$ROR\alpha$ inhibits adipocyte-conditioned medium-induced colorectal cancer cell proliferation and migration and chick embryo chorioallantoic membrane angiopoiesis

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¹Beijing Key Laboratory of Gene Resource and Molecular Development and College of Life Sciences, Beijing Normal University, Beijing, People's Republic of China; ²Cardiovascular Research Center, School of Medicine, Xi'an Jiaotong University, Xi'an, China; ³The Second Artillery General Hospital of Chinese People's Liberation Army, Beijing, China; and ⁴Institute of Aging Research, Hangzhou Normal University, Hangzhou, China

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Xiao L, Wang J, Li J, Chen X, Xu P, Sun S, He D, Cong Y, Zhai Y. RORa inhibits adipocyte-conditioned medium-induced colorectal cancer cell proliferation and migration and chick embryo chorioallantoic membrane angiopoiesis. Am J Physiol Cell Physiol 308: C385-C396, 2015. First published December 10, 2014; doi:10.1152/ajpcell.00091.2014.-Lipid metabolic disturbances are related to many diseases, such as obesity, diabetes, and certain cancers. Notably, lipid metabolic disturbances have been reported to be a risk factor for colorectal cancer. Nuclear receptors act as ligand-dependent transcription regulators and play key roles in the regulation of body lipid metabolism and the development of many cancers. Retinoic acid receptor-related orphan receptor α (ROR α) is a nuclear receptor and can regulate several lipid metabolism genes in certain cancers. Herein, we demonstrate that the conditioned medium from adipocytes has a proproliferative and promigratory effect on colorectal cancer cells and enhances angiogenesis in chicken embryonic chorioallantoic membranes. In addition, the conditioned medium leads to a decrease in the expression of $ROR\alpha$ and its target genes. Meanwhile, ROR α and its target gene expressions are lower in human colorectal tumor tissue compared with control colorectal tissue. Activation of RORa inhibits the effect of conditioned medium on the proliferation and migration of colorectal cancer cells as well as the angiogenesis in chicken embryonic allantoic membranes. In colorectal cancer cells, the putative ligand of ROR α , cholesterol sulfate (CS), prevents cell cycle progression at the G1/S boundary and concurrently modulates the expression of cell cycle-regulatory genes in colorectal cancer cell. CS inhibits angiogenesis in chicken embryonic chorioallantoic membranes and concurrently decreases the mRNA expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1a as well as the secretion of VEGF. In addition, lipogenic gene expression is higher in human colorectal tumor tissue compared with control colorectal tissue. CS inhibits the expression of lipogenic genes in colorectal cancer cells. These results suggest that RORa could represent a direct link between local lipid metabolism of colorectal tissue and colorectal cancer. Therefore, the reduction of the expression of ROR α could represent a potential warning sign of colorectal cancer.

colorectal cancer; retinoic acid receptor-related orphan receptor α ; lipid metabolism

COLORECTAL CANCER IS one of the most prevalent malignancies in the world (35), with more than 1,000,000 new cases of colorectal cancer diagnosed each year (39). Numerous factors, including genetic abnormalities and environmental factors, have been implicated in the etiology of colorectal cancer (37). Recently, increasing evidence suggests that a lipid metabolic disturbance is a critical risk factor in the occurrence and development of colorectal cancer (5). Adipose tissue is the main organ in lipid metabolic homeostasis. Until recently, adipocytes were mainly considered as an energy storage depot, but there is now clear evidence suggesting that adipocytes act as endocrine cells, producing hormones, growth factors, cytokines, and other molecules named adipocytokines (36). Notably, increased adipose tissue mass provokes adipocytokine dysregulation, which results in metabolic complications, such as type 2 diabetes, and certain cancers, such as colorectal cancer (21). Taken together, these compelling observations suggest that excess adiposity may favor tumor invasiveness via a yet uncharacterized mechanism(s). However, the link between the local lipid metabolism of colorectal tissue and the development of colorectal cancer remains unclear.

3T3-L1 is an immortalized cell line that is committed to the adipocyte lineage (14–16). In the presence of a hormonal cocktail consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), and insulin (INS), 3T3-L1 preadipocytes differentiate into adipocyte-like cells, expressing adipocyte-specific genes, accumulating triacylglycerol-rich lipid droplets, and secreting several adipocytokines (9). Herein, we used the conditioned medium of 3T3-L1 cells, which contain similar factors to the adipocytokines in the surrounding colorectal tumor milieu, to explore the link between the local lipid metabolism of colorectal tissue and the development of colorectal cancer (40, 42).

Members of the orphan nuclear receptor family play various roles in signal integration, including the modulation of neurogenesis, homeostasis, and disease by regulating subsets of gene expression patterns both positively and negatively (13, 24). The retinoic acid-related orphan nuclear receptor α (ROR α) is a member of the orphan nuclear receptor family and can functionally interact with potent oncogenic systems (34). Compared with other classes of nuclear receptors, the function and related signaling pathways in the development of colorectal cancer for ROR α have not yet been studied extensively. Meanwhile, the effects of ROR α on colorectal cancer cell proliferation, migration, and angiopoiesis are largely unclear.

In this study, we investigated the effect of the conditioned medium of 3T3-L1 on the proliferation and migration of

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colorectal cancer cells and revealed that the conditioned medium promoted the proliferation and motility of colorectal cancer cells and significantly enhanced the formation of blood vessels (BVs) in chicken embryonic chorioallantoic membranes (CAMs) in vitro. Next, we demonstrated that the conditioned medium led to a decrease in the expression of ROR α . In addition, activating ROR α with cholesterol sulfate (CS), putative ligand of ROR α , inhibited the proliferation and motility of colorectal cancer cells, which was induced by conditioned medium via the regulation of cell cycle-regulatory molecules. Furthermore, CS inhibited the formation of BVs in chicken embryonic CAMs via the regulation of the vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF-1 α) signaling pathway. The expression of lipogenic genes was elevated in colorectal tumor tissue compared with control colorectal tissue, and CS inhibited the expression of lipogenic genes in colorectal cancer cells.

