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Primary Culture of Human Cumulus Cells Requires Stearoyl-Coenzyme A Desaturase 1 Activity for Steroidogenesis and Enhancing Oocyte In Vitro Maturation

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Abstract

Stearoyl-coenzyme A desaturase 1 (SCD1) is a key enzyme in lipid metabolism and is expressed in cumulus cells. The objective of the present study was to evaluate the effect of SCD1 inhibition in human cumulus cells on triglyceride content, steroidogenesis, and oocyte in vitro maturation. Human cumulus cells were exposed to SCD1 inhibitor CAY10566 (SCDinhib) alone or in combination with oleic acid in primary culture. The SCDinhib markedly suppressed triglyceride accumulation (−47%, $P = .01$), aromatase gene expression (−36%, $P = .02$), and estradiol production (−49%, $P = .01$) even at a dose not affecting cell viability and apoptosis. Human immature oocytes at the germinal vesicle (GV) stage were cocultured with pretreated cumulus cells. The rate of oocytes reaching the metaphase II stage was significantly lower in coculture with SCDinhib-treated cumulus cells than with control cumulus cells (−18%, $P < .01$), which recovered by oleic acid supplementation. This finding on in vitro maturation rate was also reproducible with mouse GV oocytes. The results suggest that SCD1 activity is required for cumulus cell lipid storage and steroidogenesis. In addition, oocyte maturation is negatively affected by SCD1 inhibition in cumulus cells, possibly due to a deficient lipid-mediated paracrine support.

Keywords

germinal vesicle, lipid, paracrine, steroid hormone

Introduction

Stearoyl-coenzyme A desaturase 1 (SCD1; EC 1.14.19.1) is a microsomal membrane enzyme that is involved in de novo fatty acid synthesis.[1] Stearoyl-coenzyme A desaturase 1 produces oleic acid by inserting a double bond in the most prevalent substrate stearic acid. On one hand, inhibition of SCD1 activity has been considered as a potential therapeutic strategy for lipid-related metabolic disorders,[2] while on the other hand, its inhibition induces apoptosis[3] and decreases cell proliferation via blocking progression of the cell cycle.[4] In addition, a chemical SCD1 inhibitor (SCDinhib) compromised mouse embryonic development by cytotoxic effects on the inner cell mass cells.[5] More recent data indicate the role of SCD1 in preserving the pluripotency potential.[6,7]

Stearoyl-coenzyme A desaturase 1 as a metabolic enzyme marker is expressed in cumulus cells and at a higher level in the cumulus associated with metaphase II (MII) oocytes than those associated with germinal vesicles (GVs).[8] Moreau et al[9] have shown that SCD1 is expressed in rat granulosa cells of antral follicles but not in primordial follicles. Moreover, follicle-stimulating hormone (FSH), which initiates and supports follicular development, significantly increased SCD activity in rat ovary.[9] The SCD1 expression downregulation induced by in vitro supplementation of polyunsaturated fatty acids has been associated with lower bovine embryo survival and development.[10]

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Immature oocytes are not competent metabolically, so their maturation is dependent on exogenous supports.[11] Inside each follicle, an immature oocyte is surrounded by a relatively large number of cumulus cells. Paracrine factors derived from these cells provide a functional microenvironment for oocyte maturation.[12] Current data suggest that SCD1 activity of cumulus cells can contribute to the ability of them to support oocyte maturation. Approaches so far to identify the role of SCD1 in female fertility have rather been descriptive, linking SCD1 expression profiling data with oocyte maturation and developmental capacity,[8,9] with no evidence thus far of its functional role. The objective of the present study was to evaluate the effect of SCD1 inhibition by specific chemical inhibitors on triglyceride content and steroidogenesis in primary culture of cumulus cells isolated from human GV oocytes. In addition, effects of SCDinhib-treated cumulus cells were tested on human and mouse oocyte maturation in vitro. A coculture system was used instead of the conventional cumulus-oocyte complex (COC) culture to distinguish effects mediated by surrounding cumulus cells from direct effects on oocytes. This system also allowed us to obviate the ethical concerns surrounding testing chemicals in direct contact with germ cells or embryo.

Materials and Methods

Materials

Cell culture materials, media, and TRIzol were obtained from Invitrogen/Gibco Company (Carlsbad, California). The SCD1 inhibitors were purchased from Cayman Chemicals (CAY10566; Ann Arbor, Michigan) and Focus Biomolecules (MF-438; Plymouth Meeting, Pennsylvania). Deoxyribonuclease I, human fatty acid–free albumin, hyaluronidase, testosterone, and standard fatty acid methyl esters were obtained from Sigma Chemicals (St Louis, Missouri). ISM embryo culture mediums and hyaluronidase enzyme were purchased from Medicult (Lyon, France). All other chemicals used were of analytical grade.

Isolation of Cumulus Cells and GV Oocytes

Cumulus-enclosed GV-stage oocytes were obtained in accordance with the approved guidelines; the donated cells were consecutively collected from women during their first cycle of in vitro fertilization (IVF) using intracytoplasmic sperm injection (ICSI) in the IVF Center at the Women University Hospital in Tabriz from September 2014 to August 2016. In accordance with the Declaration of Helsinki, the study protocol was approved by the ethics committees of Shahid Beheshti University of Medical Sciences and Tabriz University with the institutional review board permission numbers 400/3226 and 89141, and all patients provided written informed consent. There was no deviation from the standard of care or practice for all participants. All patients were asked to participate irrespective of the regimen for pituitary suppression. Cells were obtained from a total of 185 women, 140 of whom donated only cumulus cells, 42 of whom donated both cumulus cells and GV oocytes, and 3 of whom donated only GV oocytes (Figure 1). No patient had previously undergone radiation, surgery, or cytotoxic chemotherapy. Patients older than 35 years and younger than 20 years and those with a body mass index greater than 32, a smoking history, endometriosis, history of endocrine disease, and diabetes were excluded from the study. According to our clinical protocol, indications for IVF-ICSI include 2 failed rounds of intrauterine insemination, tubal disease, and male factor infertility. For the above reasons, the average age of our study participants was relatively younger than typical population in most IVF-ICSI studies.

