Oncogenic retinoic acid receptor α promotes human colorectal cancer growth through simultaneously regulating p21 transcription and GSK3 β / β -catenin signaling

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Abstract

Retinoic acid receptor α (RAR α) plays important roles in the progression of several cancers such as leukemia, breast cancer, and lung cancer. In this study, we demonstrated that RAR α protein was frequently overexpressed in human CRC specimens and CRC cell lines. RAR α knockdown decreased cell survival, proliferation, and colony formation in vitro and tumorigenic potential in nude mice. Specifically, RAR α knockdown inhibited cell cycle progression at G1 phase through upregulation of cell cycle inhibitor p21, and downregulation of cyclinD1. Furthermore, RAR α was directly recruited to the p21 promoter to inhibit the expression of p21. Simultaneously, RAR α contributed to the progression of CRC cells in part due to upregulation of cyclinD1 via activation of GSK3 β / β -catenin pathway. Molecular mechanism studies revealed RAR α interacted with GSK3 β and led to decreased expression of GSK3 β at ser9, followed by increased β -catenin expression. Taken together, our results signified the importance of RAR α in CRC and demonstrated that RAR α promotes CRC progression through suppressing p21 transcription and enhancing GSK3 β / β -catenin signaling. RAR α might become a potential molecular target for the treatment of CRC.

Key Words: colorectal cancer, retinoic acid receptor α , GSK3 β , p21

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer-related mortality around the world [1]. Although surgery combined with radiotherapy and chemotherapy is a predominant therapeutic strategy for early stage tumors [2], the majority of patients are not diagnosed until the late stage [3]. CRC is characterized by difficulty of early diagnosis, poor prognosis and 5 year survival rate of only 11.7% [3, 4]. Despite of much advances in screening, diagnosis and treatment of CRC in past decades [5], CRC remains a severe disease. Hence it is urgent to define the molecular mechanisms underlying the progression of CRC for developing novel therapeutic strategies.

Retinoic acid receptors (RARs) are transcription factors that are essential in embryonic development, metabolism, maintenance of differentiated cellular phenotypes, and cell death [6]. RARs are encoded by three distinct genes, α , β , and γ [7]. RAR α , a member of the nuclear receptor superfamily [8, 9], plays an important role in many biological processes including carcinogenesis [10-12]. All-trans-retinoic acid (ATRA) and several polyunsaturated fatty acids, which can bind to RAR α , are currently in clinical trials for the treatment of leukemia, breast cancer, and hepatocellular carcinoma [13]. Am580, a selective agonist of RAR α , exerted significant inhibitory effects on breast tumor growth and endometrial cancer cell proliferation [14, 15], suggesting the suitability of targeting RAR α for cancer therapy. Consistently, the oncogenic potential of RAR α has been demonstrated. Genetic dysfunction of RAR α enhances tumorigenesis [16, 17]. The expression of a

PML/RAR α fusion protein is essential for the development of acute promyelocytic leukemia [18, 19]. High intratumoural RAR α protein levels correlate with reduced relapse-free survival in breast cancer patients [20, 21]. Accumulating evidence indicated that RAR α plays important roles in various human cancers such as leukemia, breast cancer, lung cancer, osteosarcoma, and gastric cancer through multiple signaling pathways including estrogen receptor α , p38 α mitogen-activated protein kinases, protein kinase B, phosphoinositide 3-kinase, nuclear factor (erythroid-derived 2)-like 2, E2F2, nuclear factor- κ B, tumor necrosis factor receptor-associated death domain, G α q, and Krueppel-like factor 5 [16, 18, 21-27]. These results indicated that RAR α is a bona fide oncogene. However, the expression profile and the function of RAR α in CRC still remain unknown, and are worthy of identifying in order to develop improved target-based drugs for cancer therapy.

In the present study, we demonstrated that RAR α protein is frequently overexpressed in human CRC specimens and CRC cell lines. RAR α knockdown in CRC cells reduced cell proliferation through regulation of p21 expression and GSK3 β / β -catenin pathways.

2. Materials and methods

2.1. Reagents

Wnt3a was from R&D systems (Minneapolis, MN, USA). Propidium iodide (PI) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma (St. Louis, MO, USA). 5-Ethynyl-2-deoxyuridine (EdU) was obtained from RiboBio (Guangzhou, China). IPTG was purchased from Thermo Scientific (Wilmington, DE, USA). Polyclonal antibodies against RAR α , phosphorylated β -catenin, GSK3 β and Flag and monoclonal antibodies against phosphorylated GSK3 β , β -catenin and HA were from Abcam Ltd. (Cambridge, United Kingdom). Polyclonal antibody against β -actin was from Cell signaling Technology (Danvers, MA, USA). Monoclonal antibodies against cyclinA, cyclinB, cyclinD1, cyclinE1 and p21 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase and Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). PVDF membrane was obtained from Millipore (Billerica, MA, USA). The EliVision Plus kit was from Maixin Bio (Fuzhou, China).

2.2. Patients and tumor specimens

Tumorous and their adjacent non-tumorous colorectal cancer tissues were collected from 32 patients who underwent surgery at the First Affiliated Hospital of Xiamen University. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institute Research Ethics Committee of the First Affiliated Hospital of Xiamen University. Tissue microarrays with colorectal cancer were

purchased from Shanghai Xinchao Biotech Co. Ltd.