MATERIALS AND METHODS

Reagents. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), TO-901317, CS, IBMX, DEX, and INS were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). TRizol, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and an antibiotic mixture (penicillin-streptomycin) were purchased from GIBCO-BRL (Grand Island, NY). The BCA-100 protein quantitative analysis kit was purchased from Pierce (Rockford, IL). ROR α rabbit polyclonal antibody, β -actin rabbit polyclonal antibody, and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein molecular weight markers were obtained from Pharmacia (Saclay, France). The polyvinyldifluoride (PVDF) membrane for Western blot analysis was purchased from Millipore (Bedford, MA).

Cell culture and preparation of conditioned medium. The human colon cancer SW480 cells were generously provided by Dr. Junjie Zhang (Beijing Normal University, College of Life Science). The mouse colon carcinoma cell line C26 and the murine 3T3-L1 preadipocyte cell line was purchased from the Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). SW480 and C26 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO2 atmosphere. Assays were conducted with cells harvested at 80-90% confluence. 3T3-L1 preadipocytes were cultured in DMEM containing 10% FBS. To induce 3T3-L1 cell differentiation, 2-day postconfluent preadipocytes were cultured in DMEM containing 10% FBS, 10 mg/ml INS, 0.5 mmol/l DEX, and 0.5 mmol/l IBMX for 2 days. Cells were then cultured in DMEM supplemented with 10% FBS and 10 mg/ml INS for an additional 2 days, after which cells were refed every other day with DMEM containing 10% FBS. After 8 days, the cells fully differentiated. During the differentiation of 3T3-L1, the entire replacement medium during 8-day differentiation (days 2, 4, 6, and 8 postdifferentiation) of 3T3-L1 was collected every time and then mixed together and filtered via a 0.22-µm filter. This medium was used as the supernatant medium (termed mCM).

Tissue samples. Samples of human colon cancer and adjacent grossly normal-appearing tissue were obtained at the time of surgery from four patients undergoing colon surgical resection at the general hospital of the People's Liberation Army (PLA) Second Artillery Corps. Control specimens were collected from the accompanying normal mucosa 5–10 cm distal to the carcinoma. Informed consent was obtained from each subject before surgery. The tissue was cut into small pieces and either snap-frozen in liquid nitrogen and stored at -80° C or fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at 4°C for histological analysis.

Oil Red O staining. For the Oil Red O staining, the cells were washed three times with PBS and fixed with 3.7% formaldehyde for 1 h. Oil Red O (0.5% in isopropanol) was diluted with water (3:2), filtered two times with a 0.45-µm filter, and added to the fixed cells for 15 min at room temperature (RT). Cells were washed with 70% ethanol and water before visualization via light microscopy and photographed.

Quantitative real-time PCR analysis. Total RNA was isolated with TRizol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed to cDNA using Moloney murine leukemia virus transcriptase (Promega, Madison, WI) and oligo(dT)₁₈ primer (Takara, Shuzo, Japan). cDNA were subsequently subjected to SYBR Green-based real-time PCR using an ABI 7500 real-time PCR System (Applied Biosystems, Alameda, CA). Primers used in real-time PCR are shown in Table 1. All results were obtained from at least three independent experiments. The gene expression was normalized against the expression of cyclophilin B (15).

Western blot analysis. The protein expression of ROR α was determined via Western blot analysis. Tissues or cells were harvested with cell lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing freshly added protease inhibitor cocktail (Sigma). Fifty micrograms of protein per lane were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. Protein detection was performed with anti-ROR, anti- β -actin, and horseradish peroxidase-conjugated secondary antibodies. Immuno-complexes were detected with enhanced chemiluminescence detection reagent (Millipore). Protein band densities were analyzed using the spot density analysis software (ImageJ software). Values were normalized by β -actin.

Chick embryo CAM assay. The chick embryo CAM assay was performed as previously described (4). Three-day-old fertilized white eggs were cracked, and chick embryos with intact yolks were carefully placed in 100-mm plastic petri dishes. After 24 h of incubation at 37° C with 60% humidity, filter paper (5 mm in diameter) containing various volumes of CM dried on plastic petri dishes (60 mm) was implanted on the CAM of individual embryo avascular zones. After 72 h of incubation, embryos and CAMs were photographed with a Nikon digital camera, and the area of newly formed vessels oriented toward the substances on the filter paper (1 cm² in diameter) was counted using image pro plus 5.0 software by two observers in a double-blind manner. Assays for each test sample were carried out using four to six eggs.

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was performed using a commercial VEGF ELISA kit (R&D Systems). Assays were performed in duplicate, and readings were compared with standard curves obtained with standard proteins provided with the kits.

Transfection of small-interfering RNA duplexes. The small-interfering RNA (siRNA) duplexes targeting all isoforms of human ROR α and mouse ROR α as well as control nonspecific siRNA were synthesized and purified by Genepharma. SW480 and C26 cells were transfected with siRNAs using the Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol. The oligonucleotide sequences used in siRNA assays are listed in Table 2.

Overexpression transfection assays. In overexpression experiments, SW480 or C26 cells were transfected by p3xFLAG-CMV10-ROR α (ROR α) or control p3xFLAG-CMV-10 (p3X) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After 72 h, they were assessed by quantitative real-time PCR and Western blot analysis.

