specific inhibition of the cholesterol biosynthesis branch of the mevalonate/isoprenoid pathway also induces cancer cell death. These findings significantly underscore the importance of *de novo* cholesterol synthesis for cancer cell biology and suggest that SQS is a potential novel target for antineoplastic intervention.

Cholesterol is a major structural lipid. It intercalates with phospholipids in cellular membranes and drives domain organization, giving rise to discrete structures that are mobile in the plane of the membrane and are referred to as lipid rafts (1–4). The distinct nature of these rafts or membrane microdomains is supported by the insolubility of lipid rafts in cold non-ionic detergents such as Triton X-100, resulting in their low buoyant density in sucrose gradients (2). The association of specific subsets of proteins with cholesterol-rich membrane microdomains allows for functional compartmentalization of membranes and is thought to play a crucial role in key cellular functions, including signal transduction, cell growth, cell polarization, migration, and survival (2–6). Consistent with this concept, it has been shown that cholesterol is required for the growth of cancer cells (7, 8). Recent findings have linked these effects to changes in lipid rafts and have shown that elevated levels of circulating cholesterol increase the cholesterol content of lipid rafts, promote tumor growth, and reduce apoptosis in prostate cancer xenografts (6, 9).

Cells acquire cholesterol from the circulation but also have the ability to synthesize cholesterol *de novo* via the mevalonate/isoprenoid pathway (Fig. 1) (7, 10). One of the first and rate-limiting steps in this pathway is the formation of mevalonate by β-hydroxy-β-methylglutaryl-CoA (HMG-CoA)[4] reductase, the enzyme that constitutes the target of statins (11), cholesterol-lowering drugs that are commonly used to prevent cardiovascular diseases (12). Interestingly, several large population-based studies have shown that statins also reduce the risk of developing various cancers (13). Furthermore, statins have been demonstrated to induce growth arrest and apoptosis in cancer cell cultures *in vitro* (14–21). Whether inhibition of cholesterol synthesis is the main trigger of these effects remains, however, a matter of debate. In fact, mevalonate is further metabolized to farnesyl diphosphate, which functions as a precursor for cholesterol but can also be used for isoprenylation (farnesylation and geranyl-geranylation) of proteins and for the synthesis of heme, dolichol, and ubiquitin (11, 22, 23). As a consequence, inhibition of HMG-CoA reductase by statins not only inhibits cholesterol synthesis but also blocks protein isoprenylation, which is essential for the function of several cancer-associated signaling proteins, including members of the ras/Rho family (24). Accordingly, the anticancer effects of statins have been largely attributed to inhibition of this post-translational mechanism (13, 24–27).

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[1] A postdoctoral fellow of the Fund for Scientific Research-Flanders (Belgium).
[2] Research assistants of the Fund for Scientific Research-Flanders (Belgium).
[3] To whom correspondence should be addressed: LEGENDO, Gasthuisberg, O&N1, Herestraat 49, bus 902, B-3000 Leuven, Belgium. Tel.: 32-16-330533; Fax: 32-16-345934; E-mail: Johan.Swinnen@med.kuleuven.be.

[4] The abbreviations used are: HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; RNAi, RNA interference; siRNA, small interfering RNA; GFP, green fluorescent protein; BrdUrd, bromodeoxyuridine; SQS, squalene synthase; SRE, sterol regulatory element; SREBP, SRE-binding protein.

Uptake and synthesis of cholesterol are tightly controlled by sterol regulatory element-binding proteins (SREBPs), transcription factors that are synthesized as inactive endoplasmic reticulum membrane-associated precursors (28, 29). When cells are depleted of cholesterol, SREBPs migrate to the Golgi where they are proteolytically cleaved; then SREBPs translocate to the nucleus and stimulate the expression of proteins involved in the uptake and synthesis of cholesterol (28–30). Remarkably, in prostate cancer cells, the feedback regulation of these transcription factors is partially lost due to activation of the SREBP maturation mechanism by androgens (31, 32). These are male sex steroid hormones that play a major regulatory role in the development and progression of prostate cancer (33). In fact, androgens enhance the expression of SREBP cleavage-activating protein, a key protein involved in the sensing of intracellular sterol levels, thereby disturbing the delicate balance between sensor and retention proteins present in the regulatory complex that governs SREBP maturation (30, 31). As a consequence, androgens induce activation of SREBPs in prostate cancer cells even in the presence of cholesterol, thereby stimulating the expression of various enzymes of the mevalonate/isoprenoid pathway, such as HMG-CoA synthase, HMG-CoA reductase, and farnesyl diphosphate synthase (32, 34).

Squalene synthase (SQS) catalyzes the first reaction of the mevalonate/isoprenoid pathway committed exclusively to cholesterol biosynthesis (7, 35) (Fig. 1) and hence plays a crucial role in directing intermediates to either sterol or non-sterol branches of this metabolic pathway. As the expression of this enzyme is also regulated by the cholesterol feedback mechanism and is responsive to SREBPs in other cell systems (several SREBP-binding sites (SREs) have been identified in the human SQS promoter) (35–38), we investigated in the present work whether in prostate cancer cells SQS expression is also induced by androgens and whether this regulation has an impact on the cholesterol content of raft and non-raft membrane fractions. Moreover, by targeting SQS using small interfering RNA technology or chemical inhibitors, we explored the role and importance of SQS activity and cholesterol synthesis in the growth and survival of cancer cells.

