Estrogen Receptor-β as a Potential Target for Colon Cancer Prevention: Chemoprevention of Azoxymethane-Induced Colon Carcinogenesis by Raloxifene in F344 Rats

Naveena B. Janakiram,[1] Vernon E. Steele[2] and Chinthalapally V. Rao[1]

Abstract Raloxifene, selective estrogen receptor (ER) modulator, is not fully explored in colorectal cancer. In the present study we, (*a*) investigated the effect of raloxifene on ER-positive colon cancer HCT-116 cell growth, (*b*) assessed the relevance of ER-β in colon tumorigenesis, and (*c*) assessed the chemopreventive efficacy of raloxifene against azoxymethane (AOM)-induced colon carcinogenesis using aberrant crypt foci (ACF) as surrogate end point marker. HCT-116 cells treated with raloxifene showed a significant decrease in cell growth associated with a decrease in ER-β expression levels. AOM-induced colon adenocarcinoma showed significant up-regulation of ER-β expression at both the protein and mRNA levels compared with normal mucosa, suggesting that ER-β is positively associated with colon cancer. An assay using five different dietary dose levels (0.31, 0.62, 1.25, 2.5, or 5 ppm) of raloxifene for 6 weeks in male F344 rats found the maximum tolerated dose to be 5 ppm. To evaluate inhibitory properties of raloxifene on colonic ACF, 7-week-old rats were fed experimental diets containing 0, 0.625, 1.25, and 2.5 ppm of raloxifene. After 1 week, rats received s.c. injections of AOM, 15 mg/kg body weight, once weekly for 2 weeks. Rats continued to receive respective experimental diets and sacrificed 8 weeks after the last AOM treatment. Raloxifene given in the diet significantly inhibited AOM-induced total colonic ACF (31-40%; $P < 0.001$-0.0005) and multicrypt (four or more) aberrant foci (23-50%; $P < 0.05$-0.005) in F344 rats. Our findings suggest that ER-β acts as a colon tumor promoter and raloxifene as an antagonist to ER-β, providing protection against colon carcinogenesis.

Colon cancer includes both hereditary and nonhereditary types with incidence rates increasing in age groups above 50 (1). Reducing the number of deaths from colorectal cancer depends on detecting and removing precancerous colorectal polyps as well as detecting and treating the cancer in its early stages. Given the high incidence rates of colon cancer in the aging population and high mortality rates for the advanced disease, new agents for prevention are needed. Expression patterns of different molecules in various signaling pathways in carcinogenic tissue and normal tissues help one to understand their positive/negative role in carcinogenesis. This will help in choosing a potential chemopreventive agent.

Estrogen signaling is complicated and influences several physiologic and pathologic outcomes. Estrogen receptor (ER) signaling pathways regulate important physiologic processes, such as cell growth and apoptosis (2). Raloxifene is the prototypical selective ER modulator (SERM) that has been shown to prevent osteoporosis and breast cancer (3, 4). SERMs are synthetic estrogen ligands that can exhibit either an estrogenic or an antiestrogenic effect depending on the tissue types (5, 6). Specifically, SERMs are usually ER agonists in bone, liver, and the cardiovascular system; ER antagonists in the brain and breast; and mixed ER agonists/antagonists in the uterus (5, 7). Previous studies have shown that raloxifene binds to both ER-α and ER-β with high affinity (6, 8); however, the most predominant receptor in the colon is ER-β (9). Among the SERMs, raloxifene is unique in that it is an estrogen antagonist in the uterus (10). The mechanism for the observed tissue-specific effect of SERMs is attributable to differences in coregulator recruitment in a tissue-specific manner (11). Despite the widespread clinical application of raloxifene, very little is understood about how this might affect colon cancer.

Since the discovery of ER-β, no evidence has suggested a fixed role for estrogen/ER in colon cancer. Studies to elucidate the role of ER-β association with colon cancer remain

Authors' Affiliations: [1]Department of Medicine, Hem-Onc Section, OU Cancer Institute, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma and [2]Division of Cancer Prevention, Chemopreventive Agent Development Research, Rockville, Maryland
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Requests for reprints: Chinthalapally V. Rao, Department of Medicine, Hem-Onc Section, OU Cancer Institute, University of Oklahoma Health Sciences Center, 975 Northeast 10th Street, BRC Building II, Room 1203, Oklahoma City, OK 73104. Phone: 405-271-3224; Fax: 405-271-3225; E-mail: cv-rao@ouhsc.edu.

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inconclusive. Cancer epidemiologic studies in postmenopausal women show an inverse relationship between the use of hormonal replacement therapy (HRT) and colorectal adenoma and colorectal cancer development (12). This observation was further confirmed in the Women's Health Initiative, a prospective, randomized clinical trial of 16,608 postmenopausal women designed to determine the effects of HRT on multiple health outcomes. Women's Health Initiative participants who received HRT over an average follow-up of 5.2 years showed a relative risk of colorectal cancer of 0.63 (95% confidence interval, 0.43-0.92) compared with women who received the placebo (13). Moreover, SEER data from 2000 to 2003 show that cumulative colorectal cancer incidence and mortality are delayed in women by 4 to 8 years, relative to men (14). These intriguing findings lead us to ask whether ER-β and/or mediated signaling are associated with colon carcinogenesis and whether SERMs can be used to prevent colorectal cancer.