Cell viability test (MTT assay). To determine cell viability, cells $(5 \times 10^3/\text{well})$ were seeded in sterile 96-well tissue culture plates and treated as indicated. Cell viability was evaluated in each well via the addition of 20 µl of MTT (2.5 mg/ml in PBS) 4 h before the reading. For analysis, the cell-free supernatants were removed completely from each well, and 100 µl of DMSO were added. The optical densities of

Table 1. Primers used for real-time PCR

Gene	Species	Sequence (5'-3')			
LXR	Human	Forward: 5'-CCCTTCAGAACCCACAGAGATC-3'			
		Reverse: 5'-GCTCGTTCCCCAGCATTTT-3'			
PPARγ	Human	Forward: 5'-CAGGAAAGACAACAGACAAATCA-3'			
		Reverse: 5'-GGGGTGATGTGTTTGAACTTG-3'			
FABP4	Human	Forward: 5'-CCTTTAAAAATACTGAGATTTCCTTCA-3'			
		Reverse: 5'-GGACACCCCCATCTAAGGTT-3'			
	Mouse	Forward: 5'-AGCATCATAACCCTAGATGGCG-3'			
		Reverse: 5'-CATAACACATTCCACCACCAGC-3'			
C/EBPa	Human	Forward: 5'-AGCAGGAGAAGGCCAAGG-3'			
		Reverse: 5'-CCCGGGTAGTCAAAGTCG-3'			
RORα	Human	Forward: 5'-CTCCGCACCGCGCTTAAAT-3'			
		Reverse: 5'-TTGTCTCCACAGATCTTGCATGG-3'			
	Mouse	Forward: 5'-AGGCAGAGCTATGCGAGC-3'			
	110450	Reverse: 5'-TCAAACAGTTCTTCTCGACGAGG-3'			
SCD-1	Human	Forward: 5'-CTGCCCCTACGGCTCTTT-3'			
565 1	Tumun	Reverse: 5'-CCTCCCCCAATTATCACCATCA-3'			
	Mouse	Forward: 5'-CCCCACACCCCTTACATCCA-3'			
	Wiouse	Reverse: $5'$ -TACCCTCTAAAACATTTCTCCAAAACC_3'			
ACC	Humon	Forward: 5' TETTECTECACCCCAATE 3'			
ACC	Tuman	P everse: $5'$ TOTATOOTOOTOOTOOTOOTOOTOOTOOTOOTOOTOOTOO			
	Mouse	Forward: 5' TCACACACACTCATCCCACACA 3'			
	Wiouse	Polyana, 5' TECACACCCCCACACACACA			
	Humon	Forward: 5' TOTAGAGOOOOAAAAAA			
ADKP	numan	POIWARD: 5 - ICITICOIGCAGUCUAAIG-5			
-21	11	Reverse: 5 - TGTUAGUUTUTGTTUUAAUTG-5			
p21	Human	Porward: 5 -TGGAGAGTUTUAGGGTUGAAA-5			
	Manag	Reverse: 5 -UGGUGTTTGGAGTGGTAGA-5			
	Mouse	Forward: 5'-TUCACAGUGATATUCAGAGA-3'			
		Reverse: 5 - CAGGGCAGAGGAAGTACTGG-3			
Cyclin D1	Human	Forward: 5'-AATGACCCCGCACGATTTC-3'			
		Reverse: 5'-TCAGGTTCAGGCCTTGCAC-5'			
	Mouse	Forward: 5'-CATCAAGTGTGACCCCGGACTG-3'			
		Reverse: 5'-CCTCCTCCTCAGTGGCCTTG-3'			
ApoC3	Human	Forward: 5'-CGAGCTTCAGAGGCCGAG-3'			
		Reverse: 5'-GGTCTTGGTGGCGTGCTT-3'			
	Mouse	Forward: 5'-GTACAGGGCTACATGGAACAA-3'			
D 14		Reverse: 5'-TATCGGACTCCTGCACGCTACT-3'			
Bmall	Human	Forward: 5'-TGTGGGCGCTCACTGTGT-3'			
		Reverse: 5'-TTCTGCCTGATCCTGTCATCTCT-3'			
	Mouse	Forward: 5'-GCAGTGCCACTGACTACCAAGA-3'			
		Reverse: 5'-TCCTGGACATTGCATTGCAT-3'			
HIF-1a	Human	Forward: 5'-CCATTAGAAAGCAGTTCCGC-3'			
		Reverse: 5'-TGGGTAGGAGATGGAGATGC-3'			
	Mouse	Forward: 5'-CGGCGAAGCAAAGAGTCTGAAGT-3'			
		Reverse: 5'-TCGCCGTCATATGTTAGCACCAT-3'			
VEGF	Human	Forward: 5'-GGAGTGTGTGCCCACCGAGGAGTCCAAC-3'			
		Reverse: 5'-GGTTCCCGAAACCCTGAGGGAGGCT-3'			
	Mouse	Forward: 5'-GGAGATCCTTCGAGGAGCACTT-3'			
		Reverse: 5'-GGCGATTTAGCAGCAGATATAAGAA-3'			
Cyclophilin B	Human	Forward: 5'-TTTCATCTGCACTGCCAAGA-3'			
		Reverse: 5'-TTGCCAAACACCACATGCT-3'			
	Mouse	Forward: 5'-GGAGATGGCACAGGAGGAA-3'			
		Reverse: 5'-GCCCGTAGTGCTTCAGCTT-3'			

LXR, liver X receptor; PPAR γ , peroxisome proliferator-activated receptor- γ ; FABP4, fatty acid-binding protein-4; C/EBP α , CAAT/enhancer-binding protein α ; ROR α , retinoic acid receptor-related orphan receptor α ; SCD-1, stearoyl-CoA desaturase-1; ACC, acetyl-CoA carboxylase; ADRP, adipose differentiation-related protein; HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor.

the wells were measured using a spectrophotometric multiwell microplate reader (Multiskan MS; Thermo Electron, Waltham, MA) at a wavelength of 570 nm.