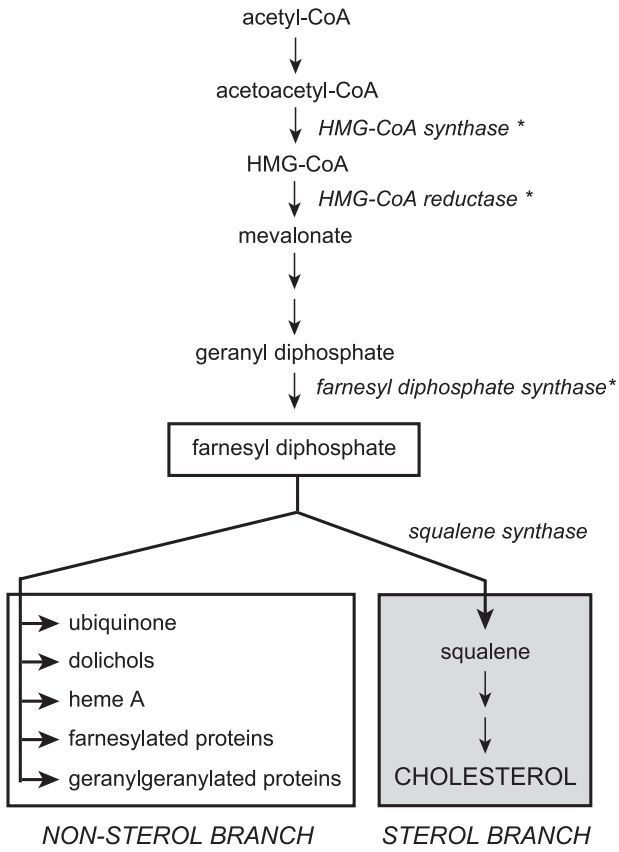


FIGURE 1. **The isoprenoid biosynthetic pathway.** The rate-limiting step in the isoprenoid biosynthesis is the formation of mevalonate. Mevalonate is then converted to farnesyl diphosphate, which can be used for the synthesis of non-sterol isoprenoids or for the synthesis of sterols by the action of squalene synthase. Enzymes the expression of which is known to be stimulated by androgens are indicated with an *asterisk*.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—The human prostate carcinoma cell lines LNCaP, PC-3, and DU-145 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human prostate carcinoma cell lines MDA-PCa-2a and PC-346c were provided by Dr. N. Navone (University of Texas M. D. Anderson Cancer Center, Houston, TX) and Dr. W. Van Weerden (Erasmus University, Rotterdam, The Netherlands), respectively. Cells were cultured as previously described (30, 39, 40). Where indicated, R1881 (PerkinElmer Life Sciences) was added to the culture medium. To assess the effects of R1881, charcoal-treated fetal calf serum was used to reduce the background levels of steroids. Zaragozic acid A (Squalestatin S1), mevastatin, FTI-277, and water-soluble cholesterol were purchased from Sigma. Transient transfections were performed using Transfast reagent (Promega). The plasmid pAcGFP1-F was purchased from Clontech.

RNA Interference—Transfection procedures with small interfering RNA (siRNA) using Oligofectamine (Invitrogen) have been described previously (41, 42); siRNA oligonucleotides were purchased from Dharmacon (Lafayette, CO). The sequences of siRNA oligonucleotides targeting luciferase have been described (41, 42). The siRNA oligonucleotides targeting SQS were sense, GAGGUUUGGAGCAGGUAUGdTdT, and antisense, CAUACCUGCUCCAAACCUCdTdT.

Northern Blot Analysis—RNA preparation and Northern blot analysis were performed as described (34). A cDNA probe for squalene synthase was synthesized by PCR on human cDNA (generated by reverse transcription as described) (34) using 5′-GGCAGTGAAGATTCGGAAAG and 5′-GCAGCGACTTCACCTAAACC as forward and reverse primers, respectively; PCR products were cloned into the PGEM-T vector (Promega). Equal loading of RNA was verified using a probe for 18 S ribosomal RNA. Relative quantitation of mRNA levels was performed using a PhosphorImager screen (GE Healthcare).

Immunoblot Analysis—For immunoblot analysis cells were washed with phosphate-buffered saline and lysed in a reducing buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 0.715 M 2-mercaptoethanol, and 8.7% glycerol. Nuclear extracts were prepared as described (34). For the preparation of membrane fractions, cells were lysed in buffer containing 50 mM Tris, pH 6.8, and 150 mM NaCl; homogenates were cleared from debris (cen-

trifugation at 3,000 × *g*, 5 min) and subjected to ultracentrifugation (100,000 × *g*, 1 h) to separate the membrane fraction. Complete protease inhibitor mixture (Roche Applied Science) was added to all protein buffers. Protein concentrations were determined using the BCA assay (Pierce). Western blots were performed as described (34, 41) using antibodies against human SQS (43), flotillin-2 (BD Biosciences), transferrin receptor (Calbiochem), GFP (BD Biosciences), SREBP-1 (34), α-tubulin (Sigma), or cytokeratin-18 (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blot analysis on cellular fractions separated by sucrose gradient ultracentrifugation (see below), equal volumes of each fraction were used for gel electrophoresis.

Promoter-Reporter Assays—The promoter-reporter constructs pSQSwt-luc, pSQSmut124-luc, and pSQSmut123-luc have been described previously (37). The pSQSmut124-luc construct harbors mutations in three SREBP-binding sites (two SRE-1 sequences and an inverted SRE-3), whereas in the pSQSmut123-luc construct two SREBP-binding sites (one SRE-1 and the inverted SRE-3) and the auxiliary-binding site for NF-Y are mutated (Fig. 3*A*). LNCaP cells were transfected with promoter-reporter constructs using Transfast transfection reagent (Promega). Where indicated, an expression vector encoding a dominant-negative form of SREBP-1, encoding amino acids 90–460 of SREBP-1 (30), or an empty expression vector was added. The next day, medium was replaced and cells were incubated with 10^{-8} M R1881 or with vehicle for 48 h. Then, cells were washed with phosphate-buffered saline and lysed; cleared lysate was analyzed for luciferase activity using a luciferase reporter kit (Promega).

Quantification of 2-[^{14}C]Acetate Incorporation in Cholesterol—After 48 h of incubation with R1881, at 72 h after transfection with siRNA, or after 72 h of incubation with zaragozic acid A, 2-^{14}C-labeled acetate (57 mCi/mmol; Amersham Biosciences) was added to the culture medium of LNCaP cells (2 μCi/100-mm dish). After 4 h of incubation at 37 °C, cells were washed, scraped in phosphate-buffered saline, and pelleted by centrifugation. Lipids were extracted using the Bligh Dyer method as previously described (44). Acetate incorporation into cholesterol was analyzed after separation of lipids by thin layer chromatography (TLC) using silica gel G plates (Merck). To separate neutral lipids, plates were developed in hexane-diethyl ether-acetic acid (70:30:1, v/v/v). Radiolabeled lipids were quantitated using a PhosphorImager screen (GE Healthcare). Values were normalized for sample protein content.

For quantification of 2-[^{14}C]acetate incorporation in lipid fractions separated by sucrose gradient centrifugation, ~3 million cells (exposed to 2-^{14}C-labeled acetate for 4 h at 37 °C) were pelleted by centrifugation and resuspended at 4 °C in a detergent lysis solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 (Merck), and protease inhibitors (Complete mini; Roche Applied Science). Detergent-soluble and detergent-insoluble lipids were separated by sucrose gradient ultracentrifugation as described (45). Lipids of each fraction were extracted using the Bligh Dyer method, and incorporation of radiolabeled acetate into cholesterol was analyzed by TLC (see above).

Quantitation of Cholesterol Content in Lipid Fractions Separated by Sucrose Gradient Centrifugation—Cellular fractions from sucrose density gradients (see above) were extracted using the Bligh Dyer method. Quantitation of free cholesterol in cellular fractions was based on the use of cholesterol oxidase and coupling of the produced hydrogen peroxide to the formation of the fluorescent dimer of homovanillic acid by means of peroxidase (45).