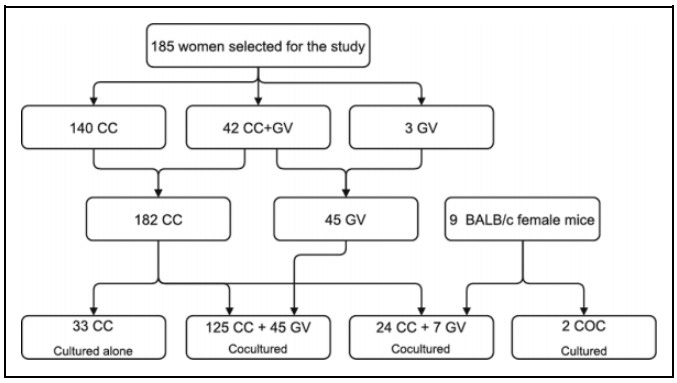

Figure 1. Distribution of all 185 women and 9 BALB/c female mice according to the donated and obtained cells, respectively. Values indicate number of women or mouse. COC indicates cumulus–oocyte complex; CC, cumulus cells from germinal vesicle (GV) oocyte.

The mean age of participants was 27.60 years and mean body mass index was 26.91 kg/m^2. They had been diagnosed with tubal disease (n = 47), male factor infertility (n = 92), and unexplained infertility (n = 46). Follicle-stimulating hormone, anti-Müllerian hormone (AMH), and estradiol were assayed in serum samples collected within 3 to 5 days of the menstrual cycle before the initiation of IVF treatment using enzyme-linked immunosorbent assay (ELISA) kits (Monobind, Lake Forest, California). The mean serum concentrations of FSH, AMH, and estradiol were 6.87 mIU/mL (range: 4.41-9.93 mIU/mL), 2.36 ng/mL (range: 1.02-4.84 mIU/mL), and 53.78 pg/mL (range: 25.11-81.92 mIU/mL), respectively. Participants underwent a standard IVF using recombinant FSH (rFSH, Gonal-F; Serono, Geneva, Switzerland) on day 3 of menstrual cycle in combination with decapeptyl (DebioPharm, Geneva, Switzerland) or cetrorelix (Serono, Geneva, Switzerland) for controlled ovarian stimulation.[13] The dose of rFSH was chosen between 150 and 300 IU/d, depending on body mass index, age of the women, and the anticipated ovarian response. The ovarian response was monitored by follicular development on ultrasound and serum estradiol concentration every 2 days from stimulation days 6-7. Human chorionic gonadotrophin (HCG, 10 000 IU, Choriomon; Meizler, São Paulo, Brazil) was administered as an intramuscular injection when sonography revealed 3 preovulatory follicles with an average diameter of 18 to 20 mm. Oocytes were collected 36 hours after HCG administration by vaginal ultrasound-guided puncture of the ovarian follicles.[13]

Culture of Cumulus Cells

Human cumulus cells were dissected from the GV-stage COC obtained from a total of 182 women undergoing IVF-ICSI using a sterile needle and rinsed in TCM199 medium. The cells were dispersed by gentle pipetting in 1% hyaluronidase enzyme. Isolated cumulus cells pooled from 3 women were cultured in TCM199 medium supplemented with rFSH (75 IU/mL) and 10% fetal bovine serum, containing vehicle, SCDinhib (5-100 nM), or SCDinhib and oleic acid (50-100 μM) for 48 hours. Nontoxic concentrations with at least 50% change in the SCD1 activity index were selected for the subsequent experiments. Cells were maintained at 37°C in ambient O_2 and 5% CO_2 in a humidified incubator. Pretreated cumulus cells were cultured alone for cell viability and molecular experiments and were cocultured after 48 hours with heterologous immature GV-stage oocytes for in vitro maturation (IVM) experiments. To check recovery from SCDinhib-induced effects, pretreated cumulus cells were cultured in the IVM media without any treatment for an additional 24 hours corresponding to IVM period.

Stearoyl-Coenzyme A Desaturase 1 Inhibition

To inhibit cumulus cells' SCD1 activity, 3-[4-(2-chloro-5-fluorophenoxy)-1-piperidinyl]-6-(5-methyl-1,3,4-oxadiazol-2-yl)-pyridazine (CAY10566) and/or 2-methyl-5-(6-(4-(2-trifluoromethyl)phenoxy)piperidin-3-yl)-1,3,4-thiadiazole (MF-438), potent and selective inhibitors of SCD1, were added at the concentrations indicated to culture medium for 48 hours. The reason for the use of 2 different inhibitors in some experiments was to ensure selectivity of inhibition. Albumin-bound oleic acid[14] in combination with simultaneous SCDinhib treatment was used in rescue experiments. The vehicle consisted of dimethyl sulfoxide/albumin (<0.05% vol/vol) was used as mock-treated control. Cell viability and cytotoxicity were quantified using Trypan blue exclusion and sulforhodamine B assay,[15] respectively. Induction of cell apoptosis was evaluated by annexin V-fluorescein isothiocyanate/propidium iodide staining and analyzed according to the manufacturer's protocol (eBioscience, San Diego, California) on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California). The SCD1 inhibition was checked at the end of inhibitor treatment by examining cellular fatty acids using gas–liquid chromatography technique.

Production of Estradiol

Aromatase activity was evaluated by measuring the conversion of exogenous testosterone (0.5 μM) to estradiol. The levels of secreted estradiol into 24-hour aliquots of cumulus cell culture supernatants were measured using a commercial ELISA kit (Diagnostics Biochem Canada Inc, Ontario, Canada) with a detection limit of 10 pg/mL (coefficient of variation 1.52%).