Cell count. Harvested cells were washed with 1× PBS and mixed with trypan blue dye, and the number of viable cells was determined manually using a hemocytometer. Briefly, a suspension of $\sim 1 \times 10^6$ cells/ml was prepared, and a 1:1 mixture of the cell suspension and the 0.4% trypan blue solution was made. The solution was gently mixed and allowed to stand for 5 min at RT. Cell suspension (15 µl) was applied to the edge of the chamber between the cover slip and the V-shaped groove in the chamber. The cell suspension was drawn into

the chamber by capillary action and then allowed to sit for 1-2 min and counted.

Cell cycle analysis. At 48 h after induction, SW480 and C26 cells were harvested and fixed with 75% cold ethanol. The fixed cells were stained with propidium iodide (Calbiochem, San Diego, CA), and the cell cycle distribution was determined using a FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Transwell assay. A Transwell system (8 μ m pore size; Corning, Corning, NY) was used to evaluate the cell migration. First, a 100- μ l cell suspension (1 × 10⁶ cells/ml) in FBS-free medium was added to the upper compartment while 500 μ l of growth medium (GM),

Table 2. Oligonucleotide sequences used in siROR α assays

Species	Sequence (5'-3')		
Human	5'-CGCUGCCAACACUGUCGAUUATT-3' 5'-UAAUCGACAGUGUUGGCAGUGTT-3'		
Mouse	5'-GAGCUCCAGCAGAUAACGUTT-3' 5'-ACGUUAUCUGCUGGAGCUCTT-3'		

adipocyte-conditioned medium (mCM), or mCM with CS was placed in the lower compartment. The total number of cells that migrated into the lower chamber was counted after 48 h of incubation at 37° C with 5% CO₂. The filters were fixed with methanol and stained with 0.1% Giemsa. After being gently rinsed with water, the cells on the upper surfaces of filters were removed by wiping the filters with a cotton swab. Cells remaining on the lower surface of the filters were photographed under a microscope. The number of migrated cells was counted with 10 randomly selected fields. Experiments were performed in triplicate.

Hematoxylin and eosin staining. Tumor and normal colon tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections $(4-5 \ \mu m)$ were prepared and stained with hematoxylin and eosin according to standard protocol.

Immunohistochemical staining. Human colon sample paraffin-embedded sections of 5 μ m thickness were obtained from the Second Artillery General Hospital of Chinese PLA. For villin staining, paraffin-embedded tissues were first deparaffinized in xylene for 10 min, followed by 5 min each in serial dilutions of ethanol (100, 95, and 75%). After antigen retrieval, sections were incubated for 1 h at RT with antivillin antibody at then counterstained with hematoxylin, dehydrated, and mounted. Negative control studies were done by incubating samples without the primary antibody.

Statistical analysis. The data were presented as means \pm SE. The significance of paired data was determined by Student's *t*-test. Data with more than two groups were analyzed by ANOVA followed by the post hoc Dunnett's multiple-comparison test. Differences were considered significant at P < 0.05 and 0.01.

RESULTS

The distribution of adipocytes in human colorectal cancer tissue is elevated compared with normal colorectal tissue. Many studies in lipid metabolism and cancer have shown that an obvious correlation between lipid metabolism disorders and cancer exists. However, whether the local lipid metabolic disorder induces colorectal cancer has not been reported. To explore the relationship of local colorectal lipid metabolism disorders and colorectal cancer, we analyzed the fat cell distribution in the colorectal tissue, since the fat tissue is an essential organ in lipid metabolic homeostasis. Histological analysis confirmed the presence of adipocytes (Bodipy staining, green) surrounding the colorectal cancer in a human patient sample (Fig. 1, A and B). Figure 1C showed the villin staining of colorectal cancer tumor cells surrounding the adipocytes in the human samples. In addition, detailed patient information, including body weight, body mass index, cholesterol, low density lipoprotein, high density lipoprotein, and triglyceride level, is included in Table 3. These observations suggest that adipocytes are clinically relevant cells that may provide a physiological basis for the occurrence of colorectal cancer. Here, we investigated the possible association of adipocytes with colorectal cancer.

Colorectal cancer cells cultured with mCM exhibit an enhanced proliferative and motile phenotype. The results in Fig. 1 suggest that the occurrence of colorectal cancer may be

linked to fat cells. Because adipose tissue is a functional part of the endocrine system, and because of the increased number and size of adipocytes, colorectal cancer tissue will have a higher level of secreted adipocytokines. Previous studies reported the secretion profiles during adipocyte differentiation involving a complex and dynamic process, mostly adipokines, such as adiponectin and other inflammation-related molecules, exhibit a temporally ordered pattern during differentiation (41, 42). To better define how adipocytokines in local colorectal tissue may induce colorectal cancer, we collected the entire replacement medium during 8-day differentiation (days 2, 4, 6, and 8 postdifferentiation) of 3T3-L1 and mixed them together, termed mCM (as mentioned in MATERIALS AND METHODS). Next, we cultured colorectal cancer cells (SW480 and C26) with mCM for 24 or 48 h. Upon incubation of SW480 and C26 cells with mCM, the proliferation of the cells was greatly increased (Fig. 2A) compared with GM. In addition, cell migration is an additional critical step in tumor metastasis (6). Thus, we determined the effect of mCM on cell migration. When cell motility was examined using a standard Transwell assay (28), microscopy (Fig. 2B) and quantification (Fig. 2C) of migrated cell numbers showed that more numbers of mCM-treated SW480 and C26 cells than GM-treated cells migrated toward the bottom chamber in a time-dependent manner. Tumor progression is closely linked with microvessel formation (2). Overgrowth of BVs may lead to the development and progression of tumor growth (3, 20, 22, 27). The avian egg offers an efficient model to assess new compounds and scaffolds, particularly on the avian CAM, a well-known site of experimentation. Therefore, we examined whether mCM could affect the progress of angiogenesis using a chick CAM assay. As demonstrated in Fig. 2D, relative to GM, the mCM-treated CAM significantly enhanced the formation of BVs in vitro, and the tube area increased three times as shown in Fig. 2E. Both obesity and a high-fat diet have been proposed to be critical risk factors for the occurrence and development of colorectal cancer. Consistent with these reports, the results of our study, as demonstrated in Figs. 1 and 2, suggest that the adipocytokines that are secreted by adipocytes in the local colorectal tissue have an important function in the proliferation and migration of colorectal cancer cells and the angiogenesis observed in tumor formation and growth.