Proliferation/Cytotoxicity Assays—At the indicated time after transfection with siRNA or after incubation with zaragozic acid A, cells were collected and cell number and viability were determined using a trypan blue dye exclusion assay as described previously (46). Cell proliferation was quantitated using a bromodeoxyuridine (BrdUrd) labeling and detection kit (Roche Applied Science).

RESULTS

Androgens Markedly Stimulate SQS Expression in Androgen-responsive Prostate Cancer Cells—To evaluate whether expression of the gene encoding SQS is regulated by androgens, different human prostate cancer cell lines were incubated for 48 h with or without R1881 (10^{-8} M), a synthetic androgen that, unlike the natural androgens testosterone and 5α-dihydrotestosterone, is metabolically stable. As shown in Fig. 2*A*, SQS mRNA expression was detected in all prostate cancer cell lines under androgen-deprived conditions. Exposure to R1881 induced a marked increase of SQS mRNA in the androgen-responsive cell lines LNCaP, PC-346c, and MDA-PCa-2a. In contrast, R1881 had no effect on SQS transcript levels in the androgen receptor-negative PC-3 and DU-145 cells (Fig. 2*A*).

Androgens Increase SQS mRNA and Protein Levels in a Dose- and Time-dependent Manner—To analyze the androgen regulation of SQS gene expression in more detail, LNCaP cells were incubated with R1881 at different concentrations or for different periods of time. Northern and Western blot analyses demonstrated that R1881 increased SQS expression at concentrations starting from 10^{-10} M. Effects were maximal at 10^{-9} M (Fig. 2*B*). At a concentration of 10^{-8} M R1881, elevation of SQS expression occurred between 8 and 12 h after the addition of the steroid and reached a plateau between 48 and 72 h (Fig. 2*C*).

Involvement of SREBP-binding Sites in the Androgen-induced Activation of SQS Promoter Activity—The human SQS promoter harbors three SREBP-binding sites (two SRE-1 motifs and an inverted SRE-3) and an auxiliary-binding site for NF-Y (inverted Y-box). The two SRE-1 sequences and the inverted SRE-3 have been demonstrated to bind SREBP-1 and/or SREBP-2 (37, 38), whereas the inverted Y-box is also involved in sterol-mediated regulation of the SQS promoter (37, 38). Previous promoter-reporter studies have revealed that mutation of the three SREBP-binding sites (two SRE-1 sites and the inverted SRE-3) in the SQS promoter (Fig. 3*A*) severely decreases reporter expression after SREBP overexpression (37, 38). Mutation of the first SRE-1 and the inverted SRE-3 together with the inverted Y-box (Fig. 3*A*) even further decreased SREBP-mediated reporter expression, which was almost completely abolished (37, 38), indicating that these three elements are essential for SREBP-mediated induction of SQS expression. To investigate whether the effects of androgens on SQS expression were

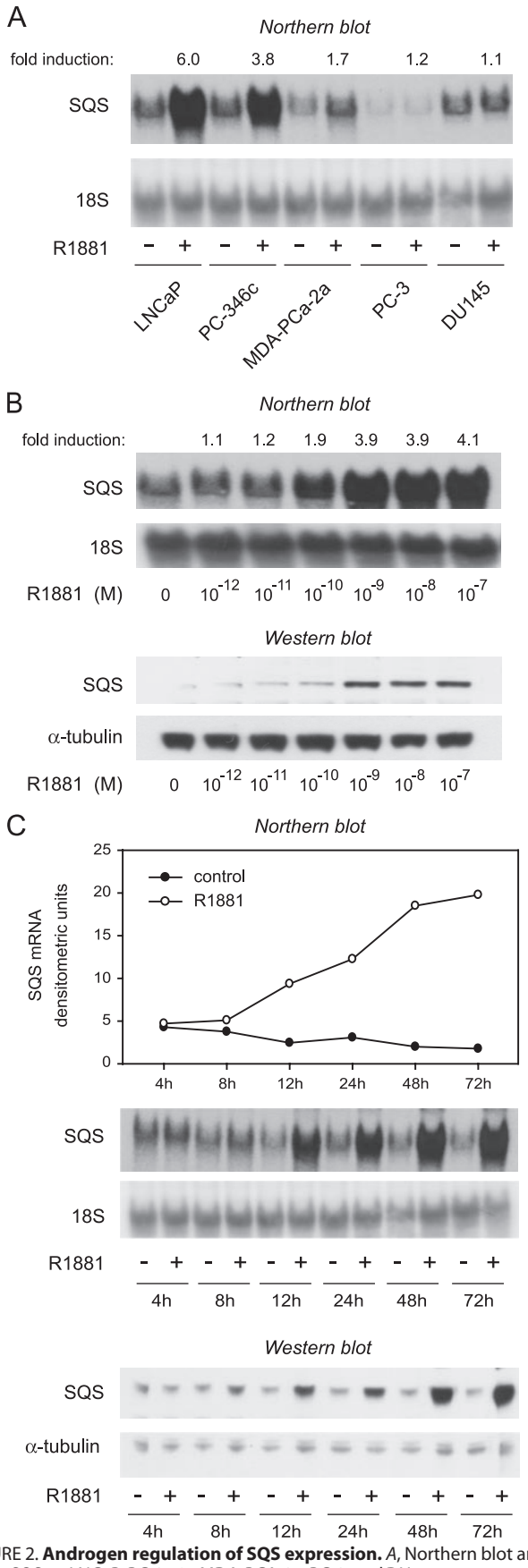


FIGURE 2. **Androgen regulation of SQS expression.** *A*, Northern blot analysis for SQS on LNCaP, PC-346c, MDA-PCA-2a, PC-3, and DU145 prostate cancer cells, cultured for 48 h with or without R1881 (10^{-8} M). *B*, Northern blot and Western blot analyses for SQS on LNCaP cells cultured for 48 h with increasing concentrations of R1881. *C*, Northern blot and Western blot analyses for SQS on LNCaP cells cultured with R1881 (10^{-8} M) or vehicle for different periods of time. 18 S and α-tubulin expression were used as internal control for Northern blot and Western blot analysis, respectively. Phosphorimaging was used for relative quantitation of mRNA levels (expressed as arbitrary densitometric units or indicated by -fold induction compared with the control (without R1881)).

mediated by SREBPs, we performed transient transfection experiments using promoter-reporter constructs derived from the human SQS gene (Fig. 3*A*). First, LNCaP cells were transiently transfected with an expression construct containing a 1-kb wild type promoter fragment of the human SQS gene in front of a luciferase reporter gene (pSQSwt-luc). Treatment of transfected cells with R1881 caused a marked increase in reporter activity (Fig. 3*B*). Then, LNCaP cells were transiently transfected with the pSQSmut124-luc construct in which the three SREBP-binding sites (two SRE-1 sites and the inverted SRE-3) were mutated, or with the pSQSmut123-luc construct in which one SRE-1, the inverted SRE-3, and the inverted Y-box were mutated (Fig. 3*A*). Compared with the wild type construct pSQSwt-luc, androgen stimulation of the SQS promoter was decreased after transfection with pSQSmut124-luc (Fig. 3*B*). In agreement with previously obtained data (37, 38), androgen stimulation of the SQS promoter was even further reduced after transfection with the pSQSmut123-luc construct (Fig. 3*B*). These findings point to a role for SREBPs in the androgen regulation of SQS transcription.