Gene Expression Analysis

Total RNA extraction was carried out using the TRIzol, and residual genomic DNA contamination was removed by digestion with deoxyribonuclease. RNA was quantified by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, Delaware) at 260 nm, and the ratio of absorbance at 260 nm/280 nm was >1.8 for all samples. Two micrograms of total RNA were used to generate complementary DNA (cDNA) using a first-strand cDNA synthesis kit for reverse transcription polymerase chain reaction (PCR; Roche, Hertfordshire, United Kingdom). Quantitative PCR was carried out in duplicate using a Rotor-Gene 3000 real-time PCR system (Corbett Robotics, Brisbane, Australia) and the SYBR Premix Ex Taq (Takara Bio Inc, Shiga, Japan). The sequences of primers are presented in Supplemental Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA transcript was quantified as an endogenous reference according to the manufacturer's instructions. The RNA samples without reverse transcriptase were included as control. The PCR reaction conditions were 30 seconds at 95°C, then 40 cycles at 95°C for 5 seconds, and a final incubation at 60°C for 30 seconds. Standard curves for products were linear (correlation coefficients >0.99; slope range: −3.21 to −3.68). Relative gene expression was determined by using the comparative cycle threshold (ΔΔCT) method and normalized by the amount of GAPDH for each sample. All quantities were expressed as an x-fold difference relative to control.

Lipid Analysis

For fatty acid measurement, 24-hour serum-free conditioned media or washed cells were collected in methanol extraction solution (80% methanol and 20% H_2O) containing n-tridecanoic acid as an internal standard. Insoluble material was removed by centrifugation at 10 000 rpm for 5 minutes at 4°C. The supernatant was transferred in a glass vial, and the lipids were methylesterified with an acetyl chloride/methanol solution[16]; the products were extracted twice with 1 mL of hexane, dried under N_2, and reconstituted into 0.5 mL hexane for quantitative analysis. Gas–liquid chromatography was carried out using a Buck Scientific GC-610 gas chromatograph equipped with a flame ionization detector and split injection system, fitted with a capillary column (TR-CN100 column; 60 m length × 0.25 mm inside diameter; Teknokroma, Barcelona, Spain). The oven temperature program was 170°C to 210°C, 2°C/min, and then isothermal for 20 minutes. The fatty acids were identified and quantified relative to the corresponding standards as percentages of total fatty acids by integrating the peak areas using PeakSimple software version 3.59 (SRI Instruments, Torrance, California). The coefficient of variation was 2.5% for palmitic acid (16:0), 3.2% for palmitoleic acid (16:1n-7), 4.5% for stearic acid (18:0), and 4.1% for oleic acid (18:1n-9).

To biochemically analyze the triglyceride accumulation, total lipid was extracted from cumulus cell lysate by Bligh and Dyer

Table 1. Characteristics of the 45 Oocyte Donor Women.[a]

Age, years	26.55 ± 4.80 (20-35)
Body mass index, kg/m^2	26.47 ± 3.06 (21-32)
Basal FSH, mIU/mL[b]	6.63 ± 1.69 (4.42-9.23)
Anti-Müllerian hormone, ng/mL[b]	2.32 ± 1.01 (1.07-4.84)
Basal estradiol, pg/mL[b]	54.39 ± 21.49 (25.16-81.92)
Cause of infertility	
Tubal, n (%)	16 (35.6)
Male, n (%)	21 (46.7)
Unexplained, n (%)	8 (17.8)
Oocytes	
Mature, n	10.91 ± 3.65 (5-19)
Germinal vesicles, n	3.68 ± 0.76 (3-5)

Abbreviations: IVF, in vitro fertilization; SD, standard deviation.
[a]Unless otherwise noted, values are in mean ± SD (range).
[b]Measured in serum samples collected within 3 to 5 days of the menstrual cycle before the initiation of IVF protocol.

method using chloroform/methanol mixture[17] and assessed using a triglyceride quantification kit (Pars Azmun, Tehran, Iran) with a detection limit of 1.0 mg/dL. The triglyceride concentration was normalized to the protein content determined by the Folin-Lowry method,[18] with bovine serum albumin as a standard.

Oocyte IVM

The IVM of 3 sibling GV oocytes from the same donor was carried out on collections from a total of 45 women. Demographic characteristics of oocyte donors are reported in Table 1. In order to avoid the spontaneous GV breakdown, obtained oocytes were immediately cultured as described below.

Within 20 minutes after collection, GV oocytes were denuded enzymatically by a brief exposure to hyaluronidase and were individually transferred to 96-well plate containing mock-, SCDinhib- or SCDinhib, and oleic acid pretreated heterologous cumulus cells (5×10^3 per well). Maturation was done in fresh TCM199 culture medium containing 75 m IU/mL rFSH, 0.5 IU/mL HCG, 10 mg/mL human serum albumin (Octapharma AG, Lachen, Switzerland), and 0.5 μM testosterone in a humidified incubator at 37°C, containing ambient O_2 and 5% CO_2. The nuclear maturational state of each oocyte was determined by polar body identification under a microscope after 20 hours of culture.

In order to examine whether our findings were also applicable to mouse model, we repeated IVM experiments with cocultures of mouse GV oocytes. To obtain COC at the GV stage, a total of 9 sexually matured BALB/c female mice were used in the present study (Figure 1) and were received an intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Hipra, Girona, Spain). All protocols were conducted in accordance with animal protection laws and regulations approved by the institutional ethics committee. After 48 hours of PMSG priming, the mice were killed, ovaries were dissected, and follicles were punctuated with a 27-gauge needle.[19] Mouse GV oocytes were denuded and cultured by the same way as for human GV oocytes. Only large antral follicles surrounded by cumulus cells and morphologically normal appearing GV-stage oocytes were used for IVM. Denuded mouse GV oocytes were similarly cocultured with pretreated human cumulus cells. The mouse experiment also included controls consisting of COC, maturation media in the absence of testosterone and no cumulus cells.

Human Oocyte Developmental Capacity

In vitro matured human MII oocytes following coculture in the models described above were inseminated with grade I spermatozoa, selected by swim-up procedure,[20] under a micromanipulator microscope. The ICSI of the sibling oocytes was performed using husband's spermatozoa to comply with local ethical conditions. Injected oocytes were placed in 25 μL microdrops of ISM embryo culture mediums under lightweight paraffin oil. Fertilization rate was assessed 17-19 hours after ICSI, with the appearance of 2 pronuclei. Embryo development rate was also assessed for multicell, morula, and blastocyst stages after 48 hours to 5 days of in vitro culture.

Statistical Analysis

Data presented are the mean and standard deviation of 3 separate experiments done in duplicate. Calculation of significance between groups was done according to the Fisher exact test for count data or 2-way analysis of variance with post hoc Tukey tests for multiple comparisons. A P value $<.05$ was considered statistically significant.