mCM decreases the expression of ROR α in colorectal cancer cells. ROR α , a member of the orphan nuclear receptor family, functions in lipid metabolism and certain cancers. ROR α is expressed in many tissues in mammals, including cerebellar Purkinje cells, liver, thymus, skeletal muscle, skin, lung, and kidney (17, 38). We examined the expression of ROR α in human tissue samples. Western blot and real-time PCR analyses revealed that the expression levels of $ROR\alpha$ protein and mRNA were markedly lower in colorectal cancer compared with control colorectal tissue in humans (Fig. 3, A-C). Based on the different expression patterns of ROR α between the human colorectal tumor tissue and control colorectal tissue, we concluded that ROR α might function as a warning sign for local colorectal cancer. Subsequently, we determined the expression of the ROR α target genes, ApoC3 and Bmal1, in colorectal tissues. Real-time PCR analysis revealed that the expression patterns of these genes were similar to those observed for ROR α in human colorectal cancer tissue as well as normal tissue distant from the tumor (Fig. 3C).



Fig. 1. Tumor cells and surrounding adipocytes in human colorectal cancer tissue. Representative Bodipy staining lipid droplets (LD, green) in human colorectal cancer tissue (*A*) and hematoxylin and eosin staining after immunofluorescence staining show the tumor and adipocytes in situ (*B*). *C*: villin staining of colon tumor cells (brown staining; arrow) and tumor surrounding adipocytes (arrow) in the human samples. Scale bar, 100 µm.

To further examine the role of ROR α in the development of colorectal cancer, we determined whether mCM modulated the expression of ROR α and its target genes. Real-time PCR analysis revealed that the expression levels of ROR α , ApoC3, and Bmal1 were decreased in both SW480 and C26 cells upon culture in mCM compared with culture in control medium (GM) (Fig. 3D). These results provide a basis for the study of the role of ROR α in the secretion of the adipocytokines linked to colorectal cancer. Importantly, nuclear receptors are major

targets for drug discovery and have key roles in cell development and homeostasis, as well as in many diseases such as obesity, diabetes, and cancer. Therefore, we chose ROR α as a potential target to test whether it has a role in antitumorigenesis.

CS (a putative ROR α ligand) inhibits proliferation and motility in colorectal cancer cells. Based on the results in Fig. 3, we hypothesized that decreasing levels of ROR α are associated with the development of colorectal cancer. To investi-

Table 3. Patient characteristics

General				Lipid Profile				
Patient No.	Age, yr	Gender	Body Weight, kg	BMI, kg/m ²	CHOL, mM	LDL, mM	HDL, mM	Triglycerides
1	84	М	65	28.9	4.67	3.1	0.94	1.24
2	68	М	75	24.2	4.82	3.36	0.84	0.95
3	55	М	52	22.2	4.63	3.02	0.78	1.98
4	58	М	78	26.1	5.63	3.99	1.12	1.16

M, male; BMI, body mass index; CHOL, cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein.



Fig. 2. Adipocyte conditioned medium (mCM) increases proliferation and motility of SW480 and C26 cells and angiogenesis in chicken embryonic chorioallantoic membranes (CAMs). A: time-dependent effect of mCM on the growth of SW480 and C26 cells. Viable cells were counted at each time point of incubation with growth medium (GM) or mCM. A value of 100% was assigned for the cell number before incubation with GM or mCM. B: time-dependent effect of mCM on the motility of SW480 and C26 cells. C: quantitative Transwell migration assay. The y-axis represents the no. of migrating cells. Data are the average number of cells that migrated in a representative experiment, measured in triplicate and presented as means \pm SE (magnification, $\times 200$) (*P < 0.05 and **P < 0.01 vs. GM). D: representative photographs of filter paper soaked with the indicated substance (n = 4) and surrounding CAMs. E: quantification of the area of newly formed blood vessels (**P < 0.01 vs. PBS group).

gate the roles of ROR α in the development of colorectal cancer, we examined whether pharmacological activation of ROR α affects the growth of colorectal cancer cells. Thus, we treated SW480 and C26 cells with mCM alone or mCM in the presence of different concentrations of CS (a putative RORa ligand, 5 and 10 µM) and examined the cell growth. As demonstrated in Fig. 4A, when SW480 and C26 cells were incubated with mCM and CS for 48 h, the proliferation of SW480 cells was greatly decreased in a dose-dependent manner. Treatment with CS at concentrations of 1-100 µM had little impact on the SW480 and C26 viability, indicating that CS inhibited cell proliferation at these concentrations without affecting cell viability (Fig. 4B). Meanwhile, when SW480 and C26 cells were grown in the presence of mCM, a significant stimulation of tumor motile abilities was observed compared with cells grown in GM. When SW480 and C26 cells were grown in the presence of mCM with different concentration of CS, fewer cells migrated toward the bottom chamber compared with cells treated with mCM alone (Fig. 4, C and D). The

observation that CS perturbed the colorectal cancer cell proliferation led us to examine whether CS might modulate cell cycle progression in colorectal cancer cells. SW480 and C26 cells were incubated with mCM and different concentrations of CS, and the cell cycle states were analyzed using flow cytometry. At 48 h after induction, 45.68% of mCM-treated SW480 cells entered the S phase, whereas only 39.99% of 5 µM and 34.44% of 10 μ M CS-treated SW480 cells entered the S phase. Meanwhile, at 48 h after induction, 39.28% of mCM-treated C26 cells entered the S phase, whereas only 36.08% of 5 μ M and 33.83% of 10 µM CS-treated C26 cells entered the S phase. Therefore, compared with cells treated with mCM alone, SW480 and C26 cells treated with mCM in addition to CS exhibited a larger portion of the population in the G_1 phase and a smaller fraction of the population in the S phase in a dose-dependent manner (Fig. 4, E and F). Thus, ROR α induced cell cycle arrest at the G₁/S boundary in colorectal cancer cells. Because CS arrested cell cycle progression at the G_1 phase in colorectal cancer cells, we asked the question