2.3. Plasmids construction

The coding regions of human RAR α was retrieved from the cDNA of human liver cell LO2 by PCR, and then cloned into Xho I/Bam HI sites of pIRES-EGFP vector or Bam HI/Xho I sites of pCDNA3.0-flag vector to generate constructs for expression of RAR α or flag-RAR α . The coding region of human GSK3 β was cloned into pCDNA3.0-HA or pGEX-4T-1 vector to generate the constructs for expression of HA-GSK3 β or GST-GSK3 β . Five RAR α truncated fragments (A/B, C, D, E and F) were amplified by using pCDNA3.0-RAR α as a template and cloned into pCDNA3.0-flag vector, respectively. RAR α -specific targeting sequence (19 nt sequences GCTTCCAGTTAGTGGATAT of human RAR α cDNA separated by a 9 nt spacer TTCAAGAGA from the reverse complement of the same sequence) were cloned into pll3.7 vector to generate pll3.7-shRAR α . For lentivirus production, 293T cells were transfected with pll3.7-neo-shRAR α and lentivirus packaging plasmids (VSVG/PMDL/REV) using Lipofectamine 2000 according to the manufacturer's instruction. The transfected cells were cultured at 37 °C for 48 h and the virus generated was used for infection.

2.4. Site-directed mutagenesis of the RARE element of p21 promoter

The p21 promoter was inserted into the pGL3-basic vector to generate the pGL3-p21-Luc promoter/reporter plasmid. Site-directed mutagenesis of RARE of p21 promoter was RARE (5'-CAAAGGTGAAGTCCAGGGGAGGTCAG-3' to 5'-CAAAGGCGAAGTCCAGAGGAGGTCAG-3').

2.5. Cell culture and stable cell lines

HT29 and HCoEpiC were grown in RPMI-1640 medium (Hyclone, Logan, UT, USA). HCT116, RKO and SW480 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA). Both media were supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 100 U/mL of penicillin-streptomycin at 37 °C in a humidified incubator containing 5% CO₂. After infection of lentivirus with pll3.7-neo-shRAR α or with pll3.7-shCtrl, stably transfected RKO and HCT116 cells were established by treatment with geneticin (250 μ g/mL) for 3 weeks.

2.6. Immunohistochemistry (IHC)

IHC was performed as described previously [28]. Briefly, after antigen retrieval using pressure cooker boiling for 2 min, paraffine-embedded human CRC tissue sections were washed and incubated with anti-RAR α antibody (1:200) overnight at 4°C followed by incubation with corresponding secondary antibody for 1 h. After washing, the slides were detected with the EliVision Plus Kit (Maixin Bio, Fuzhou, China) according to the manufacturer's instructions and then co-stained with hematoxylin to visualize nuclei.

2.7. MTT assay

Cell viability was analyzed by using MTT assay, as previously describe [29]. Briefly, a total of 2×10^3 cells were seeded in each well of 96 well plate and MTT was added to each well every 24 h. The plates were incubated for 4 h before addition of solubilization solution (150 μ L DMSO). The absorbance was measured at 490 nm

using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

2.8. 5-Ethynyl-2-deoxyuridine (EdU) labeling assays

A total of 4×10^3 cells were seeded in each well of a 96 well plate. Twenty-four hours after transfection, 100 μ L EdU solution (50 μ M) was added and the cells were cultured for an additional 2 h at 37 °C. The cells were fixed in 4% paraformaldehyde at room temperature for 30 min, followed by permeabilization with 0.5% Triton X-100. Then the cells were stained with 1 $\times$ Apollo reaction mixture for 30 min. Finally, Hoechst 33342 solution was added to visualize the nuclei. Images were taken using a Leica TCS SP5 II laser confocal microscope (Leica, Barcelona, Spain).

2.9. Colony formation assays

Five hundred cells were cultured in 6-well plate. After 14 days, the cells were washed twice with PBS, fixed in 4% paraformaldehyde for 20 min, stained with crystal violet for 30 min, and the colonies were counted visually.

2.10. Cell cycle analysis

The cells were synchronized by serum starvation for 24 h and induced to re-enter cell cycle by culturing in medium plus 10% fetal bovine serum for 24 h. And then the cells were harvested, and fixed in 75% ethanol at 4 °C for 40 min. Cells were incubated with RNase A at 37 °C for 30 min, and then stained with propidium iodide for 30 min at room temperature. Cell cycle was measured by flow cytometry. The data were analyzed with the ModFit 3.3 software (Verity Software House, Topsham, ME, USA).

2.11. Quantitative real-time PCR

Total RNA was isolated by using the RNeasy kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out by using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as the controls for normalization. Primers used for qPCR were listed on Supplementary Table S1.

2.12. Dual-luciferase reporter assays

The cells were transfected with reporter plasmids in the presence or absence of RAR α expression plasmid by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were harvested after 48 h post-transfection, the activities of both firefly luciferase and renilla luciferase were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The renilla luciferase was used as an internal standard for transfection efficiency.

2.13. Western blotting

Equal amounts of protein lysates were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA). The membrane was probed with the specific primary antibodies, and then incubated with horseradish peroxidase-conjugated secondary antibody. The signal was finally visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA).

2.14. Co-immunoprecipitation (Co-IP) and GST pulldown assay

For Co-IP, Co-IP was performed mainly as described [30]. Briefly, Cells were lysed in modified lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40) 100 mM NaF, and 1 mM PMSF). The lysates were mixed and precipitated with the appropriate antibody together with dynabeads protein G (Life Technologies, Norway) by incubation at 4 °C for 3 h. After extensive washing, the protein-antibody complexes on the beads were analyzed by Western blotting. For GST pulldown assays, GST or GST-fusion proteins were expressed in E. coli strain BL21 and incubated with Glutathione Agarose (Pierce, Rockford, IL, USA) followed by the addition of cell lysates according to the manufacturer's instructions. Unbound proteins were removed after several washes. The proteins were eluted by boiling in loading buffer for 10 min for SDS-PAGE.