Results

Stearoyl-Coenzyme A Desaturase 1 Inhibitor Reduced Viability and Increased Apoptosis in Cumulus Cells

The dose–response experiments using sulforhodamine B and Trypan blue assays were performed to determine the nontoxic concentrations of SCDinhib toward cumulus cells for subsequent experiments. The cumulus cell number and viability were significantly decreased in a dose-dependent manner following the treatment with high concentrations of SCDinhib CAY10566, demonstrating toxic effects (Figure 2A). Concentrations of $\leq$25 nM for 48 hours, however, showed no toxicity nor a reduction in the number of cells in cultured cumulus cells. The results of flow cytometry analysis also showed that at 50 nM of SCDinhib, the ratio of early (Annexin V+/PI−) and late (Annexin V+/PI+) apoptotic cells was significantly increased 4- and 2-fold, respectively (Figure 2B and C). However, the ratio of apoptotic cells was not significantly changed in the cells treated with SCDinhib at concentrations of 25 nM or lower compared with the control group.

Stearoyl-Coenzyme A Desaturase 1 Inhibitor Reduced Monounsaturated Fatty Acids and Triglyceride Content in Cumulus Cells

Treatment of cumulus cells with SCDinhib for 48 hours led to decreases in monounsaturated fatty acids, palmitoleate (16:1n-7),

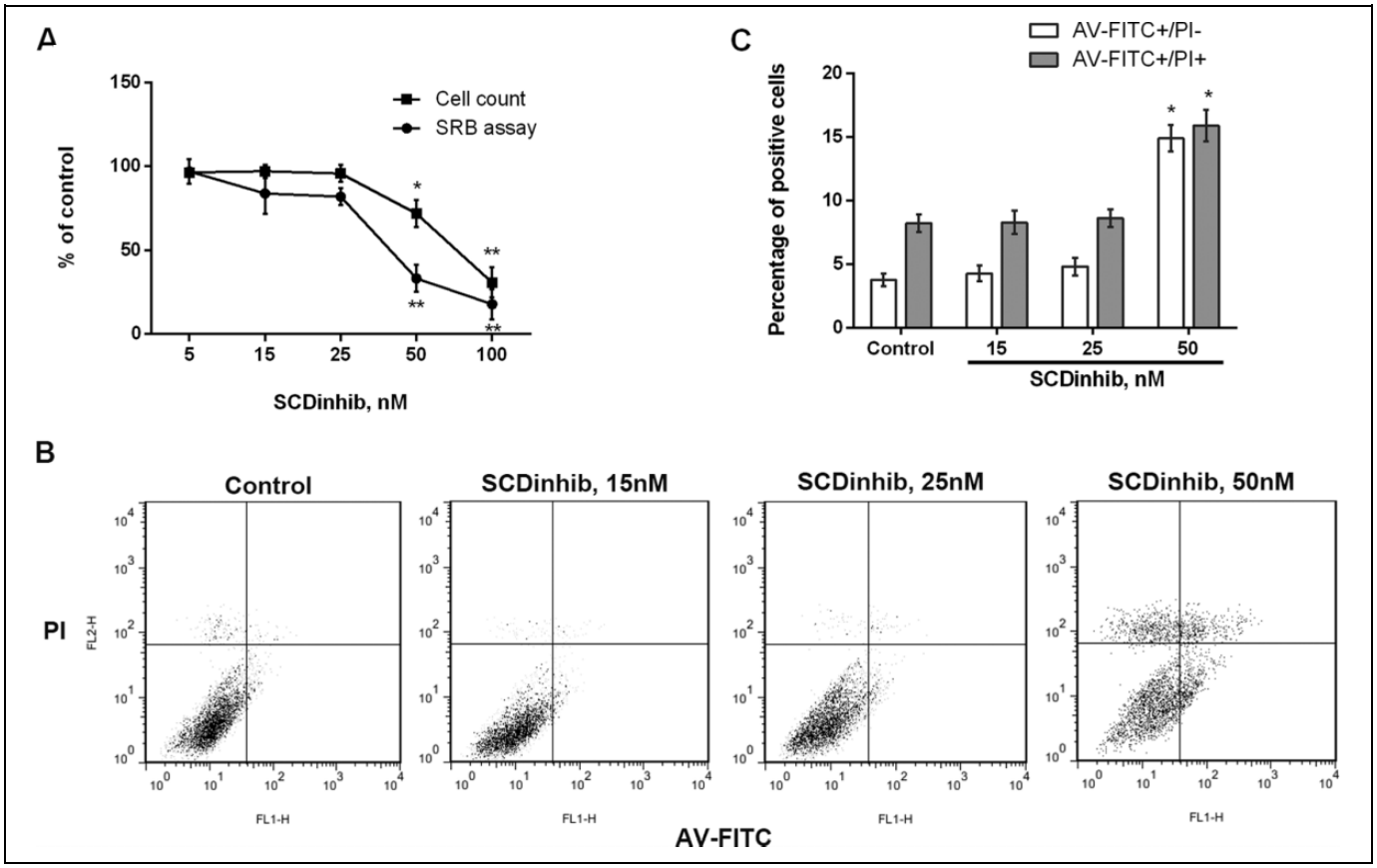

Figure 2. Effect of stearoyl-coenzyme A desaturase 1 (SCD1) inhibition on cumulus cell viability and apoptosis. A, Sulforhodamine B (SRB) and Trypan blue exclusion growth (cell count). B, Fluorescein isothiocyanate (FITC)-conjugated annexin V (AV) apoptosis analyses were done following mock treatment with dimethyl sulfoxide/albumin (<0.05%), treatment with the indicated doses (5-100 nM) of SCD1 inhibitor CAY10566 (SCDinhib) for 48 hours. C, Summary of the apoptosis data in histogram form. For apoptosis assay, cells were harvested and double stained with AV-FITC and propidium iodide (PI), and flow cytometric analysis was performed. The percentage changes are presented as mean and standard deviation from 3 independent experiments done in duplicate on cumulus cells pooled from 3 women. *$P < .05$ and **$P < .01$ versus control.

and oleate (18:1n-9) in cellular lipids. The ratio of palmitoleate/palmitate (16:1n-7/16:0) and oleate/stearate (18:1n-9/18:0) was calculated as validated metabolic indices of SCD1 activity.[21] Changes in activity indices of SCD1 following treatment with different doses of SCDinhib CAY10566 are shown in Figure 3A and B. Both showed significant and dose-dependent reductions at 48 hours following SCDinhib treatment or alone after additional 24 hours, with 22% to 59% decrease at a range of 5.0 to 25 nM inhibitor in growth media compared with control ($P < .05$). In an effort to better characterize the effects of SCDinhib on the paracrine function, we also analyzed fatty acids content in the serum-free conditioned media of cumulus cells (Figure 3C). The SCDinhib significantly reduced both SCD1 activity indices in media by approximately 45% in comparison with control mock-treated cells.