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Fig. 3. mCM decrease the expression of retinoic acid receptor-related orphan receptor a (ROR α) and the target genes ApoC3 and Bmal1 in colorectal cancer cells. A: Western blot analysis of RORa expression in human colorectal adenocarcinoma tissues (C) and adjacent normal (N) tissues. B: RORa levels were normalized to the amount of β -actin. C: real-time PCR results evaluating mRNA levels of RORa, ApoC3, and Bmal1 expression in human colorectal adenocarcinoma tissues and adjacent normal tissues. D: real-time PCR analysis to measure relative mRNA expression of RORa, ApoC3, and Bmal1 in mCM-treated SW480 and C26 cells. The real-time PCR data are representative of 3 independent experiments. The blot shown is representative of 4 independent experiments. Data are means ± SE. The results are from 3 independent experiments. *P < 0.05 and **P < 0.01 vs. normal control (GM).

whether CS might affect the expression of cyclin-dependent kinase inhibitors, thus resulting in G_1/S arrest. As shown in Fig. 4G, CS substantially promoted the expression of p21 and decreased cyclin D1 mRNA in SW480 and C26 cells. These data suggest that p21 and cyclin D1 are likely targets for ROR α in the inhibition of cell proliferation and cell cycle arrest since they induce quiescent cells to progress through the cell cycle.

Angiogenesis is a key step in tumor growth, invasion, and metastasis. Thus, the antiangiogenic therapy was postulated to be an attractive approach for antitumor treatment (11). To examine the ability of CS to inhibit angiogenesis, we used a CAM assay. Three days after hatching, the BVs on the slides were counted. As shown in Fig. 4*H*, relative to the mCMtreated group, CS significantly inhibited the formation of BVs. In addition, SW480 and C26 cells also displayed a downregulation of VEGF and HIF-1 α mRNA expression in mCM with CS compared with mCM treatment alone (Fig. 4*I*). ELISA results demonstrated that CS treatment inhibited the secretion of VEGF in SW480 and C26 cells (Fig. 4*J*).

Genetic ROR α inhibits proliferation and motility in colorectal cancer cells. To investigate whether genetic activation of ROR α also affects the growth of colorectal cancer cells, we overexpressed ROR α and knocked down ROR α in SW480 and C26 cells, respectively.

Figure 5A suggested we have succeeded in knocking down and overexpressing ROR. As shown in Fig. 5B, when SW480 cells knocked down ROR α , 41.09% of SW480 cells entered the S phase, whereas only 36.69% of negative control SW480 cells entered the S phase. When SW480 cells overexpressed ROR α , 26.66% of SW480 cells entered the S phase, whereas 31.37% of vehicle control SW480 cells entered the S phase. ROR α in SW480 cells were knocked down, the mRNA expression of p21 was decreased, and the mRNA expression of cyclin D1 was increased (Fig. 5*C*). In contrast, overexpression of ROR α increased the expression of p21 mRNA and decreased the mRNA expression of cyclin D1 (Fig. 5*D*). Accordingly, RNAi knockdown of ROR α significantly increased tumor motile abilities. Overexpression of ROR α inhibited the tumor motile abilities (Fig. 5*E*).

CS decreases the expression level of key transcriptional regulators of angiogenesis. Several lipogenic genes, including sterol-regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS), are upregulated in certain obesitylinked cancers, such as breast, prostate, endometrial, and colorectal (1, 8, 10, 23, 43). Consistent with these reports, the levels of lipogenic genes, such as liver X receptor α , SREBP-1c, peroxisome proliferator-activated receptor γ , fatty acid-binding protein-4 (aP2/FABP4), stearoyl-CoA desaturase-1 (SCD-1), acetyl-CoA carboxylase (ACC), CAAT/enhancer-binding protein α , and adipose differentiation-related protein were elevated in human colorectal cancer tissues compared with normal tissues from the same colon cancer patients (Fig. 6A). To explore the molecular mechanism through which CS inhibits angiogenesis in chicken embryonic CAM, we induced 3T3-L1 to differentiate via incubation in differentiation medium (DM) in the presence or absence of CS. 3T3-L1 cells



Fig. 4. Cholesterol sulfate (CS) inhibits the proliferation and motility of SW480 and C26 cells and angiogenesis in chicken embryonic chorioallantoic membrane. A: SW480 and C26 cells were incubated with GM, mCM, or mCM with CS (5 and 10 μ M) for 48 h. The dose-dependent effect of CS on the growth of SW480 and C26 cell data is shown as percentages of the GM-treated control, set as 100% (*P < 0.05 and **P < 0.01 vs. mCM). B: SW480 and C26 cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. C: dose-dependent effect of CS on the motility of SW480 and C26 cells using Transwell assay. D: quantification of 10 randomly selected fields shown in C (*P < 0.05 vs. mCM). E and F: CS induces cell cycle arrest at the G₁ phase in SW480 and C26 cells. Flow cytometric analysis of cell cycle regulation by ROR α in SW480 and C26 cells. SW480 and C26 cells were incubated with GM, mCM, or mCM with CS (5 and 10 μ M) for 48 h. After incubation, the cells were collected and stained with propidium iodide for flow cytometric analysis. G: CS modulates the expression of cell cycle-regulatory genes in colorectal cancer cells. SW480 and C26 cells were incubated with CS for 48 h. Real-time PCR analysis to measure the relative mRNA expression levels of p21 and cyclin D1. The values shown are expressed as means \pm SE of 3 independent experiments. *P < 0.05 and **P < 0.01 vs. CM group. H: CS inhibited chicken embryonic chorioallantoic membrane microvessel formation. Representative photographs of fliter paper soaked with the indicated substance (n = 4) and surrounding CAMs. Quantification of the area of newly formed blood vessels (**P < 0.01 and *P < 0.05 vs. mCM). I: real-time PCR analysis to measure the relative mRNA expression levels of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF)-1 α in SW480 and C26 cells. (*P < 0.05 and **P < 0.01 vs. mCM). J: SW480 and C26 cells were incubated with GM, mCM, or mCM with CS for 48 h. Cell supernatants were prepare