In the present investigation, azoxymethane (AOM)-induced control rat colon tumors and normal mucosa were tested for ER-β (protein and mRNA) expression to further understand the role of ER-β in colon tumor biology. Because of the low prevalence of ER-α expression in colorectal cancer, we studied the potential role of ER-β in the development of colorectal cancer. Raloxifene (antagonistic to ER signaling in the colon) was tested for its efficacy on human colon cancer cell line (HCT-116) for the modulation of ER-β, proliferating antigen, proliferating cell nuclear antigen (PCNA) expressions, and growth. Furthermore, we assessed the maximal tolerated dose (MTD) of raloxifene and chemopreventive efficacy in AOM-induced rat colon carcinogenesis model using aberrant crypt foci (ACF) as a surrogate end point marker.

Materials and Methods

Cell lines and reagents

HCT-116, human colon cancer cell line, was obtained from the American Type Culture Collection and maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin). Cells were incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2. The origin of HCT-116 cells is from adult male colorectal carcinoma patient HCT-116 cells, and these cell lines express ER-β. Human colon cancer DLD-1, ER-β–negative cell line, was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin). DLD-1 was studied to compare its sensitivity/resistance on raloxifene treatment with ER-β–positive cell line HCT-116. HCT-116 cells were chosen for further *in vitro* experiments. All experiments were carried out in 70% confluent cell growth in duplicates and each set of experiments was run thrice. Raloxifene was kindly provided by the National Cancer Institute Chemopreventive Drug Repository (Rockville, MD). A 2 mg/10 mL stock was prepared in DMSO.

Effect of raloxifene on cell growth

Viability assay for HCT-116 and DLD-1 cells was done by trypan blue exclusion method. Exponentially growing HCT-116 and DLD-1 cells were seeded in complete medium along with raloxifene, and after 24 h, floating and adherent cells were collected and suspended in 25 μL PBS. These cells were mixed with 0.4% trypan blue, and the stained and unstained cells were counted using hemocytometer. Percentage of unstained cells represents the percentage of viable cells.

Detection of ER-β and PCNA by Western blotting

Expression of ER-β and PCNA protein was analyzed in HCT-116 cells. After treatment with various concentrations of raloxifene (0-5 μmol/L) for 24 h, cells were harvested and lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] on ice. After centrifugation, supernatants were collected and the protein content was measured using a Bio-Rad protein assay kit. Equal amounts of protein from each extract were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Toyo Roshi) using the Bio-Rad electrotransfer system. Blots were blocked by incubating in 5% milk with Tris-HCl (pH 7.5) and 0.1% Tween 20 for 1 h at room temperature and probed overnight at 4°C with rabbit antiestrogen β polyclonal antibody (Santa Cruz Biotechnology) for ER-β protein and mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology) for PCNA. Antibodies were diluted 1:1,000 with 5% milk in Tris-HCl (pH 7.5) and 0.1% Tween 20. The immunoblots were then probed with horseradish peroxidase–conjugated anti-rabbit IgG for ER-β and horseradish peroxidase–conjugated anti-mouse IgG for PCNA [1:2,000 diluted with 5% milk in Tris-HCl (pH 7.5)]. After the final wash, the signal was detected with an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.).

Immunocytochemistry

HCT-116 cells were grown on slides and treated with 0 or 5 μmol/L of raloxifene. After 24 h of treatment, the slides with the cells were fixed in 3% formaldehyde for 15 to 20 min and washed in PBS for 5 min thrice. The slides were rehydrated and washed with PBS for 15 min at room temperature. Specimens were treated in water bath in 0.01 mol/L citrate buffer (pH 6.0) for 30 min at 100°C, slowly cooled to room temperature, and washed with PBS for 5 min at room temperature. After quenching endogenous peroxide with 3% hydrogen peroxide in PBS for 10 min at room temperature, the sections were incubated with a blocking solution (supplied by Zymed kit) for 60 min at room temperature. Then, the slides were incubated overnight at 4°C with a 1:200 dilution of anti-ER-β and 1:300 dilution of anti-PCNA (Santa Cruz Biotechnology). After washing with PBS thrice, the slides were incubated with secondary antibody for ER-β and PCNA for 2 h. The color reaction was developed by 3,3′-diaminobenzidine according to the manufacturer's instructions given in the kit supplied by Zymed Laboratories.

In vivo experiments

Animals, diet, and care. All animal experiments were done in accordance with the NIH guidelines and the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee–approved protocol. Male F344 rats were obtained from Charles River Laboratories, housed under standardized conditions (21°C, 60% relative humidity, 12-h light/12-h dark cycle, 20 air changes/h), and fed a standard laboratory rodent chow and drinking water until initiation of the experiment. Diets were prepared based on the modified AIN-76A containing 5% corn oil by weight (American Institute of Nutrition). Raloxifene was premixed with a small quantity of casein and then blended into bulk diet using a Hobart Mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Raloxifene content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. Rats were allowed *ad libitum* access to the respective diets and tap water.