Indirectly, SCD1 activity plays a key role in triglyceride synthesis by controlling the availability of monounsaturated fatty acid substrates. We assessed whether SCDinhib treatment concomitantly affected triglyceride content as determined chemically in cumulus cells extract. At 48 hours following treatment, the total triglyceride content was significantly decreased by −47% ($P = .01$) in cells treated with SCDinhib (Figure 3D). The combination of oleate with SCDinhib resulted in recovery of triglyceride in cumulus cells.

Stearoyl-Coenzyme A Desaturase 1 Inhibitor Reduced Steroidogenesis in Cumulus Cells

Our preliminary experiments showed that treatment with oleate alone at 50 μM caused no significant changes in aromatase gene expression (Figure 4A) and estradiol production (Figure 4B) of cultured cumulus cells. So it was cotreated with SCDinhib as rescue therapy in subsequent experiments. To investigate whether SCD1 affects estradiol production by cumulus cells, the effect of treatment with a nontoxic dose of SCDinhib was examined alone or in combination with oleate. Following 48-hour treatment with SCDinhib CAY10566, quantitative PCR showed −36% reduction in aromatase gene expression ($P = .02$, Figure 4A) and analysis of conditioned

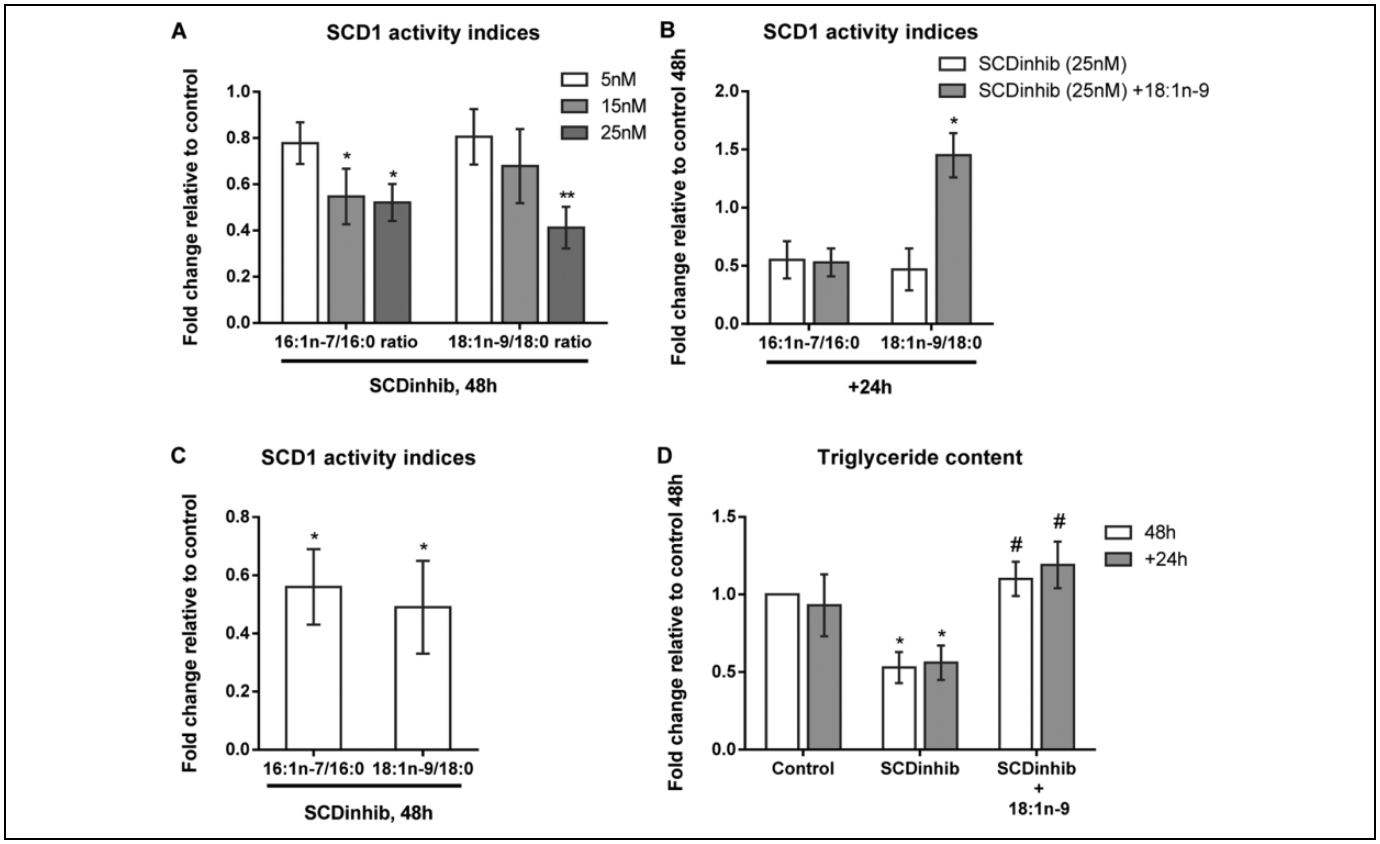

Figure 3. Effect of stearoyl-coenzyme A desaturase 1 (SCD1) inhibition on SCD1 activity and triglyceride content in cumulus cell. Activity indices of SCD1 in cellular extract following treatment with different doses of SCDinhib for 48 hours (A) and after additional 24 hours without treatment (B). Activity indices of SCD1 in conditioned medium (C) and total triglyceride content (D) following treatment with 25 nM SCDinhib. Cells were cultured in media supplemented with testosterone (0.5 μM), being mock treated with dimethyl sulfoxide/albumin (<0.05%), SCDinhib CAY10566 at indicated doses alone or in combination with oleic acid (18:1n-9, 50 μM). Assays for conditioned medium included an incubation for 24 hours in fetal bovine serum free medium. Conditioned medium or cell lysates were collected and analyzed for palmitate (16:0), palmitoleate (16:1n-7), stearate (18:0), or oleate (18:1n-9) by gas–liquid chromatography and for triglyceride by enzymatic method. Individual fatty acids were calculated as percentage of total fatty acids, and total triglyceride content was normalized to protein concentrations for each condition. The fold changes are presented as mean and standard deviation from 3 independent experiments done in duplicate on cumulus cells pooled from 3 women. *$P < .05$ and **$P < .01$ versus mock control; #$P < .05$ versus SCDinhib.

medium showed an estradiol decrease of -49% ($P = .01$, Figure 4B) in SCDinhib-treated cells compared to control. Control experiments with SCDinhib MF-438 that is chemically distinct from CAY10566 similarly caused decreases in cumulus cell aromatase gene expression (-43%, $P = .01$) and estradiol production (-45%, $P = .02$) in the low dose of 5 nM (Figure 4C), further demonstrating the SCD1-specific effects. The SCDinhib did not significantly change SCD1 gene expression (Figure 4D).

In rescue experiments using oleate together with SCDinhib, although SCD1 expression was decreased (-39%, $P = .03$, Figure 4D), the reductions in aromatase expression and estradiol production were efficiently recovered (>20%, $P < .05$) when compared to SCDinhib alone treatment (Figure 4A and B). However, only nonsignificant trends for further increase in aromatase expression and estradiol production were seen after additional 24-hour culture without treatments, indicating the importance of accessibility to exogenous oleate in cells with a deficiency in SCD1 activity.

Stearoyl-Coenzyme A Desaturase 1 Inhibitor Pretreatment Abolished the Enhancing Effect of Cumulus Cells on Oocyte Maturation in Coculture

To investigate whether SCD1 activity in cumulus cells affects oocyte maturation, pretreated cumulus cells were cocultured with human immature GV-stage oocytes for 20 hours. In the extended culture of pretreated cumulus cells alone for additional 24 hours in IVM media, SCDinhib-induced effects including changes in activity indices of SCD1 (Figure 3B), triglyceride content (Figure 3D), aromatase gene expression (Figure 4A), and estradiol production (Figure 4B) remained much the same, indicating that these changes were maintained over the coculture. The rates of oocytes reaching the MII stage were significantly lower in immature oocytes cocultured with SCDinhib-treated cumulus cells than with control cumulus cells (-18%, $P = .001$; Table 2).

Consistent with the above findings, ICSI-fertilized human oocytes from cocultures with SCDinhib-pretreated cumulus