2.15. Chromatin immunoprecipitation assay (ChIP)

ChIP assay was processed according to the manufacturer's instructions (Millipore, Billerica, MA, USA). RKO cells at 80% confluence were cross-linked with 1% formaldehyde at 37 °C for 10 min, lysed, and sonicated 14 times each for 10 s at 4 °C to reduce the average DNA length. The samples were diluted 10-fold and then precleared with protein A-agarose/salmon sperm DNA for 1 h at 4 °C, followed by an overnight incubation at 4 °C with anti-RAR α or anti-mouse IgG (as a negative control). The complexes were precipitated with protein A-agarose for 2 h. After reversal washes, the genomic region of the p21 flanking the potential RAR α -binding sites was amplified by PCR with the following primer pairs:

5'-AACTGCAAATGTTTCAGGCACA-3' (sense) and

5'-CTCCCATCTACCTCACACCC-3' (antisense).

2.16. Animal Model

Healthy BALB/c male nude mice (4 weeks old) were obtained from the Xiamen University Laboratory Animal Center in Xiamen, China. Animal experiments were performed in accordance with ethical guidelines of animal experimentation. 3×10^6 shRAR α -RKO cells and control cells were subcutaneously injected into the dorsal flanks of mice respectively. After the establishment of the tumors, tumor volume (V) was monitored by measuring the length (L) and width (W) with vernier caliper and calculated with the formula $V = 0.5 \times (L \times W^2)$. After 24 days, the mice were sacrificed and the tumors were photographed and processed for western blot and immunohistochemistry analysis.

2.17. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (San Diego, CA, USA). Survival curves were analyzed by Kaplan-Meier using a log-rank test. Data are represented as means $\pm$ SEM from at least three independent experiments performed in triplicate. One-way analysis of variance (ANOVA) or Student's *t*-test was used for comparison of two groups. P value ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Clinical significance and overexpression of RAR α in human CRC

To evaluate the expression of RAR α in CRC, western blotting was performed to assess the levels of RAR α protein in a set of 32 tumor and adjacent non-tumorous colorectal tissues. The levels of RAR α were significantly higher in 21CRC specimens versus the non-tumorous tissues (Fig. 1A, B). In comparison with normal colon epithelial cells (HcoEpiC), all four human CRC cell lines (HT29, HCT116, RKO and SW480) expressed elevated levels of RAR α (Fig. 1C), suggesting a plausible role of RAR α in CRC cells.

To verify if the above findings were consistent with a different analysis platform and patient cohort, immunohistochemistry (IHC) was used to analyze RAR α expression on 80 paired paraffin-embedded CRC tissues and non-tumorous colorectal tissues and another 20 CRC tissues on tissue microarrays (Fig. 2A). The majority of tumors had a high percentage of cells expressing RAR α (56/100) (Tab. 1).

Representative staining of tumors classified as low ($\pm$, +) and high (++, +++) are shown in Figure 2B. The clinical data in Table 2 showed that RAR α overexpression was significantly associated with poor differentiation, pathological types and clinical stage ($P < 0.05$), whereas no association was found with age, sex, tumor size, lymph node metastasis and distant metastasis ($P > 0.05$). Furthermore, the postoperative overall survival rate in patients with high RAR α expression (median survival time, 52 months) was lower than that in patients with low RAR α expression (median survival time, 76.5 months) (Fig. 2C). These results demonstrated the clinical significance of

RAR α serving as a potential molecular target for CRC patients.

3.2. RAR α knockdown suppresses CRC cell proliferation

To investigate the role of RAR α in the growth of CRC cells, stable knockdown of RAR α in RKO and HCT116 cells, in which elevated RAR α protein level was observed, significantly decreased cell survival (Fig. 3A, upper panel). In contrast, overexpression of RAR α in HT29 cells, which express relatively less RAR α protein, significantly increased cell survival (Fig. 3A, lower panel). Knockdown and overexpression of RAR α were further confirmed by western blotting. The protein expression of RAR α was significantly reduced by stable knockdown and was potently enhanced by overexpression (Fig. 3B). To further determine the role of RAR α in proliferation of CRC cells, cultures were subjected to EdU incorporation analysis. The percentage of EdU-positive cells was significantly decreased in shRAR α -RKO cells, and remarkably increased in RAR α -overexpressed HT29 cells (Fig. 3C). Furthermore, RAR α knockdown in RKO and HCT116 cells significantly decreased focus formations, whereas RAR α overexpression in HT29 cells significantly increased focus formations (Fig. 3D). These results indicated that RAR α is vital for the proliferation of CRC cells.

The effects of RAR α on the growth of cancer cells were further validated in animals by injection of the same number of shRAR α -RKO cells or shCtrl-RKO cells into BALB/c mice. RAR α knockdown significantly decreased tumor growth (Fig. 4A), and the mean weight of RAR α -knockdown tumors was about four-fold smaller than that from control tumors (Fig. 4B, C). Furthermore, Ki67 staining for tissue sections

from shRAR α -RKO and control tumors was performed to determine the effects of RAR α knockdown on cell proliferation in vivo. Ki67 is a marker for tumor cell proliferation. RAR α knockdown significantly reduced the number of Ki67-positive cells (Fig. 4D, E). These results suggested that RAR α knockdown could suppress the growth of CRC cells in vivo.

3.3. RAR α knockdown induces CRC cell cycle arrest

To explore the mechanism by which RAR α knockdown inhibited proliferation of CRC cells, cell cycle analysis was performed to test whether RAR α -knockdown cells were arrested in a specific phase of the cell cycle. Flow cytometric analysis showed that RAR α knockdown induced G1 arrest and a concomitant decrease of cell number at the S phase (Fig. 5A). Thus RAR α seemed to regulate the G1/S phase transition.

To address the molecular mechanism underlying the cell cycle arrest, the gene and protein levels of several cell cycle-related genes were measured. RAR α knockdown did not cause significant changes in the mRNA expression of cyclinA, cyclinB, and cyclinE1, but led to a significant decrease in the expression of cyclinD1 and a concomitant increase in the expression of the cell cycle inhibitor p21 (Fig. 5B). The expression of cell cycle-related proteins was examined at different time after serum addition following synchronization by serum starvation for 24 h. The results showed that the expression of G1-related factor cyclinD1 recovered more slowly and less and the cell cycle inhibitor protein p21 decreased more slowly and less in RAR α -knockdown RKO cells than in control cells, whereas the expression of mitosis-promoting factors including cyclinA, cyclinB, cyclinE1 was comparable in

both cells (Fig. 5C).