were maintained in DMEM containing 10% FBS. To induce differentiation, 2-day postconfluent preadipocytes were cultured in DMEM containing 10% FBS, 10 mg/ml INS, 0.5 mmol/l DEX, and 0.5 mmol/l IBMX (termed DM) for 2 days. The medium was then replaced with DMEM containing INS, and the cells continued to differentiate until *day* 8 (still termed DM). On *day* 8 after induction, the cells were stained with the lipophilic dye Oil Red O to demonstrate triacylglycerol accumulation. DM-treated cells resembled differentiated adipocytes and accumulated triacylglycerol as expected. In contrast, CS-treated cells had a reduced level of Oil Red O staining, indicating that CS reduced lipid accumulation in the cells (Fig. 6*B*). Thus, adipogenesis is tightly associated with angiogene-

sis, as shown by the findings that adipose tissue explants trigger BV formation, whereas adipose tissue endothelial cells promote preadipocyte differentiation (7). As shown above, angiogenesis can be enhanced by mCM. The expression levels of VEGF and HIF-1 α were examined during adipocyte differentiation in the presence and absence of CS because of their known proangiogenic functions. Notably, CS treatment reduced the mRNA expression level of VEGF and HIF-1 α in a dose-dependent manner in 3T3-L1 cells (Fig. 6C). Furthermore, CS treatment also reduced the secretion of VEGF in a dose-dependent manner (Fig. 6D). Thus, the suppression of VEGF and HIF-1 α expression in adipocytes and colorectal cancer cells might reduce microvessel formation in tumor



Fig. 5. Effects of ROR α overexpression and knockdown for cell proliferation and migration in SW480 cells. *A*: protein level of ROR α analyzed by Western blotting 48 h after transfection of siROR α or p3xFLAG-CMV10-ROR α and quantification of ROR α protein levels, which were normalized to the amount of β -actin. Data represent means \pm SE of the no. experiments in each group. **P* < 0.05 compared with control. *B*: after transfection of siROA α or p3xFLAG-CMV10-ROR α (ROR α), the SW480 cells were collected and stained with propidium iodide for flow cytometric analysis. *C* and *D*: real-time PCR analysis to measure the relative mRNA expression levels of p21 and cyclin D1 in siRNA (*C*) or p3xFLAG-CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -CMV10-ROR α (ROR α) SW480 cells (*D*). *E*: representative images of migratory cells in siRNA or p3xFLAG-CMV10-ROR α -transduced SW480 cells and the corresponding quantification mentioned above (**P* < 0.05 compared with the respective negative control). All experiments were performed 3 times.

tissues. We examined whether CS was able to suppress the expression of lipogenic genes in colorectal cancer cells. In SW480 and C26 cells, CS decreased the mRNA expression of ACC, SCD-1, and FABP4 (Fig. 6*E*). Meanwhile, SW480 cells also displayed a reduction of VEGF and HIF-1 α mRNA level in overexpressed ROR α and significant upregulations of the expression of VEGF and HIF-1 α mRNA level when ROR α was knocked down(Fig. 6, *F* and *G*).

DISCUSSION

The dysregulation of adipocytokines in adipose tissue is involved in several pathological conditions, including diabetes and certain cancers. We examined the presence of adipocytes in human colorectal tissue and demonstrated a significant increase in both the number and size of the adipocytes in colorectal cancer tissue compared with normal colorectal tissue. In the present study, using the 3T3-L1 cells as a model of adipocytes, we created conditioned medium (mCM) to imitate the secretion of adipocytokines from the adipocytes to mimic the natural condition surrounding colorectal tissue. Considering the complexity of adipocyte tissue (different stage adipocytes or/and preadipocytes), we used the entire replacement medium during 8-day differentiation (*days 2, 4, 6*, and 8 postdifferentiation) of 3T3-L1 in all of our experiments. We observed that mCM promoted the proliferation and motility ability of colorectal cancer cells and enhanced the formation of BVs in chicken embryonic allantoic membranes in vitro. In addition, we checked the effects of different postdifferentiation day conditioned medium (*days 0, 2, 4*, and 8) on the growth and migration of C26 cells (data not shown). The results showed different stages of adipocyte media enhanced the growth and migration of colonic cells, particularly *day 4* and *day 8* medium. Thus, we concluded that adipocytokines that are secreted by the adipocytes in the local colorectal tissue contributed to the progression of colorectal cancer.