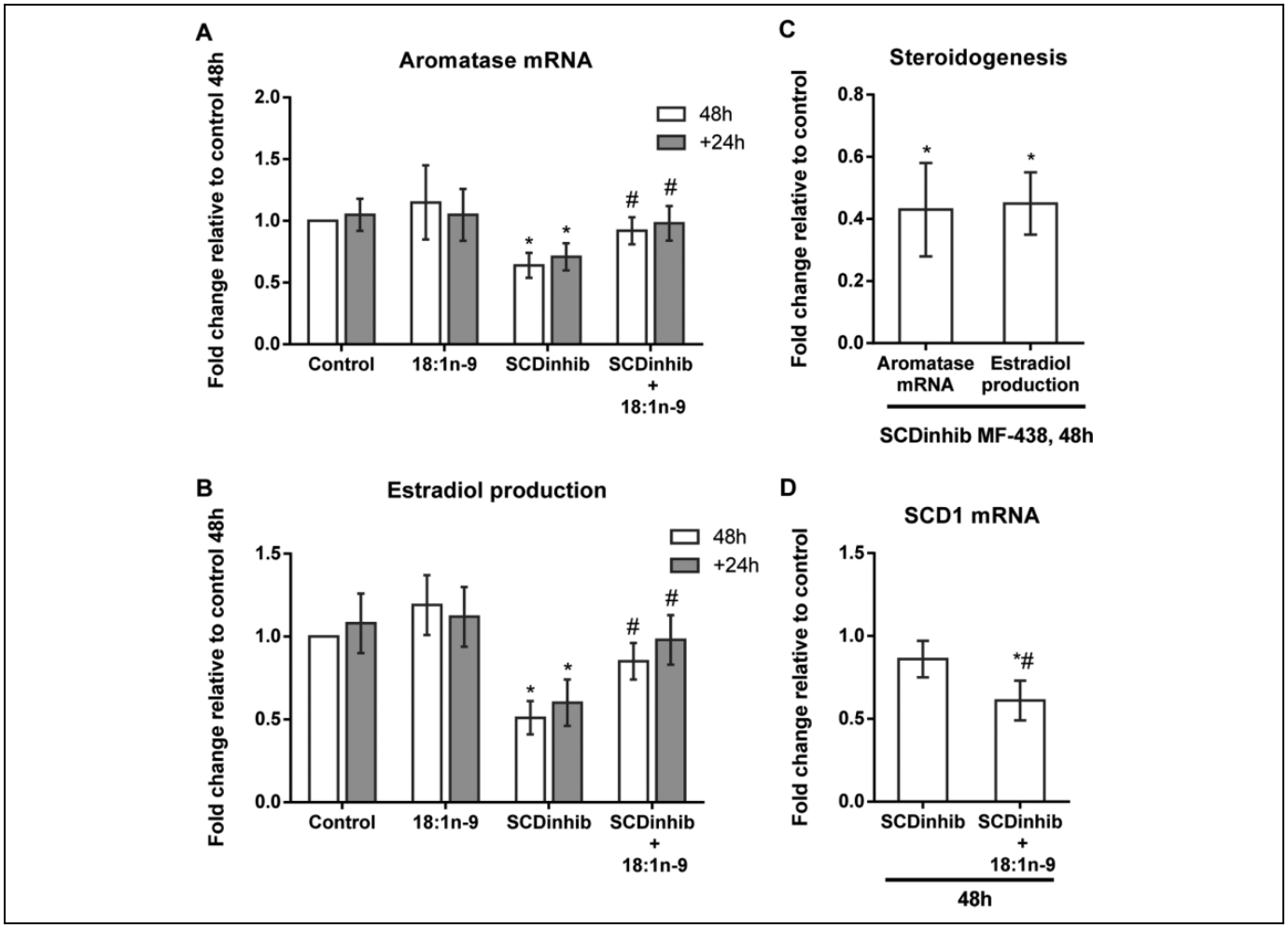

Figure 4. Changes in aromatase gene expressions, estradiol production (A-C), and stearoyl-coenzyme A desaturase 1 (SCD1) mRNA (D) in cultured cumulus cells after SCD1 inhibitor (SCDinhib) treatment. Cells were cultured in media supplemented with testosterone (0.5 μM), being mock treated with dimethyl sulfoxide/albumin (<0.05%), SCDinhib CAY10566 (25 nM), or MF-438 (5 nM) alone or in combination with oleic acid (18:1n-9, 50 μM) for 48 hours or following additional 24 hours without treatment. Cell content of genes mRNA was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene for each condition. Estradiol concentration was measured in 24-hour aliquots of conditioned medium. The fold changes are presented as mean and standard deviation from 3 independent experiments done in duplicate on cumulus cells pooled from 3 women. *$P < .05$ versus mock control; #$P < .05$ versus SCDinhib. mRNA indicates messenger RNA.

cells showed reduced in vitro survival and developmental capacity. These oocytes produced fewer 2-pronuclear zygote (-16%, $P = .005$) and multicell embryos (-18%, $P = .01$), with nonsignificant trends toward reduction in morula (-16%, $P = .36$) and blastocyst (-9%, $P = .39$) stage embryos compared to the control group (Table 2). The reduction in maturation percentage and fertilization rate was efficiently rescued by oleic acid supplementation in cumulus cell culture ($+18\%$, $P = .001$ and $+11\%$, $P = .03$, respectively).

To check whether changes in GV maturation rate can be replicated in mouse model, the effects of coculture with SCDinhib-pretreated cumulus cells on IVM were also examined in mouse immature oocytes. These experiments were limited to assessing IVM rate because of inaccessibility to instrumental facilities for mouse ICSI. In a preliminary experiment, the direct effect of 25 nM SCDinhib CAY10566 on mouse COC at the GV stage was compared to that on denuded GV oocytes cultured alone (n = 20 in each group). However, the 2 groups showed no statistically significant differences in MII (38% vs 35% of GV oocytes, respectively). To specifically characterize cumulus cell-mediated effect and to discern the effects on cumulus cell versus oocytes, experiments were repeated with cumulus cells-denuded GV oocytes coculture (n = 40 for each condition). In order to minimize any possible differences in species and in compatible with human oocytes tests, human cumulus cells were used for these experiments. Nuclear maturation of mouse GV oocytes as progression to MII was profoundly lower in immature oocytes cultured alone (n = 21, 52%) or cocultured with SCDinhib-treated cumulus cells (n = 23, 57%) than those cocultured with control (n = 29, 72%) and oleate-supplemented (n = 27, 67%) cumulus cells. In order to evaluate whether treatment with testosterone was