Induction of colon tumors in F344 rats. To assess the expression levels of ER-β in colon tumors and normally appearing mucosa, rats were fed control AIN-76A diet and treated with AOM (15 mg/kg body weight once weekly for 2 wk). Colonic tumors and normal tissues were harvested 40 wk after the AOM treatment.

Western immunoblotting for ER-β. Colon tumor and normal mucosa were homogenized in a tissue homogenizer in lysis buffer

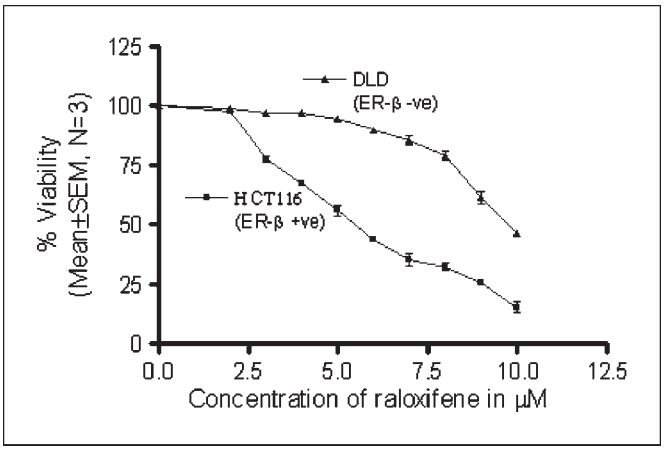

Fig. 1. Raloxifene-induced cytotoxicity determined by trypan blue assay on HCT-116 (ER-β positive) and DLD (ER-β negative) cells. Cells were exposed to 2.0 to 10.0 μmol/L of raloxifene for 24 h. IC_{50} is calculated based on 50% inhibition of cell proliferation at a specific concentration of raloxifene. Points, mean of three independent experiments; bars, SE. *, *P* value. Values are significantly different from control by unpaired two-tailed *t* test (*, $P < 0.106$; **, $P < 0.007$-0019; ***, $P < 0.0001$) near the points. DLD cell line was observed to be more resistant compared with HCT-116 cell line.

[50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] on ice. After centrifugation, supernatants were collected and the protein content was estimated as described above under *in vitro* experiments.

Reverse transcription-PCR for ER-β. Total RNA from samples (tumor and mucosa) was extracted using Totally RNA kit for isolation of total cellular RNA (Ambion) as per the manufacturer's instructions. Equal quantities of DNA-free RNA were used for reverse transcription reactions for making cDNA using SuperScript reverse transcriptase (Invitrogen). PCR was done for ER-β using the following conditions: denaturation at 94°C for 3 min, followed by 32 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min. Oligonucleotide primer sequences used were as follows: 5′-TTCCCGGCAGCACCAGTAA-3′ (sense) and 5′-TCCCTCTTTGCGTTTGGACTA-3′ (antisense). PCR was done using the Taq polymerase master mix (Qiagen, Inc.). The PCR products were visualized and photographed under UV illumination.

Immunohistochemistry. Rat colon tumor and normal tissue sections (obtained by AOM treatment) were dried at 56°C, deparaffinized in xylene, rehydrated, and washed with PBS for 15 min at room temperature. Specimens were treated as explained above under *in vitro* experiments. Expression of ER-β was observed and pictures were photographed under Olympus IX71 microscope connected to a digital imaging system with SPOT RT software version 3.0.

Determination of the MTD of raloxifene. The purpose of this MTD study was to determine the maximum tolerable dose of raloxifene in F344 rats. MTD is defined as the highest dose that causes no more than 10% weight loss, compared with the appropriate control diet group, and does not produce mortality or any clinical signs of toxicity that would be predicted to shorten the natural life span of the animal. At 7 wk of age, groups of male F344 rats (six rats per group) were fed the AIN-76A diet containing 0.31, 0.625, 1.25, 2.5, or 5 ppm of raloxifene. Body weights were recorded once weekly for 6 wk. All animals were killed after 6 wk, and the organs were examined grossly for any abnormalities.

Experimental design for efficacy of raloxifene. The experiment was designed to evaluate the efficacy of 062, 1.25, and 2.5 ppm of raloxifene administered continuously from 1 wk before carcinogen treatment until the end of the study. The dose selection was based on our MTD study. At 7 wk of age, groups of rats ($n = 18$ rats per group; AOM-treated 12 rats plus vehicle (saline)-treated 6 rats) were fed either the control diet or experimental diet containing 0, 0.62, 1.25, or 2.5 ppm of raloxifene. At 8 wk of age, rats intended for carcinogen treatment were injected s.c. with AOM (Midwest Research Institute) at a dose rate of 15 mg/kg body weight once weekly for 2 wk, and those intended for vehicle treatment received an equal volume of normal saline. These dietary regimens were continued until termination of the experiment (i.e., 8 wk after the second AOM treatment). Rats were killed by CO_2 euthanasia, and all organs were examined grossly. Colons were evaluated for ACF. For this evaluation, they were slit open lengthwise from the anus to the cecum and then fixed flat with mucosa on the upper side between filter papers in 10% buffered formalin.