ROR α is a member of the orphan nuclear receptor family and has been implicated in the regulation of several genes involved in lipid metabolism and tumorigenesis. Herein, we reveal that ROR α is expressed in human colorectal tissues and that the adipocyte conditioned medium led to a decrease in the



Fig. 6. Effects of ROR α overexpression and knockdown for lipogenesis in SW480 cells. *A*: real-time PCR analysis to measure the relative mRNA expression levels of some lipogenic genes in siROR α or p3xFLAG-CMV10-ROR α (ROR α) SW480 cells. Data represent means \pm SE of 3 experiments in each group. **P* < 0.05 and ***P* < 0.01 compared with normal groups. *B*: 3T3-L1 preadipocytes were induced to differentiate in the presence of CS (5 and 10 μ M). On *day* 8, cytoplasmic triacylglycerol was stained with Oil Red O. DM, differentiation medium. *C*: 3T3-L1 preadipocytes were stimulated to differentiate in the presence of CS (5, 10, and 15 μ M). Cell lysates were prepared on *day* 8 and subjected to real-time PCR to detect VEGF and HIF-1 α (***P* < 0.01 and **P* < 0.05 vs. DM group). *D*: 3T3-L1 preadipocytes were stimulated to differentiate in the presence of CS (5, and 10 μ M). Cell lysates were stimulated to differentiate in the presence of CS (5 and 10 μ M). Cell supernatants were prepared on *day* 8 and subjected to real-time PCR to detect VEGF and HIF-1 α (***P* < 0.01 and **P* < 0.05 vs. DM group). *D*: 3T3-L1 preadipocytes were stimulated to differentiate in the presence of CS (5 and 10 μ M). Cell supernatants were prepared and subjected to ELISA to detect VEGF. *E*: quantification of acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1), and fatty acid-binding protein (FABP) mRNA levels in SW480 and C26 cells. *F* and *G*: real-time PCR analysis to measure the relative mRNA expression levels of p21, cyclin D, HIF-1 α , VEGF, and ACC in p3xFLAG-CMV10-ROR α (ROR α) SW480 cells (*F*) or ROR α silenced cells (*G*).

expression of ROR α . Meanwhile, expression levels of ROR α and its target genes are lower in human colorectal tumor tissue compared with control colorectal tissue. Because of the difference in expression patterns in colorectal tumor and normal tissues, we concluded that ROR α might have a potential role in the progression of colorectal cancer. Subsequently, we observed that CS, a putative ligand of ROR α , inhibited SW480 and C26 cell proliferation and induced G₁ arrest in colorectal cancer cells in a dose-dependent manner via the modulation of the expression of cell cycle-regulatory genes. Using a Transwell assay, we demonstrated that CS inhibited cell motility in colorectal cancer cells. Furthermore, angiogenesis is a key step in tumor growth and cancer development. VEGF and HIF-1 α are the two important angiogenic cytokines that have not only angiogenic properties but also an autocrine ability to regulate the synthesis of multiple angiogenic proteins in multiple cell

types (12, 25, 30, 44). Thus, the level of VEGF and HIF-1 α could directly and indirectly indicate the ability of a tumor to cause angiogenesis. In fact, we observed that CS inhibited angiogenesis in chicken embryonic allantoic membranes by regulating the expression of both VEGF and HIF-1 α . In addition, we examined the ROR α agonist CS under regular growth condition on colorectal cancer cell proliferation and migration using flow cytometric and Transwell migration assay. The results showed there were no significant effects of the ROR α agonist CS under regular growth conditions on the proliferation and migration and migration and migration of C26 cells (data not shown).

Many cancer cells exhibit an increased lipogenic activity related to rapid cell growth and division. Because the cell growth and division rates are higher in cancer cells than in normal cells, lipid metabolites such as phospholipids are crucial for maintaining membrane integrity in cancer cells (19). There is a renewed interest in the ultimate role of FAS, a key lipogenic enzyme catalyzing the terminal steps in the de novo biogenesis of fatty acids in cancer pathogenesis. Increased expression of the tumor-associated FAS gene, by conferring growth and survival advantages rather than functioning as an anabolic energy-storage pathway, appears to accompany the natural progression of most human cancers (26). Moreover, the development and progression of cancer is accompanied by marked changes in the expression and activity of enzymes involved in the cellular homeostasis of fatty acids. One class of enzymes that play a particularly important role in this process is ACC. In addition, evidence suggests that there is a critical implication of SCD-1 in the modulation of multiple biological mechanisms, specifically in lipid biosynthesis and the proliferation and survival signaling pathways that contribute to the development and progression of cancer (18). Recently, some studies have demonstrated that deficiencies in FABP4 substantially impaired metastatic tumor growth in mice, indicating that FABP4 has a key role in ovarian cancer metastasis (29). In this aspect, inhibitors of lipogenic genes have been proposed as anticancer drugs. For instance, FAS inhibitors, such as cerulenin and C75, exhibit potent inhibition of human cancer cell growth (31-33). Indeed, we observed that CS inhibited the expression of lipogenic genes, including FAS, ACC, SCD-1, and FABP4, and that this inhibition is potentially associated with retardation of colorectal cancer cell growth. When we repressed the expression of ROR α via siRNA, the expression of lipogenic genes was significantly increased.

Therefore, this work suggests a more detailed mechanism of action of adipocytokines in the development of colorectal cancer. The accumulation of adipocytes may secrete elevated levels of adipocytokines, which provide the potential of a local physiological basis in the occurrence of colorectal cancer. Moreover, the conditioned medium led to a decrease in the expression of ROR α , since ROR α expression was lower in human colorectal tumor tissue compared with control colorectal tissue. Thus, reduction of the expression of ROR α may represent a potential warning signal of colorectal cancer. Furthermore, because the antiproliferative and antimigratory effects of ROR α inhibitors affect colorectal cancer cells, our data provide substantial evidence that a ROR α agonist might be useful as a potential therapeutic target for colorectal cancer.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: L.X., J.W., and Y.Z. conception and design of research; L.X., J.W., J.L., X.C., and S.S. performed experiments; L.X., J.W., and J.L. analyzed data; L.X. interpreted results of experiments; L.X. and J.W. prepared figures; L.X., J.L., P.X., and D.H. drafted manuscript; L.X., J.W., P.X., D.H., Y.C., and Y.Z. edited and revised manuscript; L.X. and Y.Z. approved final version of manuscript.

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