Table 2. In Vitro Maturation and Developmental Capacity of Human Oocytes Cocultured With SCD1 Inhibitor-Pretreated Cumulus Cells.[a,b]

	Germinal Vesicles Cocultured With Pretreated Cumulus Cells		
	Control, n = 45	SCDinhib, n = 45	SCDinhib + 18:1n-9, n = 45
Metaphase II	32 (71)	24 (53)[c]	32 (71)[d]
Two-pronuclear zygote	17 (38)	10 (22)[c]	15 (33)[d]
4-8 cells	13 (29)	5 (11)[c]	12 (27)
Morula	9 (20)	2 (4)	5 (11)
Blastocyst	4 (9)	0 (0)	3 (7)

Abbreviation: ICSI, intracytoplasmic sperm injection.
[a]Germinal vesicles (GVs) were cocultured for 20 hours, during in vitro maturation, with heterologous cumulus cells mock treated with dimethyl sulfoxide/albumin (<0.05%), treated with SCD1 inhibitor CAY10566 (SCDinhib, 25 nM) alone or SCDinhib in combination with oleic acid (18:1n-9, 50 μM). Three sibling GV oocytes from 45 women were randomly assigned to each condition. Metaphase II matured oocytes were inseminated by ICSI and embryo development was monitored after 48 hours to 5 days of in vitro culture.
[b]Data are presented as number (percentage).
[c]$P < .05$ versus mock control.
[d]$P < .05$ versus SCDinhib.

required for the enhancing effect of cumulus cells on oocyte maturation in coculture, similar experiments were performed involving SCDinhib-treated and control cumulus cells in the absence of testosterone (n = 10 for each condition). A significantly lower mouse GV maturation into MII stage was observed in coculture with control cumulus cells in the absence of testosterone as compared to that in complete medium (n = 4, 40% vs n = 7, 70%). However, no statistically significant excess of adverse effect was observed in coculture with SCDinhib-treated cumulus cells in the absence of testosterone (n = 4, 40%), confirming that the effect of testosterone is dependent on or mediated through cumulus cells.

Discussion

Functional maturation of oocytes is mainly determined by the active interaction with the surrounding cumulus cells. This study explored the putative role of SCD1 in cumulus cells by measuring a number of metabolic and functional parameters including the production of estradiol and IVM of immature oocytes.