To examine whether RAR α has the same role in cell cycle regulation in xenograft tumors in nude mice, the gene and protein levels of cell cycle-associated gene were assessed. RAR α knockdown markedly inhibited the expression of cyclinD1 and increased the expression of p21 (Fig. 5D, E). These results were consistent with the in vitro data, implying that RAR α promotes cell cycle progression.

3.4. RAR α inhibits the transcription of p21

To further confirm the regulatory effects of RAR α on p21, western blotting was employed to measure the expression of p21. The protein expression of p21 was increased by RAR α knockdown, and was decreased by RAR α overexpression (Fig. 6A). These results were in line with the gene expression data. To identify the exact mechanism by which RAR α controlled the expression of p21 mRNA, we searched the promoter region of the human p21 gene, and cloned this region in a luciferase reporter plasmid. Dual-luciferase reporter assay showed that transfection of RAR α into 293T cells greatly attenuated the p21 promoter activity, whereas transfection of RAR α -targeted shRNA significantly activated the p21 promoter activity (Fig. 6B).

Analysis of the human p21 gene revealed one proximal RAR α -binding site (RARE) in the promoter region of p21 (Fig. 6C). The proximal RARE site was mutated and we examined whether these elements contributed to the repression of p21 by RAR α . Mutation of proximal RARE site removed the inhibitory effect of RAR α in RKO cells (Fig. 6D), suggesting that this site might be responsible for RAR α binding in vivo. To determine whether RAR α could be recruited to the proximal RARE site in

the p21 promoter, ChIP assay was performed, and the results showed that, RAR α was recruited to the RARE in the promoter region of p21, and the recruitment was increased in the presence of exogenous RAR α (Fig. 6E). Taken together, these results suggested that RAR α promoted CRC cells proliferation by inhibition of the expression of cell cycle inhibitor protein p21 through direct binding to its promoter region.

3.5. RAR α enhances GSK3 β / β -catenin pathway

CyclinD1, a typical target of the GSK3 β / β -catenin pathway [31, 32], was downregulated in shRAR α cells (Fig. 5B, C). Therefore we hypothesized that RAR α might play a role in GSK3 β / β -catenin pathway to regulate cyclinD1 transcription and subsequent cell proliferation. Dual-luciferase reporter assay showed that RAR α knockdown markedly decreased Topflash activity. Simultaneously RAR α overexpression could significantly increase Topflash activity (Fig. 7A). In agreement with previous studies, we confirmed that cyclinD1 was a target of the GSK3 β / β -catenin pathway in CRC cells (Fig. 7B). Moreover, RAR α overexpression induced the expression of cyclin D1, which could be significantly suppressed by siRNA of β -catenin (Fig. 7C). RAR α knockdown increased the expression of GSK3 β at ser9, subsequently accompanied an increase of β -catenin at ser33 and ser37 (Fig. 7D). These results suggested that RAR α might serve as a coactivator for GSK3 β / β -catenin pathway activation.

3.6. RAR α interacts with GSK3 β through the C domain of RAR α

In an effort to study the precise regulation of RAR α on GSK3 β / β -catenin

signaling, Flag-tagged RAR α and HA-tagged GSK3 β were coexpressed in 293T cells, and then Co-IP assays were performed. Anti-Flag antibodies, but not control IgG, immunoprecipitated GSK3 β from cell lysates (Fig. 8A, upper panel). Reciprocally, anti-HA antibodies could also immunoprecipitate RAR α from cell lysates (Fig. 8A, lower panel). In addition, the interaction of endogenous RAR α and GSK3 β in RKO cells and HCT116 cells could be detected by Co-IP assays by using anti-RAR α antibodies (Fig. 8B). To further confirm the association of RAR α with GSK3 β in vitro, GST pulldown assays were performed. The results showed that the GST-GSK3 β protein, but not GST, was able to pull down RAR α (Fig. 8C), indicating that RAR α can directly bind to GSK3 β .

RAR α is a multidomain protein that contains the amino terminus containing a transactivation domain (A/B), the central DNA-binding domain (C), hinge region (D), the ligand-binding domain (E), and the carboxy-terminal domain (F) (Fig. 8D, upper panel) [8]. To identify the domains within RAR α required for interaction with GSK3 β , we examined the ability of GST-GSK3 β to interact with five fragments of RAR α fused with Flag-tag. The results showed that RAR α interacted with GSK3 β through its C domain (Fig. 8D, lower panel). Collectively, these data suggested that RAR α serves as an essential coactivator for GSK3 β / β -catenin activation by physically interacting with GSK3 β and thus enhances its transcriptional activity for cyclin D1.

4. Discussion

Accumulating evidence revealed that the growth-promoting effect of RAR α in various tumors [10, 33, 34]. However, the expression profile and the role of RAR α in CRC remain elusive. In the present study, we found that RAR α was significantly elevated in the CRC tissues of two small independent cohorts and abnormally expressed in all CRC cell lines examined, suggesting that RAR α may have a role in CRC progression. Despite the small patient population, RAR α retained as an independent biomarker in a Cox multivariate analysis demonstrating an increased risk of death in patients with high RAR α expression compared to those with low RAR α expression. Therefore, RAR α could be a potential predictor of poor prognosis of CRC patients after curative resection, and its overexpression might be taken into account in the following optimal treatment scheme.

Previous studies have showed that RAR α is a key player in the regulatory network of cell proliferation and differentiation [35, 36]. Our current data suggested that CRC cell survival, proliferation, and colony formation were decreased by RAR α knockdown, and were increased by RAR α overexpression, suggesting that RAR α plays an important role in the progression of CRC. Additionally, RAR α knockdown significantly reduced CRC growth in mice in vivo.