To further corroborate the role of SREBPs in the transcriptional activation of the SQS gene by androgens, we introduced a dominant-negative form of SREBP-1 in LNCaP cells. This dominant-negative form was generated by deleting the amino-terminal transactivation domain of SREBP-1 (30). The resulting protein is still able to bind to SREs but is transcriptionally inactive and blocks the access of wild type endogenous SREBPs to SREs. Cotransfection of LNCaP cells with the pSQSwt-luc reporter gene (Fig. 3*A*) and with increasing amounts of an expression vector encoding dominant-negative SREBP almost completely abolished the stimulatory effects of androgens on SQS promoter-driven reporter expression (Fig. 3*C*). Cotransfection with empty vector had no inhibitory effect (Fig. 3*C*).

Androgens Increase Cholesterol Levels in Detergent-resistant Membrane Microdomains—Because androgens induce SQS gene expression (together with the expression of several other cholesterol biosynthetic genes) (34), we explored whether androgens also increased cellular cholesterol synthesis by measuring the incorporation of ^{14}C-labeled acetate (a precursor of all major lipid classes) into cellular cholesterol. Exposure to 10^{-8} M R1881 for 48 h induced a 2.6-fold increase in the *de novo* synthesis of cholesterol in LNCaP cells (data not shown). As cholesterol has been shown to function as a key component of detergent-resistant membrane microdomains (4), we next addressed the question whether androgens may affect the cholesterol content of these membrane microdomains. Therefore, after 48 h of incubation with 10^{-8} M R1881, LNCaP cells were exposed to ^{14}C-labeled acetate for 4 h. Afterwards, cells were collected and treated with the nonionic detergent Triton X-100 and subjected to sucrose den-

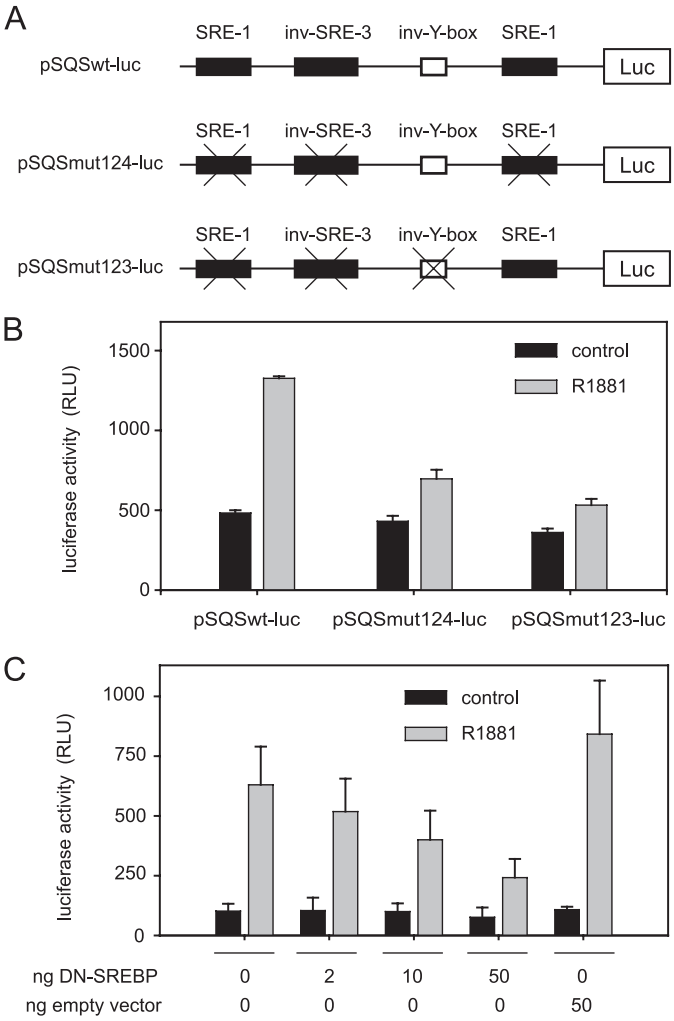


FIGURE 3. **Involvement of SREBPs in the androgen regulation of SQS expression.** *A*, reporter constructs. Plasmid pSQSwt-luc contained a luciferase reporter gene driven by a "wild type" SQS promoter fragment harboring three SREs (two SRE-1 sites and an inv-SRE-3 site) and an inv-Y-box. The pSQSmut124-luc luciferase reporter construct was driven by a SQS promoter fragment in which the three SREBP-binding sites (two SRE-1 sites and the inverted SRE-3) were mutated. The pSQSmut123-luc luciferase reporter construct was driven by a SQS promoter fragment in which one SRE-1, the inverted SRE-3, and the inverted Y-box were mutated. *B*, LNCaP cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by a wild type SQS promoter (*pSQSwt-luc*) or with similar constructs in which regulatory sequences were mutated (*pSQSmut124-luc* and *pSQSmut123-luc*). The next day, cells were treated with or without 10^{-8} M R1881 for 48 h. Then, luciferase activity, expressed as relative luciferase units (*RLU*), was measured. Data are means ± S.D. ($n = 4$) and are representative of three independent experiments. *C*, LNCaP cells were transiently transfected with wild type SQS promoter reporter construct (pSQSwt-luc) together with increasing amounts of a construct encoding a dominant-negative form of SREBP-1 (*DN-SREBP*) or with empty vector (*control*). The next day, cells were treated with or without 10^{-8} M R1881 for 48 h. Then, luciferase activity, expressed as relative luciferase units (*RLU*), was measured. Data are means *columns* ± S.D. *bars* ($n = 3$) and are representative of three independent experiments.