Quantification of ACF. Topographical analysis of the colonic mucosa was done according to Bird (15) and is routinely done in our laboratory (16). After a minimum of 24 h, fixed colons were stained with 0.2% methylene blue solution for 5 to 10 min, placed mucosal side up on a microscopic slide, and viewed under a light microscope. The total number of ACF in the entire colon was determined in every 2-cm section of the colon, starting from the distal (taken as 0 cm) to the proximal end of the colon. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells, and easily discernible pericryptal zone. The variables used to assess the aberrant crypts were incidence and multiplicity. Aberrant crypt multiplicity was determined as the number of crypts in each focus and categorized as containing up to four or more aberrant crypts per focus.

Statistical analysis. Data are reported as mean ± SE. Statistical differences between control and treated groups were evaluated using unpaired *t* test with Welch's correction. Differences between groups are considered significant at $P < 0.05$.

Results

Effect of raloxifene on HCT-116 (ER-β positive) and DLD (ER-β negative) cell growth

HCT-116 and DLD-1 cells were exposed for 24 hours to raloxifene at concentrations varying from 0 to 10 μmol/L. As shown in Fig. 1, HCT-116 cell growth inhibition was observed in a dose-dependent manner. At ~6 μmol/L, raloxifene induced 50% inhibition of HCT-116 cell growth after 24 hours of exposure. At a concentration of 8 μmol/L or above, raloxifene induced significant toxicity in HCT-116 cells, whereas raloxifene failed to induce similar growth inhibition and toxicity on DLD-1 cells at the same concentrations (Fig. 1). Thus, all the *in vitro* experiments with raloxifene were done at a concentration of <6 μmol/L with HCT-116 cells.

Raloxifene decreases PCNA and ER-β expression

The effects of raloxifene on PCNA and ER-β protein expression in HCT-116 cells were analyzed by Western blot and immunocytochemistry methods. As shown in Fig. 2A, raloxifene suppressed the PCNA expression ~75% (Western blot signal) in HCT-116 cells compared with untreated cells. Furthermore, these results are supported by immunocytochemical results as shown in Fig. 2B. The effect of raloxifene on ER-β expression is shown in Fig. 2C and D. Treatment of HCT-116 cells with 5 μmol/L raloxifene (for 24 hours) showed >90% suppression of ER-β expression by Western blot analysis (Fig. 2C). In addition, Fig. 2D immunocytochemical results furthermore confirm a significant down-regulation of ER-β expression levels in HCT-116 cells.

Overexpression of ER-β in colon tumors

To understand the relevance of ER-β in colon carcinogenesis, we assessed the levels of ER-β receptors in AOM-induced

rat colonic tumor tissues and normal-appearing colonic mucosa by immunohistochemistry methods, Western immunoblotting, and reverse transcription-PCR (RT-PCR; Figs. 2E and 3A and B). As shown in Fig. 3A, AOM-induced colonic adenocarcinomas showed significant up-regulation of ER-β receptor expression by immunohistochemistry, which was confirmed by Western immunoblotting and RT-PCR. As shown in Fig. 2E, colonic epithelial cells in tumors stained highly positive for ER-β expression, whereas normal colonic crypt epithelial cells stained very poorly with limited or no staining. Furthermore, the up-regulation of ER-β protein and mRNA levels was observed in AOM-induced colonic tumors but not in tumor-adjacent normal-appearing colonic mucosa (Fig. 3A and B). Taken together, the above results clearly suggest that ER-β expression is positively correlated with human HCT-116 colon cancer cell growth and AOM-induced colon adenocarcinomas.

MTD and dose selection of raloxifene for preclinical efficacy study

Administration of 5 ppm raloxifene in the diet for 6 weeks produced significant body weight loss (~10%; $P < 0.05$) when compared with rats fed the control diet. However, rats fed 2.5 ppm or less dose levels of raloxifene showed no effect on the body weight retardation and any observable toxicity (data not shown). Furthermore, the food intake of animals in the experimental groups did not show any variation. Thus, the MTD of raloxifene was found to be <5.0 ppm in male F344 rats when the agent was given in AIN-76A modified diet. Although 5 ppm raloxifene in the diet induced body weight loss, it failed to show any identifiable pathologic changes. Based on dose tolerability results, we have tested 0.625, 1.25, and 2.5 ppm dose levels of raloxifene in the chemopreventive efficacy study.