Besides its suppression of cell proliferation, RAR α knockdown resulted in cell cycle arrest at the G1 phase through regulating the expression of p21 and cyclinD1, two critical cell cycle regulators. Importantly, RAR α -knockdown colorectal tumors grew much slower and expressed more p21 and less cyclinD1 than control colorectal

tumors, providing genetic evidence that RAR α was indeed required for CRC growth. The transcriptional regulation of p21, a member of the Cip/Kip family of cyclin-dependent kinases inhibitors (CKIs), is tightly involved in growth inhibition [27, 37, 38]. Increased p21 levels contribute to arrest cell proliferation [38]. Previous studies revealed that RAR α recruited to its binding sites on the p21 promoter to inhibit p21 transcription in vascular smooth muscle cells (VSMCs) [27]. These results interested us about whether RAR α was involved in the genomic regulation of p21 to promote CRC progression. In agreement with previous studies, our present results confirmed that RAR α was directly recruited to p21 promoter to suppress p21 transcription in CRC cells (Fig. 8E).

An important finding of the present study was that RAR α enhanced the expression of cyclinD1 through GSK3 β / β -catenin pathway in CRC. CyclinD1, the chief mediator of cell proliferation, is a typical downstream target of GSK3 β / β -catenin pathway [31, 39]. The GSK3 β / β -catenin pathway has important roles in many biological processes including carcinogenesis, especially CRC [40-43]. Recent studies have shown that GSK3 β / β -catenin activity is required for CRC cells progression and metastasis [44]. GSK3 β , a key part of β -catenin destruction complex (APC, Axin, GSK3 β and CK1), phosphorylates β -catenin and promotes its ubiquitylation and degradation [45-48]. Our findings are meaningful with regards to the observation that RAR α knockdown inactivated GSK3 β by phosphorylation at ser9, which led to its own stabilization and subsequently phosphorylated β -catenin at ser33 and ser37. More importantly, our further study revealed that RAR α activated CRC

growth mechanisms by serving as an essential coactivator for GSK3 β activation to enhance translocation of β -catenin from the cytoplasm into the nucleus, followed by increased expression of target genes (Fig. 8E). To the best of our knowledge, this is the first time that RAR α is reported to serve as a critical coactivator for GSK3 β / β -catenin pathway. Taken together, RAR α has an essential role in the progression of CRC through enhancing GSK3 β / β -catenin pathway, and thus emerges as a potential drug target for CRC therapy.

Collectively, the results of our present study provided novel evidence showing that RAR α has an essential role in the progression of CRC. RAR α promotes CRC progression at least by two aspects. On one hand, RAR α served as a transcriptional repression protein, and was recruited to the p21 promoter and synergistically inhibited p21 expression. On the other hand, RAR α enhanced GSK3 β / β -catenin pathway by direct interaction with GSK3 β , and thus regulated the expression of target genes such as CyclinD1. Accordingly, RAR α might be an attractive therapeutic target, which warrants its further development for cancer therapy.

Acknowledgements

This work was supported by Grants from the National Nature Science Foundation of China (Nos. 81572394, 81470544), the National Science Foundation for Fostering Talents in Basic Research of the National Natural Science Foundation of China (No. J1310027), and Open Research Fund of State Key Laboratory of Cellular Stress Biology, Xiamen University (SKLCSB2016KF005).

References

- [1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2015, *CA Cancer J Clin*, 65 (2015) 5-29.
- [2] H. Brenner, M. Kloor, C.P. Pox, Colorectal cancer, *Lancet*, 383 (2014) 1490-1502.
- [3] R. Siegel, C. Desantis, A. Jemal, Colorectal cancer statistics, 2014, *CA Cancer J Clin*, 64 (2014) 104-117.
- [4] L. Li, T. Duan, X. Wang, R.H. Zhang, M. Zhang, S. Wang, F. Wang, Y. Wu, H. Huang, T. Kang, KCTD12 Regulates Colorectal Cancer Cell Stemness through the ERK Pathway, *Scientific reports*, 6 (2016) 20460.
- [5] P. Mo, Q. Zhou, L. Guan, Y. Wang, W. Wang, M. Miao, Z. Tong, M. Li, S. Majaz, Y. Liu, G. Su, J. Xu, C. Yu, Amplified in breast cancer 1 promotes colorectal cancer progression through enhancing notch signaling, *Oncogene*, 34 (2015) 3935-3945.
- [6] A. Marinelli, D. Bossi, P.G. Pelicci, S. Minucci, A redundant oncogenic potential of the retinoic receptor (RAR) alpha, beta and gamma isoforms in acute promyelocytic leukemia, *Leukemia*, 21 (2007) 647-650.
- [7] P. Kastner, M. Mark, P. Chambon, Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life?, *Cell*, 83 (1995) 859-869.
- [8] H. Gronemeyer, J.-Å. Gustafsson, V. Laudet, Principles for modulation of the nuclear receptor superfamily, *Nature Reviews Drug Discovery*, 3 (2004) 950-964.
- [9] K. Wang, Y.J.Y. Wan, Nuclear Receptors and Inflammatory Diseases, *Experimental Biology and Medicine*, 233 (2008) 496-506.
- [10] A. di Masi, L. Leboffe, E. De Marinis, F. Pagano, L. Cicconi, C. Rochette-Egly, F.