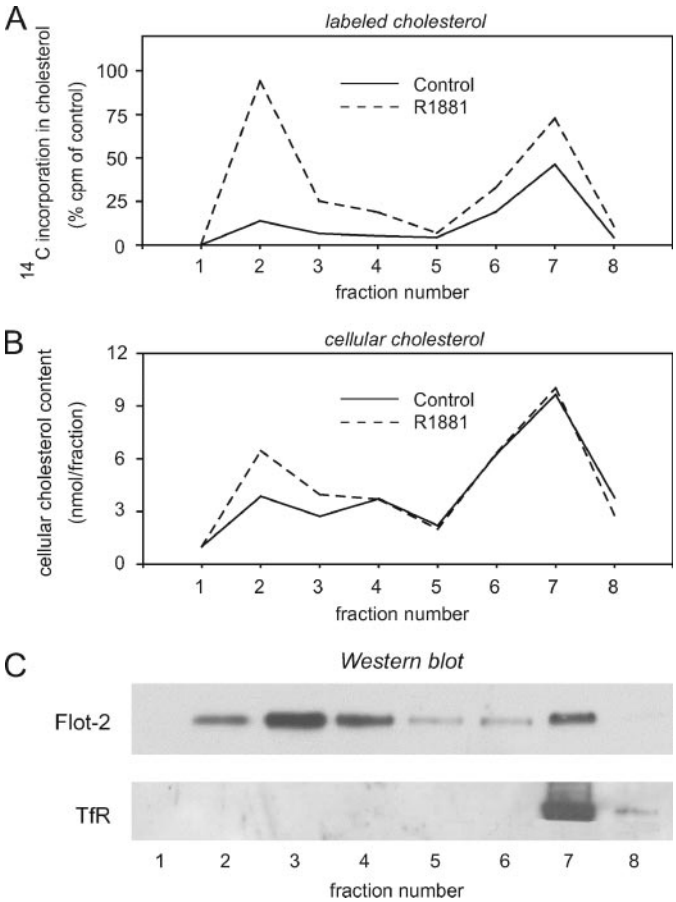


FIGURE 4. **Androgens stimulate *de novo* synthesis of cholesterol partitioning into detergent-resistant membrane microdomains.** *A* and *B*, after 48 h of exposure to R1881 (10^{-8} M) or vehicle, LNCaP cells were incubated with ^{14}C-labeled acetate for 4 h. Cells were harvested, resuspended in the presence of 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Sucrose gradient fractions were subjected to lipid extraction. Using TLC analysis and phosphorimaging, biosynthesis of cholesterol was quantitated (*A*) by measuring the incorporation of [^{14}C]acetate into cholesterol (value of each fraction is expressed as percentage of total incorporated [^{14}C]acetate (all fractions pooled) in cholesterol in control cells). Cellular cholesterol levels were determined (*B*) using an enzymatic assay. Data shown are representative of three independent experiments. *C*, untreated LNCaP cells were harvested, resuspended in the presence of 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Western blot analysis was performed on the different sucrose gradient fractions for the membrane microdomain marker flotillin-2 (*Flot-2*) and transferrin receptor (*TfR*) (a non-raft protein).

sity gradient ultracentrifugation at 4 °C. Because of their low buoyant density, this procedure allows separation of detergent-insoluble microdomains (lipid rafts) present in the "low density fractions" (fractions 1–4) from detergent-soluble lipids residing in "high density fractions" (fractions 5–8). As illustrated in Fig. 4*A*, androgens caused a 5.4-fold increase in *de novo* synthesized cholesterol in detergent-resistant membrane microdomains (fractions 1–4), whereas *de novo* synthesized cholesterol in non-raft fractions (fractions 5–8) was only slightly affected (1.7-fold increase). This stimulation of cholesterol synthesis was accompanied by a 1.6-fold increase of the cholesterol content in lipid raft fractions (fractions 1–4), whereas cholesterol levels in non-raft fractions were not or only slightly affected (fractions 5–8) (Fig. 4*B*). To confirm that fractions 1–4 and fractions 5–8 corresponded to the lipid raft fractions and the non-raft fractions, respectively, Western blot analysis for the raft marker flotillin-2 and for transferrin receptor (a protein that is not associated with membrane microdomains) was performed. As expected, flotillin-2 was predominantly present in the low density fractions (fractions 2–4), whereas expression of the transferrin receptor was only observed in the high density fractions (fractions 7–8) (Fig. 4*C*).

RNAi-mediated Inhibition of SQS Reduces Cholesterol Levels in Detergent-resistant Membrane Microdomains—To further study the role of androgen-induced cholesterol synthesis in prostate cancer cells, the SQS gene was silenced in LNCaP cells using RNA interference (RNAi) technology. Luciferase siRNA was used as a control. Northern and Western blot analyses showed that expression of SQS in LNCaP cells was markedly suppressed as early as 24 h after transfection with SQS siRNA; the silencing effect lasted at least 120 h (Fig. 5, *A* and *B*).

In agreement with the decreased SQS expression, RNAi-mediated inhibition of SQS blocked cholesterol synthesis in LNCaP cells. Radiolabeled [^{14}C]acetate incorporation assays and subsequent TLC analysis revealed that, at 72 h after transfection with SQS siRNA, cholesterol synthesis was reduced 4-fold compared with control cells (data not shown). To investigate the impact of SQS silencing on the synthesis of cholesterol that is incorporated in membrane microdomains, siRNA-transfected LNCaP cells were exposed to [^{14}C]acetate for 4 h and were then treated with Triton X-100 and subjected to sucrose gradient ultracentrifugation. Then, sucrose gradient fractions were subjected to Bligh Dyer lipid extraction. TLC analysis showed that RNAi-mediated inhibition of SQS reduced the levels of ^{14}C-labeled cholesterol both in raft fractions and in non-raft fractions (Fig. 5*C*). The inhibition of cholesterol synthesis by SQS RNAi resulted in a 50% decrease in cholesterol content of detergent-resistant membrane microdomains (fractions 1–4); cholesterol levels in non-raft fractions (fractions 5–8) were only slightly affected by SQS RNAi (Fig. 5*D*).

To investigate whether the SQS RNAi-mediated decrease in cholesterol influenced the activation of SREBPs, levels of precursor and mature SREBP were measured. Western blot analysis on total extracts and nuclear fractions of LNCaP cells revealed that SQS RNAi had no effect on SREBP maturation as levels of both precursor and mature SREBP were similar in control cells and SQS siRNA-treated cells (data not shown).