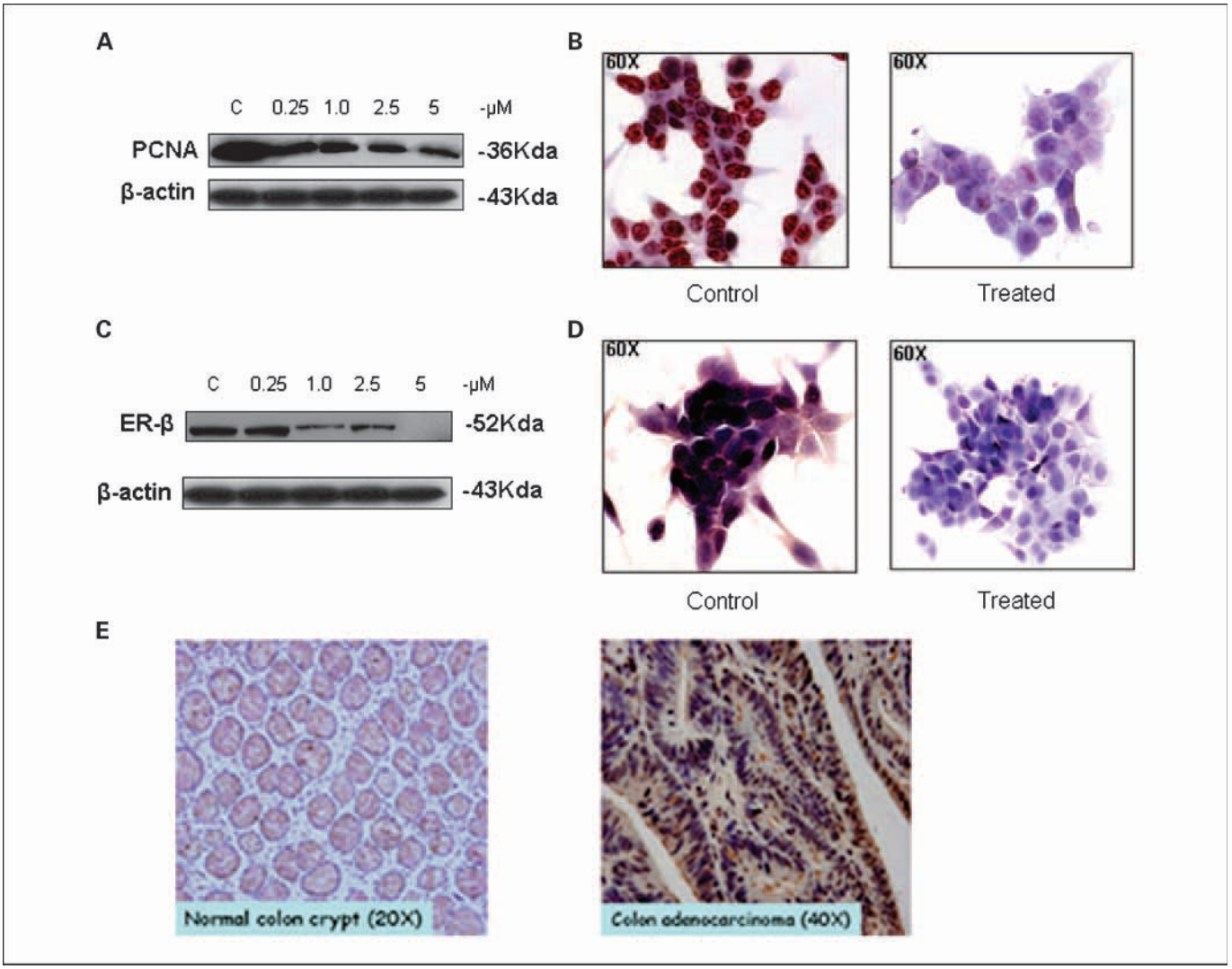

Fig. 2. *A* and *C*, protein expression levels of PCNA and ER-β were carried out by Western immunoblotting from the protein extracts of HCT-116 cells treated with various concentration of raloxifene. *B* and *D*, immunocytochemistry for PCNA and ER-β expression in raloxifene-treated and untreated HCT-116 cells. Intense positive nuclear staining (*brown stain*) is observed in control cells with diminished/spotty staining in treated HCT-116 cells. *E*, immunohistochemical analysis of ER-β protein expression in rat colon adenocarcinomas and normal colonic mucosa. An intense positive staining (*brown stain*) is observed in tumor tissue compared with normal colonic mucosa.

Effect of raloxifene on multicrypt ACF formation

The effect of different dose levels of dietary raloxifene on AOM-induced colonic ACF formation is shown in Fig. 4A and B. Rats fed diets containing 0.625, 1.25, and 2.5 ppm of raloxifene showed a significant decrease in the number of total mean ACF/colon (31-40%; $P < 0.001$-0.0005) when compared with rats fed the control diet (Fig. 4A). Rats fed with the control diet and treated with AOM induced 168 ± 11 (mean ± SE) colonic ACF containing mean average of 31 of one crypt foci, 82 of two crypt foci, 32 of three crypt foci, and 23 of four or more crypt foci (Fig. 4A). Although all the dose levels tested showed significant inhibition of total ACF suppression, they had a limited dose-dependent effect. Importantly, AOM-induced colonic aberrant crypts containing four or more were reduced significantly (23-50%; $P < 0.05$-0.005) in rats fed the raloxifene diet in a dose-response manner (Fig. 4B).