- Lo-Coco, P. Ascenzi, C. Nervi, Retinoic acid receptors: from molecular mechanisms to cancer therapy, *Molecular aspects of medicine*, 41 (2015) 1-115.
- [11] E. Garattini, M. Bolis, S.K. Garattini, M. Fratelli, F. Centritto, G. Paroni, M. Gianni, A. Zanetti, A. Pagani, J.N. Fisher, A. Zambelli, M. Terao, Retinoids and breast cancer: from basic studies to the clinic and back again, *Cancer treatment reviews*, 40 (2014) 739-749.
- [12] E. Saulle, A. Petronelli, E. Pelosi, E. Coppotelli, L. Pasquini, R. Ilari, F. Lo-Coco, U. Testa, PML-RAR alpha induces the downmodulation of HHEX: a key event responsible for the induction of an angiogenetic response, *J Hematol Oncol*, 9 (2016) 33.
- [13] N. Bushue, Y.J. Wan, Retinoid pathway and cancer therapeutics, *Adv Drug Deliv Rev*, 62 (2010) 1285-1298.
- [14] A. Bosch, S.P. Bertran, Y. Lu, A. Garcia, A.M. Jones, M.I. Dawson, E.F. Farias, Reversal by RARalpha agonist Am580 of c-Myc-induced imbalance in RARalpha/RARgamma expression during MMTV-Myc tumorigenesis, *Breast Cancer Res*, 14 (2012) R121.
- [15] Y.H. Cheng, H. Utsunomiya, M.E. Pavone, P. Yin, S.E. Bulun, Retinoic acid inhibits endometrial cancer cell growth via multiple genomic mechanisms, *J Mol Endocrinol*, 46 (2011) 139-153.
- [16] S. Hua, R. Kittler, K.P. White, Genomic antagonism between retinoic acid and estrogen signaling in breast cancer, *Cell*, 137 (2009) 1259-1271.
- [17] F. Alizadeh, A. Bolhassani, A. Khavari, S.Z. Bathaie, T. Naji, S.A. Bidgoli,

Retinoids and their biological effects against cancer, *Int Immunopharmacol*, 18 (2014) 43-49.

[18] J.V. Raelson, C. Nervi, A. Rosenauer, L. Benedetti, Y. Monczak, M. Pearson, P.G. Pelicci, W.H. Miller, Jr., The PML/RAR alpha oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells, *Blood*, 88 (1996) 2826-2832.

[19] R.H. Tao, Z. Berkova, J.F. Wise, A.H. Rezaeian, U. Daniluk, X. Ao, D.H. Hawke, J.E. Karp, H.K. Lin, J.J. Molldrem, F. Samaniego, PMLRARalpha binds to Fas and suppresses Fas-mediated apoptosis through recruiting c-FLIP in vivo, *Blood*, 118 (2011) 3107-3118.

[20] H.J. Johansson, B.C. Sanchez, F. Mundt, J. Forshed, A. Kovacs, E. Panizza, L. Hultin-Rosenberg, B. Lundgren, U. Martens, G. Mathe, Z. Yakhini, K. Helou, K. Krawiec, L. Kanter, A. Hjerpe, O. Stal, B.K. Linderholm, J. Lehtio, Retinoic acid receptor alpha is associated with tamoxifen resistance in breast cancer, *Nature communications*, 4 (2013) 2175.

[21] C.S. Ross-Innes, R. Stark, K.A. Holmes, D. Schmidt, C. Spyrou, R. Russell, C.E. Massie, S.L. Vowler, M. Eldridge, J.S. Carroll, Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer, *Genes Dev*, 24 (2010) 171-182.

[22] M. Gianni, M. Peviani, N. Bruck, A. Rambaldi, G. Borleri, M. Terao, M. Kurosaki, G. Paroni, C. Rochette-Egly, E. Garattini, p38alphaMAPK interacts with and inhibits RARalpha: suppression of the kinase enhances the therapeutic activity

of retinoids in acute myeloid leukemia cells, *Leukemia*, 26 (2012) 1850-1861.

- [23] C.F. Donini, E. Di Zazzo, C. Zuchegna, M. Di Domenico, S. D'Inzeo, A. Nicolussi, E.V. Avvedimento, A. Coppa, A. Porcellini, The p85alpha regulatory subunit of PI3K mediates cAMP-PKA and retinoic acid biological effects on MCF7 cell growth and migration, *Int J Oncol*, 40 (2012) 1627-1635.
- [24] C. Wongtrakool, S. Malpel, J. Gorenstein, J. Sedita, M.I. Ramirez, T.M. Underhill, W.V. Cardoso, Down-regulation of retinoic acid receptor alpha signaling is required for sacculation and type I cell formation in the developing lung, *J Biol Chem*, 278 (2003) 46911-46918.
- [25] H. Srinivas, D. Xia, N.L. Moore, I.P. Uray, H. Kim, L. Ma, N.L. Weigel, P.H. Brown, J.M. Kurie, Akt phosphorylates and suppresses the transactivation of retinoic acid receptor alpha, *Biochem J*, 395 (2006) 653-662.
- [26] X.J. Wang, J.D. Hayes, C.J. Henderson, C.R. Wolf, Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha, *Proc Natl Acad Sci U S A*, 104 (2007) 19589-19594.
- [27] B. Zheng, M. Han, Y.N. Shu, Y.J. Li, S.B. Miao, X.H. Zhang, H.J. Shi, T. Zhang, J.K. Wen, HDAC2 phosphorylation-dependent Klf5 deacetylation and RARalpha acetylation induced by RAR agonist switch the transcription regulatory programs of p21 in VSMCs, *Cell Res*, 21 (2011) 1487-1508.
- [28] G.L. Huang, W. Zhang, H.Y. Ren, X.Y. Shen, Q.X. Chen, D.Y. Shen, Retinoid X receptor alpha enhances human cholangiocarcinoma growth through simultaneous activation of Wnt/beta-catenin and nuclear factor-kappaB pathways, *Cancer Sci*,

106 (2015) 1515-1523.