The SCDinhib did affect various cumulus cells functions. The viability and fatty acid desaturation were significantly inhibited by SCDinhib in a dose-dependent manner, which were consistent with the induction of apoptosis. It has been shown that SCD1 participates in metabolic channeling of saturated fatty acid substrates into triglyceride, most likely by providing a more easily accessible pool of monounsaturated acyl-coenzyme As.[22] Consistent with this hypothesis, SCD1 activity was shown to be strongly associated with high fatty acid uptake rates.[23,24] Our data further suggest a functional indirect link between SCD1 activity and de novo TG synthesis in cumulus cells. Analysis by flow cytometry in cancer cells treated with SCDinhib revealed a marked apoptosis induction[3] and accumulation in the early stage of the cell cycle.[4] Since triglyceride is an important energy storage molecule, its depletion following treatment with high doses of SCDinhib may contribute to the increased apoptosis rate and decreased cumulus cells viability.

The SCDinhib markedly suppressed triglyceride accumulation, aromatase gene expression, and estradiol production in cumulus cells even at a nontoxic dose. To further examine whether these suppressions in SCDinhib-treated cumulus cells is due to the deficiency in product formation, we included oleate, a major product of SCD1, supplementation in experiment. In this rescue experiment, oleate while significantly reduced SCD1 gene expression, it efficiently reversed all suppressive effects of SCDinhib in cumulus cells. These observations suggest that SCD1-dependent triglyceride accumulation and steroidogenesis in cumulus cells is mainly mediated by its metabolic product, oleic acid. It seems that a deficiency in the metabolically active form of oleic acid that reaches a critical threshold leads to impaired steroidogenesis in cumulus cells. Reduced SCD1 expression in oleic acid supplemented cumulus cells suggests the existence of a negative feedback regulation by product.

In consistent with our findings, it has been reported that steroidogenesis significantly increased with the addition of oleic acid to the maturation medium of bovine COC.[25] Treatment of bovine cumulus cells with a high concentration of oleic acid increased estradiol secretion despite an inhibition in cell proliferation.[26] In a later study, these findings were not reproduced on theca cells.[27] Moreover, in the current study, treatment with a moderate dose of oleic acid alone does not affect steroidogenesis in cumulus cells. These indicate that the effect of oleic acid is probably not only cell type specific but also dose dependent. Notably, SCD activity was induced as a downstream of FSH signaling pathway in cumulus cells that initiates primordial follicle growth and development.[9]

The paracrine coculture with SCDinhib-treated cumulus cells during the IVM showed a significant decrease in human oocyte maturation rate compared with control cumulus cells. In addition, SCDinhib pretreatment of cumulus cells abolished the enhanced fertilization capacity and early embryo development to the multicell stage with a similar trend in morula and blastocyst stages in coculture during IVM. Mimicking the follicles microenvironment, these findings suggest that SCD1 activity is involved in the cumulus cells interaction with oocyte. Although, oleate supplementation had no significant effect on the embryo development beyond the multicell stage, it efficiently restored SCDinhib induced decrease in the rate of oocytes reaching to MII and subsequent fertilization. Accordingly, a recent study reported a positive effect of oleic acid, compared to stearic acid, on murine oocyte developmental competence using a model of in vitro-cultured murine follicles.[28] Using husbands' spermatozoa for ICSI in the present study may cause variations in developmental capacity among oocytes from distinct patients.

As an adjustment, the inclusion of sibling oocytes in all experimental groups, however, minimizes this possible confounding difference. Moreover, in order to reduce the impact of interindividual variation, cumulus cells collected from 3 individuals were pooled and identical aliquots were used in each experiment.

To address any potential for variation among participants with a relatively limited number of human GV oocytes, IVM of mouse oocytes were also tested on the same coculture system. In mouse denuded GV oocytes, SCDinhib decreased maturation rate to a similar degree as intact mouse COC, indicating that SCDinhib may also target oocyte maturation directly. Consistent with previous reports,[29,30] a positive effect in maturation rate was observed with the coculture of GV oocytes with cumulus cells. However, the current study indicated that coculture in the absence of testosterone or with SCDinhib-treated cumulus cells suppressed this positive effect.

Our results suggest that the indirect effect of SCD1 function through cumulus cells is important in oocyte maturation and subsequent fertilization. In this model, the indirect effect is supposed to be mediated by changes in factors secreted locally. Importantly, active triglyceride synthesis is essential for secretion of lipoproteins by granulosa cells that are the known carriers of steroids and vitamins to developing oocyte.[31] This mechanism may be involved in SCDinhib-induced decrease in oocyte developmental capacity via an impaired monounsaturated fatty acid and subsequent triglyceride depletion. Perhaps the key implication here is that already SCD1 expression in cumulus cells surrounding human immature oocytes has been identified as a potential predictive marker of oocyte nuclear maturation.[8] Thus, SCD1 deficiency during resumption of meiosis may represent an important therapeutic target for impaired oocyte maturation.

Due to the limited availability of human primary cells, we only tested the effect of SCD1 deficiency produced by chemical inhibitors. The complete understanding of the functional role of SCD1 will require developing relevant strategies based on overexpression and gene knockdown. Testing these strategies in immortalized cumulus cell lines will overcome some limitations with primary culture. A limitation of the present study was the modest number of formed embryos. Moreover, we could not test animal embryo development because of inaccessibility to mouse ICSI facilities. For these reasons, our results of embryo development cannot be generalized or be directly extrapolated beyond the early stages. Cumulus cells in vivo act also more generally and indirectly via action in the central nervous system to alter endocrine-related reproduction function.[32] Therefore, SCD1 activity of cumulus cells may also contribute to endocrine regulation of reproduction. This point along with local in vivo effect of SCD1 in the ovary, however, remains to be examined in future animal studies.

The results presented here suggest that SCD1 activity is required for cumulus cells viability and their triglyceride storage. In addition, induction of oocyte maturation in coculture and possibly its early developmental capacity is dependent on SCD1 activity in cumulus cells, which can be mediated by lipid-mediated paracrine support and estradiol production. Involvement of cumulus cell SCD1 activity in normal oocyte maturation may be of relevance in reproductive disorders, particularly in the pathological mechanism of impaired oogenesis.

Authors' Note

All authors participated in protocol development, data analysis, and writing and editing the manuscript. All authors have approved the final article. *Guideline/law on the care and use of laboratory animals*: This study was conducted according to the National Institutes of Health and the institutional ethical guidelines. Type of study: Experimental study of primary culture.

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Supplemental Material

The online Supplemental Table 1 is available at http://journals.sagepub.com/doi/suppl/10.1177/1933719117698578.

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