Discussion

The present investigation is part of a large-scale screening of agents in identifying and understanding the molecular targets that would help in establishing effective chemopreventive agents for the prevention and treatment of colorectal cancer. Evidence from preclinical and clinical studies supports that use of SERM (raloxifene, tamoxifen, and toremifene) provides protection against several cancers (3, 4, 17, 18). Both tamoxifen and raloxifene were shown to protect against chemically induced breast cancer (19), whereas toremifene was shown to prevent the prostate cancer in the transgenic adenocarcinoma of mouse prostate model (18). As stated in the introduction, there is evidence in support of beneficial effects of HRT in breast cancer, and also observed to have decreased risk and increase in survival of colon cancer patients (12–14). However, limited information exists on ER-α and ER-β and their modulation in the colon leading to reduced risk of colon cancer. Raloxifene has minimal adverse effects while effectively preventing osteoporosis and cancer; hence, in the present study, raloxifene is assessed for the role of ER-β in the *in vitro* and *in vivo* colon cancer models.

Our finding shows that raloxifene suppresses human colon cancer HCT-116 cell growth at low concentrations and is associated with suppression of ER-β expression. As anticipated, we detected a significant expression of PCNA in HCT-116 control cells, an important enzyme in DNA replication and a surrogate marker of proliferation, which is estrogen responsive and has been implicated in tumor growth. Whereas HCT-116 cells treated with raloxifene showed significant inhibition of PCNA expression, DLD-1 cells that lack ER-β was less sensitive to raloxifene (Fig. 1) and had limited effect on PCNA expression (data not shown). Our data suggest that the expression of ER-β may render HCT-116 colon cancer cells resistant to the growth inhibition and help in proliferation. It was reported that ER is directly involved in the regulation of colon cancer cell growth (20). Raloxifene seems to activate specific intracellular pathways by suppressing ER-β leading to cell death/growth suppression. These results are consistent with previous reports showing ER-β expression in colon cancer cells (21–23). The consistent expression of ERs in human colon cancer cells suggests that estrogen/ERs may be important targets in colon cancer patients. Furthermore, the inference about ER-β in resistance to growth inhibition and help in proliferation is supported by literature in breast and colon cancer. Jensen et al. (24) observed that ER-β was associated with breast tumors that had the highest proliferative markers (Ki67 and cyclin A), suggesting its role in proliferation. Another study reported that treatment with phytoestrogen genistein in MCF-7 breast cancer cell line caused an increase in ER-β protein before causing an increase in proliferation (25). More importantly, phytoestrogenic agents such as genistein enhance the AOM-induced colon carcinogenesis, which in part explains the importance of ER-β in colon tumor cell proliferation and tumor promotion (26). ER-β expression has been associated with elevated levels of cell proliferation markers in tumors (27). ER-β mRNA is also elevated significantly in the tamoxifen-resistant tumors compared with tamoxifen-sensitive tumors of breast cancer patients (28). Treatment by different SERMs may be more effective if any cancer is first characterized more vigorously for components within the cell (e.g., ER-α, ER-β, coactivators, and corepressors), which will induce a favorable effect by a specific SERM. Hence, in the present study, raloxifene, a SERM, is used, which functions as antagonist to ER in colon cancer, whereas phytoestrogen genistein acts as agonist. These studies suggest that ER-β may promote tumor cell survival and tumor formation as evidenced by the above *in vitro* investigations.

Previous studies have not been consistent in establishing a definitive role of ER-β in colon cancer (29, 30). However, those observations taken together suggest that measurement of ER levels is of importance when evaluating the likely response of SERMs because it is well established that response occurs selectively in ER-positive diseases. Present studies on ER-β expression were carried out to provide clear insights into its potential role in colon tumor progression. Based on the immunohistochemistry, Western blot, and RT-PCR methods, our results suggest that rat colon adenocarcinomas overexpress ER-β but not in the normal colon. Our results also suggest that ER-β is the principal ER protein found in colon cells. Unlike ER-β, we found that ER-α protein expression levels were not significantly different in rat colon adenocarcinomas compared with normal colonic mucosa (data not shown). This information suggests a major role for ER-β than ER-α in colon tumor progression. Other studies also support the above notion that colon tumor epithelial cells overexpress ER-β (23, 28). Although at present the specific functions of ER-β in colon cancer are not fully known and the exact role of estrogen and its receptors in benign and malignant colonic epithelial cells has not been fully established, there is evidence suggesting that estrogens and its receptors are important regulators of the colon physiology (30). However, these ER-β knockout mice do not develop any colonic neoplasia or precursor lesions even after 18 months, suggesting that loss of ER-β may not be associated with increased risk of intestinal neoplasia (30). Furthermore, recent studies with $Apc^{Min/+}$ and ER-$\beta^{-/-}$ compound mouse do not provide any conclusive evidence on role of ER-β by not showing a significant difference in the colon tumor formation (31, 32). In fact, ER-β was proposed to mediate estrogenic effects on colon cancer susceptibility, and the role of ER-α is expected to be minor (23). The positive association of ER-β in colon tumorigenesis suggests that ER-β may play an important role in colon tumor development and, thus, is considered to be a potential target for colon cancer prevention and treatment.