- [29] D.Y. Shen, Y.H. Zhan, Q.M. Wang, G. Rui, Z.M. Zhang, Oncogenic potential of cyclin kinase subunit-2 in cholangiocarcinoma, *Liver Int*, 33 (2013) 137-148.
- [30] G.L. Huang, Q. Luo, G. Rui, W. Zhang, Q.Y. Zhang, Q.X. Chen, D.Y. Shen, Oncogenic activity of retinoic acid receptor gamma is exhibited through activation of the Akt/NF-kappaB and Wnt/beta-catenin pathways in cholangiocarcinoma, *Mol Cell Biol*, 33 (2013) 3416-3425.
- [31] M. Shtutman, J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, A. Ben-Ze'ev, The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway, *Proc Natl Acad Sci U S A*, 96 (1999) 5522-5527.
- [32] M. Flahaut, R. Meier, A. Coulon, K.A. Nardou, F.K. Niggli, D. Martinet, J.S. Beckmann, J.M. Joseph, A. Muhlethaler-Mottet, N. Gross, The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway, *Oncogene*, 28 (2009) 2245-2256.
- [33] A. di Masi, L. Leboffe, E. De Marinis, F. Pagano, L. Cicconi, C. Rochette-Egly, F. Lo Coco, P. Ascenzi, C. Nervi, Retinoic acid receptors and cancer: From molecular mechanisms to therapy, *Molecular aspects of medicine*, (2014).
- [34] B.C. Das, P. Thapa, R. Karki, S. Das, S. Mahapatra, T.C. Liu, I. Torregroza, D.P. Wallace, S. Kambhampati, P. Van Veldhuizen, A. Verma, S.K. Ray, T. Evans, Retinoic acid signaling pathways in development and diseases, *Bioorg Med Chem*, 22 (2014) 673-683.
- [35] Y. Wang, W. Jin, X. Jia, R. Luo, Y. Tan, X. Zhu, X. Yang, X. Wang, K. Wang,

Transcriptional repression of CDKN2D by PML/RARalpha contributes to the altered proliferation and differentiation block of acute promyelocytic leukemia cells, *Cell Death Dis*, 5 (2014) e1431.

[36] X. Ye, Q. Wu, S. Liu, X. Lin, B. Zhang, J. Wu, J. Cai, M. Zhang, W. Su, Distinct role and functional mode of TR3 and RARalpha in mediating ATRA-induced signalling pathway in breast and gastric cancer cells, *Int J Biochem Cell Biol*, 36 (2004) 98-113.

[37] S. Llanos, J.M. Garcia-Pedrero, L. Morgado-Palacin, J.P. Rodrigo, M. Serrano, Stabilization of p21 by mTORC1/4E-BP1 predicts clinical outcome of head and neck cancers, *Nature communications*, 7 (2016) 10438.

[38] D. Rousseau, D. Cannella, J. Boulaire, P. Fitzgerald, A. Fotedar, R. Fotedar, Growth inhibition by CDK-cyclin and PCNA binding domains of p21 occurs by distinct mechanisms and is regulated by ubiquitin-proteasome pathway, *Oncogene*, 18 (1999) 4313-4325.

[39] H. Clevers, Wnt/beta-catenin signaling in development and disease, *Cell*, 127 (2006) 469-480.

[40] C.Y. Logan, R. Nusse, The Wnt signaling pathway in development and disease, *Annual review of cell and developmental biology*, 20 (2004) 781-810.

[41] N. Cancer Genome Atlas, Comprehensive molecular characterization of human colon and rectal cancer, *Nature*, 487 (2012) 330-337.

[42] P.J. Morin, A.B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, K.W. Kinzler, Activation of beta-catenin-Tcf signaling in colon cancer by mutations in

beta-catenin or APC, *Science*, 275 (1997) 1787-1790.

- [43] O. Voloshanenko, G. Erdmann, T.D. Dubash, I. Augustin, M. Metzger, G. Moffa, C. Hundsruker, G. Kerr, T. Sandmann, B. Anchang, K. Demir, C. Boehm, S. Leible, C.R. Ball, H. Glimm, R. Spang, M. Boutros, Wnt secretion is required to maintain high levels of Wnt activity in colon cancer cells, *Nature communications*, 4 (2013) 2610.
- [44] A. Scholer-Dahirel, M.R. Schlabach, A. Loo, L. Bagdasarian, R. Meyer, R. Guo, S. Woolfenden, K.K. Yu, J. Markovits, K. Killary, D. Sonkin, Y.M. Yao, M. Warmuth, W.R. Sellers, R. Schlegel, F. Stegmeier, R.E. Mosher, M.E. McLaughlin, Maintenance of adenomatous polyposis coli (APC)-mutant colorectal cancer is dependent on Wnt/beta-catenin signaling, *Proc Natl Acad Sci U S A*, 108 (2011) 17135-17140.
- [45] C. Yost, M. Torres, J.R. Miller, E. Huang, D. Kimelman, R.T. Moon, The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3, *Genes Dev*, 10 (1996) 1443-1454.
- [46] D. Wu, W. Pan, GSK3: a multifaceted kinase in Wnt signaling, *Trends Biochem Sci*, 35 (2010) 161-168.
- [47] U.E. Lang, P. Kocabayoglu, G.Z. Cheng, Z. Ghiassi-Nejad, U. Munoz, D. Vetter, D.A. Eckstein, R.A. Hannivoort, M.J. Walsh, S.L. Friedman, GSK3beta phosphorylation of the KLF6 tumor suppressor promotes its transactivation of p21, *Oncogene*, (2012).

[48] S.P. Monga, Role of Wnt/beta-catenin signaling in liver metabolism and cancer,
Int J Biochem Cell Biol, 43 (2011) 1021-1029.

Figure Legends

Figure 1. RAR α expression is upregulated in human CRC tissues and cell lines

(A) Expression of RAR α protein in 32 human CRC specimens and their adjacent specimens. N: non-tumorous tissue, T: tumor tissue. (B) Quantitative analysis of RAR α protein in 32 CRC specimens. (C) Western blotting analysis of expression of RAR α protein in four CRC cell lines and normal colon epithelium cells. $**P < 0.01$.