To confirm that SQS inhibition specifically inhibited the sterol branch of the isoprenoid pathway, the impact of SQS RNAi on protein isoprenylation was examined. Control and SQS siRNA-treated LNCaP cells were transiently transfected with pAcGFP1-F plasmid encoding GFP fused to a farnesylation signal, which mediates post-translational farnesylation, thereby targeting the GFP protein to the plasma membrane. 4 h later, cells were treated with vehicle, with the HMG-CoA reductase inhibitor mevastatin (10 μM), or with the farnesyl transferase inhibitor FTI-277 (10 μM). 24 h later, membrane fractions were prepared and Western blot analysis for GFP was performed. Addition of mevastatin or FTI-277 to the transfected cells resulted in a marked decrease of membrane-associated GFP (Fig. 5*E*) and an increase of cytosolic GFP (not shown), thereby indicating inhibition of protein farnesylation. However, in agreement with the fact that the enzyme SQS is unique to the sterol branch of the isoprenoid pathway, SQS RNAi had no effect on GFP farnesylation, as membrane-associated GFP levels were comparable with those of control cells (Fig. 5*E*). These findings confirm that SQS inhibition indeed specifically blocked sterol synthesis without affecting the non-sterol

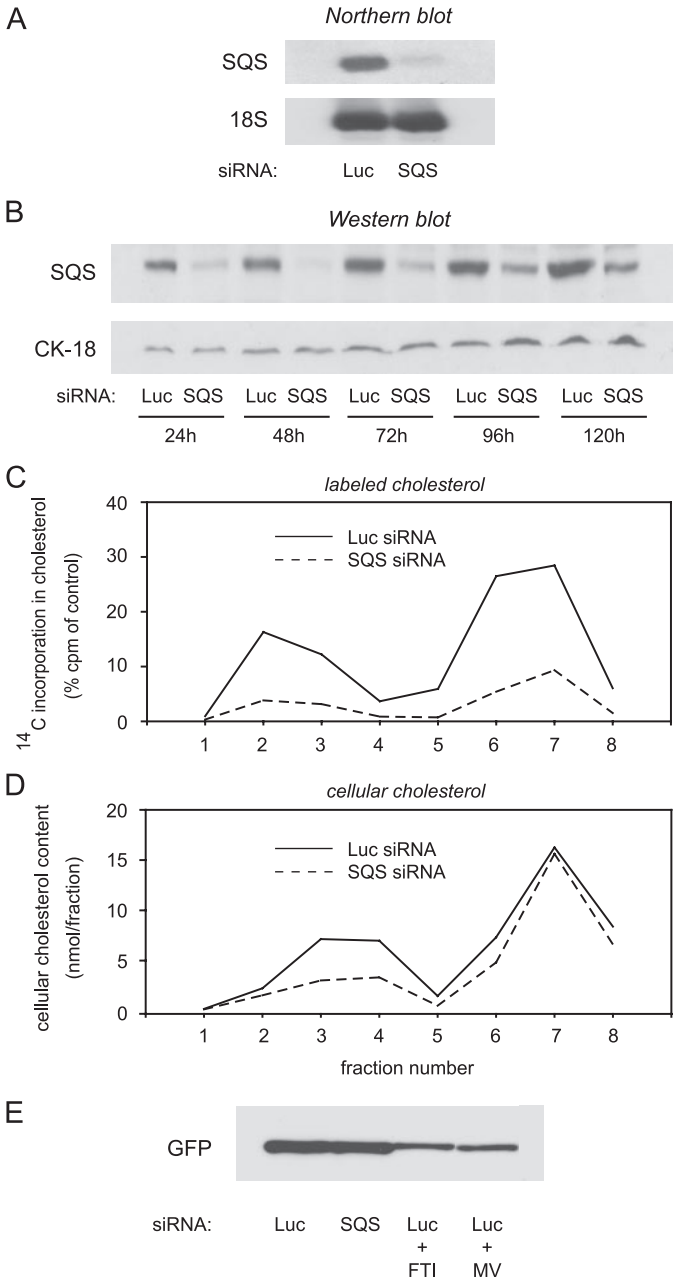
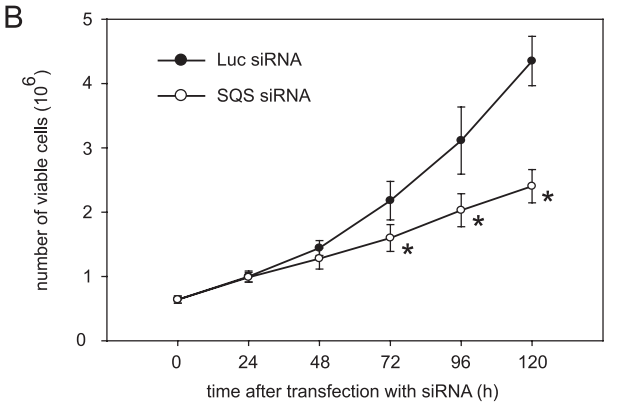
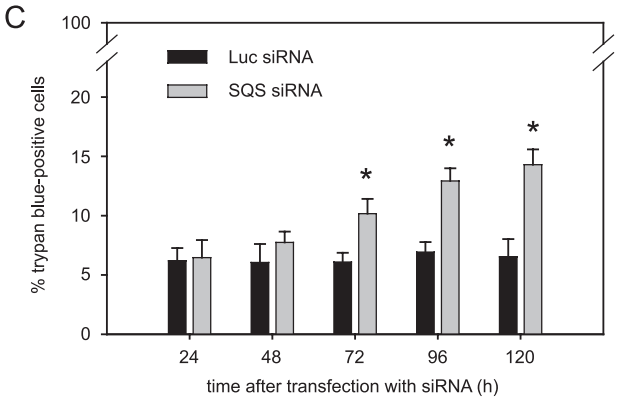


FIGURE 5. **RNAi-mediated blockage of SQS inhibits cholesterol synthesis in LNCaP cells.** *A*, expression of SQS was analyzed by Northern blot analysis at 72 h after transfection of LNCaP cells with siRNA targeting SQS or luciferase (*Luc*). 18 S RNA expression was used as internal control. *B*, expression of SQS was analyzed by Western blot analysis at 24, 48, 72, 96, and 120 h after transfection of LNCaP cells with siRNA targeting SQS or luciferase (*Luc*). Cytokeratin-18 (*CK18*) expression was used as internal control. *C* and *D*, at 72 h after transfection with siRNA targeting SQS or luciferase (*Luc*), LNCaP cells were incubated with ^{14}C-labeled acetate for 4 h; cells were harvested, resuspended in the presence of 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Sucrose gradient fractions were subjected to lipid extraction. Using TLC analysis and phosphorimaging, biosynthesis of cholesterol was quantitated (*C*) by measuring the incorporation of [^{14}C]acetate into cholesterol (value of each fraction is expressed as percentage of total incorporated [^{14}C]acetate (all fractions pooled) in cholesterol in control (*Luc siRNA*) cells). *D*, cellular cholesterol levels were determined using an enzymatic assay. Data shown are representative of two independent experiments. *E*, LNCaP cells were transfected with siRNA targeting SQS or Luc. After 48 h cells were transfected with pAcGFP1-F plasmid (containing GFP fused to a farnesylation signal). 4 h later cells were treated with vehicle or with the farnesyl transferase inhibitor FTI-277 (10 μM) or the HMG-CoA reductase inhibitor mevastatin (*MV*) (10 μM); 24 h later membrane fractions were prepared and Western blot analysis for GFP was performed (equal amounts of membrane protein were loaded).