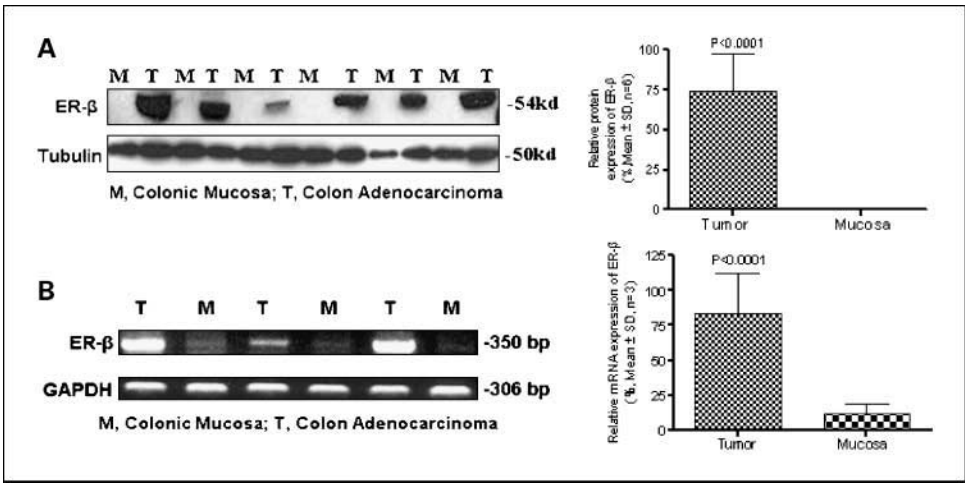

Fig. 3. *A*, up-regulation of ER-β protein expression in AOM-induced colonic adenocarcinomas in comparison with normal mucosa by Western immunoblotting. *B*, up-regulation of ER-β mRNA expression in AOM-induced colonic adenocarcinomas in comparison with normal mucosa by RT-PCR. Statistical "*P* value" is shown as a bar graph adjacent to each figure.

Hence, we tested ER-β antagonist raloxifene for its chemopreventive properties in AOM rat colon carcinogenesis.

Based on the MTD study observations, raloxifene at the dietary dose of <5 ppm in F344 male rats lacks overt toxicity. Chemopreventive efficacy of raloxifene (<5 ppm) was tested using ACFs as a surrogate end point marker. Our results suggest that raloxifene significantly suppressed AOM-induced total ACF formation. Importantly, raloxifene suppressed multicrypt ACF in a dose-dependent manner, clearly suggesting its potential for colon tumor inhibitory properties. Previous studies have shown that reduction of colon crypt multiplicity of four or more aberrant crypts per focus has been a consistent predictor of colon tumor inhibition in rats (33–35); the present study used this criterion to evaluate raloxifene for its potential chemopreventive properties. Our results are consistent with previous observations with raloxifene showing antitumor effects in 7,12-dimethylbenz(a)anthracene–induced rat mammary tumors (36) and inhibition of prostate cancer cell growth (37).

ACF has been recognized as an early preneoplastic lesion of colon cancer (38–40). It is generally observed that agents that inhibit colonic ACF formation would show chemopreventive activity against colon cancer (41). The mechanism by which raloxifene inhibits the development of ACFs is not fully known; it may be through different modes of action of this agent. AOM is a colorectal-selective carcinogen in many rodent models and particularly in F344 rat, and AOM-induced colon carcinogenesis is somewhat similar to human colorectal cancer, in regional distribution of tumors, histopathology, and progression of tumor growth, and more importantly in causing molecular changes during the development of colorectal cancers. At present, we do not have any studies that directly show that AOM (as genotoxic agent) can induce or directly up-regulate ER-β receptor. Previous studies have shown that AOMs induce mutations in APC, β-catenin, K-ras, etc., and thereby up-regulate tumor cell growth by promoting effectors such as cyclooxygenase-2, inducible nitric oxide synthase, epidermal growth factor receptor, etc., and possibly ER-β receptor, based on the present study results. ERs are reported to be present at different locations of a cell having multiple functions. For example, caveolin-1 is associated with ERs (42). Raloxifene may function by inhibiting ER-β signaling through