Figure 2. Clinical significance of RAR α in CRC

(A) Determination of RAR α performed by IHC on tissue microarrays. N: non-tumorous tissue, T: tumor tissue. (B) Representative staining intensity of tumors classified as four different grades. Bar, 50 μ m. (C) Correlations of the RAR α protein expression with overall survival rate.

Figure 3. RAR α knockdown suppresses CRC cell growth

(A) Growth of CRC cell lines RKO and HCT116 stably transfected with RAR α shRNA or control shRNA, and HT29 transiently transfected with RAR α expressing plasmid or control plasmid measured by MTT assay. (B) RAR α knockdown and overexpression confirmed by western blot analysis. (C) EdU assay used to assess DNA synthesis of shRAR α -RKO cells and RAR α -HT29 cells (left). Random ten visions were quantified (right). Bar, 100 μ m. (D) Colony formation assays used to assess the oncogenic potential of RAR α . The relative number of colonies was quantified. (A, C and D) Representatives of three independent experiments with similar results are shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. OD, optical density.

Figure 4. RAR α knockdown reduces the tumor formation

(A) shRAR α -RKO cells and control cells were injected subcutaneously into BALB/c mice, respectively. Tumor growth was monitored for 18 days. (B and C) Eighteen days after injection, mice were euthanized and tumors were harvested for imaging and weight measuring. (D) Hematoxylin and eosin, RAR α and Ki67 staining of tissue sections from control and shRAR α -RKO tumors. (E) Quantitation of Ki67-positive tumor cells. $**P < 0.01$, $***P < 0.001$.

Figure 5. RAR α knockdown induces CRC cell cycle arrest

(A) Cell cycle progress of RKO cells stably transfected with RAR α shRNA or control shRNA measured by flow cytometry. (B) The mRNA levels of cell cycle-related protein in RAR α -knockdown RKO as well as control cells determined by qPCR. A representative experiment of three independent experiments is shown. (C) The expression of RAR α , and cell cycle regulatory proteins in RAR α -knockdown RKO cells and control cells detected at different time after serum addition following synchronization of cells by serum starvation for 24 h. (D and E) The mRNA and protein levels of cell cycle-related proteins in xenograft CRC tumor tissues. $***P < 0.001$.

Figure 6. RAR α suppresses the transcription of p21

(A) Expression of p21 detected by Western blotting. (B) p21 promoter activity decreased by RAR α overexpression, and increased by RAR α knockdown in 293T cells. The activity of firefly luciferase measured and normalized to that of renilla luciferase. (C) Partial sequence of the human p21 promoter. The underlined sequences are proximal RARE sites. (D) Dual-luciferase activity measured in RKO cells as

indicated above. (E) RAR α recruited to the p21 proximal promoter in the absence and presence of ectopic RAR α as measured by ChIP assay. (B and D) A representative experiment of three independent experiments is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7. RAR α knockdown inhibits the GSK3 β / β -catenin pathway

(A) RKO and HT29 cells transfected with the indicated plasmids for 24 h followed by Wnt3a treatment for 6 h. Dual-luciferase activity was measured and normalized to that of renilla luciferase. A representative experiment of three independent experiments is shown. (B) Expression of β -catenin and cyclinD1 in RKO cells with different treatments. Wnt3a, 50 ng/mL. (C) Western blotting analysis of the indicated proteins after transfection for 24 h. (D) Protein levels of phosphorylated β -catenin (p- β -catenin) and phosphorylated GSK3 β at ser9 (pS9-GSK3 β) assessed by Western blotting. * $P < 0.05$, ** $P < 0.01$.

Figure 8. RAR α directly binds to GSK3 β

(A) Co-IP analysis of the interaction of RAR α and GSK3 β in 293T cells. (B) Co-IP analysis of the interaction between endogenous RAR α and GSK3 β in RKO and HCT116 cells. (C) GST pulldown analysis of the interaction between RAR α and GSK3 β in vitro. (D) Schematic of the RAR α protein and the interaction of RAR α with GSK3 β through its C domain. Immobilized GST-GSK3 β or GST proteins incubated with five different RAR α domain proteins overexpressed in 293T cells for GST pulldown assays. (E) Schematic model for the mechanism by which RAR α promotes CRC growth.

Figure 1

Figure 2

A

B

C

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1. Expression of RAR α in colon carcinoma tissues and the adjacent noncancerous tissues

Tissue type	N	RAR α stain grades				χ^2	<i>P</i>
		$\pm$	+	++	+++		
Cancer	100	24	20	41	15	36.98	<0.0001
Noncancer	80	44	25	11	0		

Table 2

Table 2. Associations between RAR α and clinical features of colon carcinoma

Features	n	RAR α		χ^2	P
		L	H		
Age				0.7278	0.3936
≤ 60	27	10	17		
> 60	73	34	39		
Sex				0.3649	0.5458
Male	58	27	31		
Female	42	17	25		
Differentiation				5.226	0.0223*
I-II,II	70	36	34		
II-III ,III	30	8	22		
Pathological types				4.230	0.0397*
Tubular adenocarcinoma	60	28	32		
Mucinous adenocarcinoma	19	14	5		
Adenocarcinoma (unknown)	21	2	19		
Tumor size				1.5999	0.4493
$d \leq 4$	32	12	20		
$4 < d < 10$	59	29	30		
$d \geq 10$	9	3	6		
T				6.6560	0.0359*
T2	4	4	0		
T3	64	24	40		
T4	32	16	16		
N				0.2387	0.8875
N0	52	22	30		
N1	36	16	20		
N2	12	6	6		
M				0.5468	0.4596
M0	95	41	54		
M1	5	3	2		
Clinical stage				9.5500	0.0228*
1	6	6	0		
2	45	17	28		
3	44	20	24		
4	5	1	4		