A

% BrdUrd	Luc siRNA	SQS siRNA
24 h	100 +/- 6	103 +/- 4
48 h	100 +/- 10	75 +/- 7 *
72 h	100 +/- 10	67 +/- 9 *
96 h	100 +/- 8	68 +/- 6 *
120 h	100 +/- 5	76 +/- 7 *





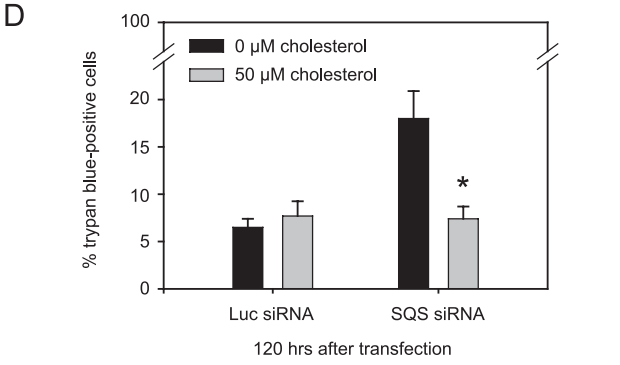


FIGURE 6. **RNAi-mediated blockage of SQS inhibits proliferation and induces cell death in LNCaP cells.** *A*, at the indicated time points after transfection with siRNA targeting SQS or luciferase (*Luc*), LNCaP cells were exposed to BrdUrd for 2 h. BrdUrd incorporation in LNCaP cells was measured colorimetrically and normalized for the number of viable cells at the start of the BrdUrd exposure (expressed as percentage BrdUrd incorporation of the control (*Luc siRNA*-transfected cells)). *B* and *C*, at the indicated time points after transfection with siRNA targeting SQS or luciferase (*Luc*), LNCaP cells were collected and stained with trypan blue, and the number of viable cells (*B*) and the percentage of dead cells (*C*) were counted. *D*, LNCaP cells were transfected with siRNA targeting SQS or luciferase (*Luc*). At 4 h after transfection, vehicle (0 μM) or cholesterol (50 μM) was added to the culture medium. At 120 h after transfection, cells were collected and stained with trypan blue, and the percentage of dead cells was counted. Data are means *columns* ± S.D. *bars* (n = 4–6) and are representative of two independent experiments. *, significantly different from control (Luc siRNA-transfected cells and 0 μM cholesterol in *panels A–C* and *panel D*, respectively) by Tukey test (p value <0.05).

branch (including protein isoprenylation) of the isoprenoid pathway.

RNAi-mediated Inhibition of SQS Induces Growth Arrest and Cell Death in Prostate Cancer Cells—In view of recent reports on the importance of raft-associated cholesterol for growth and survival of tumor cells (9, 21, 47), we examined the impact of RNAi-mediated inhibition of SQS on prostate cancer cell proliferation and viability. BrdUrd incorporation assays and trypan blue exclusion dye assays revealed that RNAi-mediated blockage of SQS significantly attenuated proliferation of LNCaP cells (Fig. 6, *A* and *B*). In addition, starting from 72 h after transfection, SQS silencing also caused significant cytotoxicity in LNCaP cells (Fig. 6*C*). Interestingly, supplementation with exogenous cholesterol (50 μM) prevented the cytotoxic effects induced by siRNA targeting SQS (Fig. 6*D*), supporting the selectivity of the RNAi-mediated SQS inhibition.

The Chemical SQS Inhibitor Zaragozic Acid A Reduces Cholesterol Levels in Detergent-resistant Membrane Microdomains and Induces Growth Arrest and Cell Death in Prostate Cancer Cells—To confirm the observed effects of RNAi-mediated SQS inhibition on prostate cancer cells, LNCaP cells were also treated with the chemical SQS inhibitor zaragozic acid A (also referred to as Squalestatin S1). After exposure of LNCaP cells to [^{14}C]acetate, total cellular lipid extracts were prepared as well as lipid extracts from sucrose gradient fractions. TLC analysis revealed that exposure of LNCaP cells to 40 μM zaragozic acid A for 72 h almost completely blocked the synthesis of both raft cholesterol and non-raft cholesterol (Fig. 7*A*). Blockage of cholesterol synthesis by zaragozic acid A particularly affected the cholesterol content of detergent-resistant membrane microdomains (fractions 1–4), which was decreased by 60%; cholesterol levels in non-raft fractions (fractions 5–8) were only slightly affected after exposure to zaragozic acid A (Fig. 7*B*). Similar to observations after RNAi-mediated inhibition of SQS, trypan blue exclusion dye assays revealed that blockage of SQS by zaragozic acid A significantly inhibited growth and induced cytotoxicity in LNCaP cells (Fig. 7*C*).

DISCUSSION

In the present work, we have demonstrated that androgens stimulate the expression of SQS in LNCaP prostate cancer cells, in a dose-dependent and time-dependent manner, through activation of SREBPs. Interestingly, the observed dose and time courses mirror those of other androgen-regulated lipogenic enzymes such as fatty acid synthase (30, 32, 40). Mutation of promoter elements previously shown to be essential for cholesterol- and SREBP-mediated stimulation of SQS expression (37, 38) potently decreased the effects of androgens, whereas co-transfection with a dominant-negative form of SREBP almost completely abolished androgen-mediated induction of the SQS promoter. These findings clearly demonstrate that the stimulatory effects of androgens on the expression of SQS are mediated by SREBPs, similar to previous observations for other lipogenic enzymes (32, 34). Because in prostate cancer cells the andro-

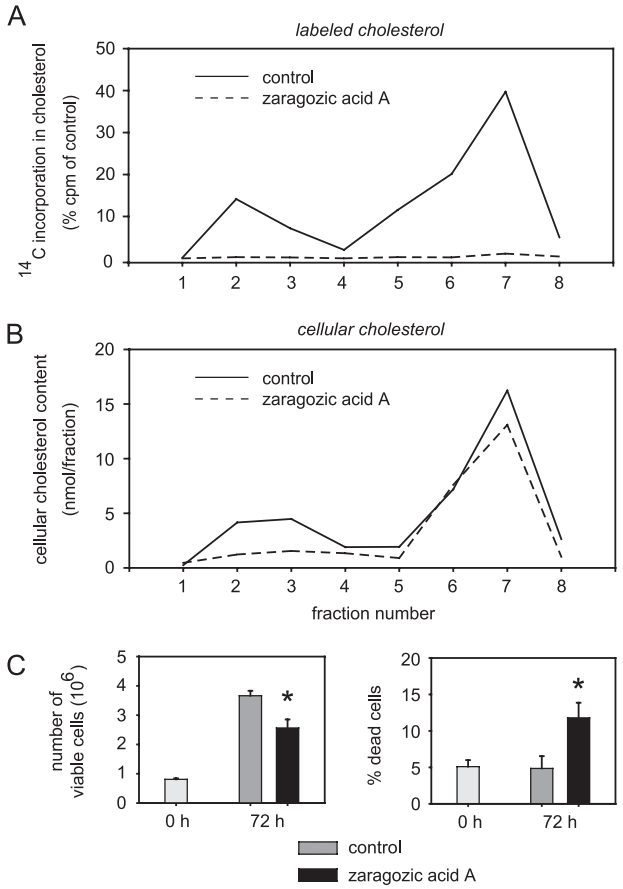


FIGURE 7. **Zaragozic acid A-mediated inhibition of SQS decreases cholesterol synthesis and induces growth arrest and cell death in LNCaP cells.** *A* and *B*, after 72 h of exposure to zaragozic acid A (40 μM) or vehicle (*control*), LNCaP cells were incubated with ^{14}C-labeled acetate for 4 h. Cells were harvested, resuspended in the presence of 1% Triton X-100, and subjected to sucrose density gradient ultracentrifugation. Sucrose gradient fractions were subjected to lipid extraction. Using TLC analysis and phosphorimaging, biosynthesis of cholesterol was quantitated (*A*) by measuring the incorporation of [^{14}C]acetate into cholesterol (value of each fraction is expressed as percentage of total incorporated [^{14}C]acetate (all fractions pooled) in cholesterol in control cells). *B*, cellular cholesterol levels were determined using an enzymatic assay. Data shown are representative of three independent experiments. *C*, after 72 h of exposure to zaragozic acid A (40 μM) or vehicle (*control*), LNCaP cells were collected and stained with trypan blue; the number of viable cells and the percentage of dead cells were counted. Numbers of viable and dead cells at the time of addition of inhibitor/vehicle are also indicated (*0 h*). Data are means *columns* $\pm$ S.D. *bars* ($n = 5$) and are representative of two independent experiments. *, significantly different from control cells (at 72 h) by Student's *t* test (p value <0.05).

gen-mediated activation of SREBPs partially overrides the cholesterol-driven negative feedback regulation of SREBP activation and actually leads to increased cholesterol synthesis (31, 32), androgen-induced stimulation of cholesterol biosynthesis may contribute to the reported accumulation of cholesterol in various tumor types, including prostate cancer (5, 6).

Although a role for cholesterol in membrane functioning has been well established (1), the recent conceptual changes regarding membrane subdomain structuring (3–6) have prompted us to explore the impact of the increased cholesterol synthesis on different membrane fractions. Interestingly, we observed that androgens predominantly increased the cholesterol content of lipid rafts, whereas cholesterol levels in non-raft fractions were barely affected in prostate cancer cells. In support of the role of cholesterol biosynthesis in the regulation of membrane microdomain cholesterol content, inhibition of SQS expression through RNAi-mediated gene silencing severely reduced the levels of raft-associated cholesterol in prostate cancer cells; non-raft cholesterol was only slightly affected. The mechanism underlying the selectivity of cholesterol partitioning remains unknown and certainly merits further investigation. One attractive hypothesis is that co-stimulation of the synthesis of raft-o-philic phospholipids, as reported previously (45), draws cholesterol preferentially to raft fractions.

Remarkably, the reduction in raft-associated cholesterol, induced by selective RNAi-mediated knock down or chemical inhibition of SQS, resulted in growth arrest and cytotoxicity in prostate cancer cells. As we targeted a cholesterol biosynthetic enzyme downstream of the isoprenoid branch point, the present study unambiguously underscores the importance of *de novo* cholesterol synthesis for cancer cell biology. Our work also corroborates previous findings that demonstrate that mechanical disruption of lipid rafts by extracting cholesterol with filipin or methyl-β-cyclodextrin reduced lipid raft cholesterol levels in cancer cells, resulting in induction of cancer cell death both in cultures *in vitro* and in xenografts *in vivo* (9, 21, 47). In contrast, treatment with exogenous cholesterol was shown to increase raft-associated cholesterol and to promote growth of cancer cells (9, 21), thereby further underlining the importance of cholesterol and membrane microdomains in tumorigenesis. Furthermore, these data also strengthen the hypothesis that the tumor-inhibiting effects of statins (13–21) may in part be mediated via blockage of *de novo* cholesterol synthesis. Importantly, because solid tumors often have limited vascular supply, they may be more dependent on *de novo* cholesterol synthesis, thereby strongly pointing to SQS (and cholesterol synthesis in general) as an interesting novel target for antineoplastic therapy. With regard to cancer prevention/treatment, besides HMG-CoA reductase inhibitors such as statins, other inhibitors that block the sterol branch of the isoprenoid pathway (including the squalene synthase inhibitors zaragozic acid A, B, and C) also merit further investigation. To our knowledge, no experimental data have been published thus far showing anti-cancer effects of zaragozic acids in animal models. However, several studies have reported that zaragozic acid A, B, and C, which inhibit squalene synthase at picomolar concentrations (with zaragozic acid B being 10 times more efficient compared with zaragozic acid A and C), efficiently inhibited cholesterol synthesis in cultured cells *in vitro* and in animals *in vivo* at very low doses (48, 49), suggesting that these compounds (in addition to statins and other inhibitors of cholesterol synthesis) may indeed have potential for use in cancer prevention/treatment.

Taken together, the present study shows that in prostate cancer cells androgens stimulate the expression of SQS, thereby funneling intermediates of the mevalonate/isoprenoid pathway toward the cholesterol synthesis branch, which results in increased levels of raft-associated cholesterol. Importantly, selective modulation of the cholesterol synthesis branch of the mevanolate/isoprenoid pathway by targeted inhibition of SQS reduces raft-associated cholesterol levels, attenuates cancer cell proliferation, and induces cancer cell death. In conclusion, our

findings show that not only exogenous cholesterol but also *de novo* synthesized cholesterol may significantly affect cancer cell biology. Moreover, these data reveal a new mechanism by which androgens may influence the development and progression of prostate cancer, provide insights into the mechanisms by which cholesterol-lowering drugs reduce cancer incidence, and, for the first time, point to SQS (and the cholesterol biosynthetic pathway) as a novel potential target for cancer prevention and anticancer therapy.

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