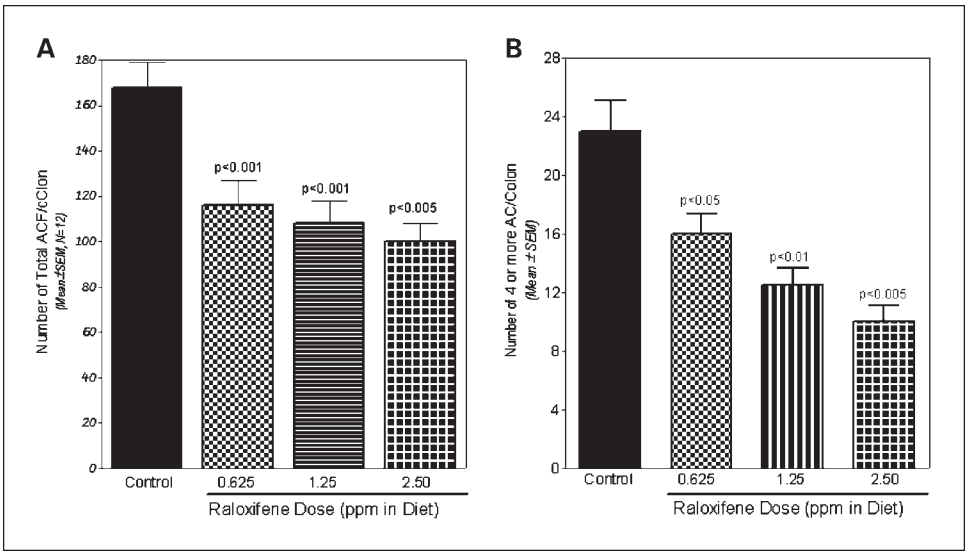

Fig. 4. *A*, effect of raloxifene on AOM-induced total mean number of colonic ACF formation in F344 rats. *B*, effect of raloxifene on AOM-induced colonic four or more aberrant crypt formation in F344 rats.

caveolin-1. Studies from our laboratory show that caveolin-1 is overexpressed in AOM-induced colonic tumor but not in normal mucosa and, importantly, is associated with human colon cancer cell proliferation rates (43). Raloxifene treatment was observed to decrease cyclooxygenase-2 and inducible nitric oxide synthase expression in inflamed areas in rats (44). This possibility of molecular mechanisms of raloxifene needs to be studied in colorectal adenoma inhibition. Further studies are required to understand exact molecular mechanisms of raloxifene or SERMs in colon cancer.

The present preclinical studies showed that treatment with raloxifene resulted in significant inhibition of ACFs in the colon carcinogenesis model that strongly express ER-β in tumors on AOM treatment. This suggests that raloxifene may have a functional relationship with ER-β in colon cancer, making ER-β a strong target for SERM therapy. Regulation/modulation of ER-β may help in prevention of carcinogenesis, which is not very well established in colon. It is unknown if the chemopreventive effects of raloxifene function directly via ER-β. As per our observation, there was no change in ER-α expression in the tumor epithelial cells; as its role in the colon was suggested to be minor, it may be possible that raloxifene is functioning through ER-β. In an exploratory trial with primary breast cancer patients, 60 mg/d raloxifene showed a significant antiproliferative effect in ER-positive breast cancer, shown by the decrease in Ki67 (45). Study of Tamoxifen and Raloxifene trial results suggest that raloxifene reduced the risk of developing invasive breast cancer by ~50%. Importantly, women who were taking raloxifene daily had 36% fewer uterine cancers and 29% fewer blood clots compared with tamoxifen group (17). Thus, development of raloxifene for colon cancer prevention possesses several advantages over the tamoxifen or the traditional nonsteroidal anti-inflammatory agents.

In summary, the present *in vivo* and *in vitro* studies suggest that ER-β is a potential target for colon cancer prevention and possibly for treatment. Raloxifene shows anticarcinogenic properties in rat colon model, further implicating the potential of SERMs in colon cancer prevention. In view of the above, a long-term chemopreventive efficacy study using adenocarcinoma as end point marker with raloxifene is needed to further establish its clinical use in high-risk colon cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. O'Shaughnessy JA, Kelloff GJ, Gordon GB, et al. Treatment and prevention of intraepithelial neoplasia: an important target for accelerated new agent development. Clin Cancer Res 2002;8:314–46.
2. Segars JH, Driggers PH. Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. Trends Endocrinol Metab 2002;13:349–54.
3. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene. JAMA 1999;282:637–45.
4. Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene in postmenopausal women. JAMA 1999;281:2189–97.
5. Katznellenbogen BS, Katznellenbogen JA. Defining the "S" in SERMs. Science 2002;295:2380–81.
6. Kuiper GGJM, Lemmen JG, Carlsson B. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor-β. Endocrinology 1998;3: 4252–63.
7. Lonard DM, Smith CL. Molecular perspectives on selective estrogen receptor modulators (SERMs): progress in understanding their tissue-specific agonist and antagonist actions. Steroid 2002;67:15–24.
8. Bryant HU, Glasebrook AL, Yang NN, Sato M. An estrogen receptor basis for raloxifene action in bone. J Steroid Biochem Mol Biol 1999;69:37–44.
9. Enmark E, Pelto-Huikko M, Randien K. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 1997;82:4258–65.
10. Bryant HU, Wilson PK, Adrian MD. Selective estrogen receptor modulators: pharmacological profile in the rat uterus. J Soc Gynecol Investig 1996; 3:152A.
11. Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002; 295:2465–8.
12. Chen MJ, Longnecker MP, Morgenstern H, Lee ER, Frankl HD, Haile RW. Recent use of hormone replacement therapy and the prevalence of colorectal adenomas. Cancer Epidemiol Biomarkers Prev 1998;7:227–30.
13. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA 2002;288:321–33.
14. Ries LAG, Eisner MP, Kosary CL. SEER cancer statistics review. Bethesda (MD): National Cancer Institute; 2003. p. 1975–2001.
15. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett 1995;93:55–71.
16. Naveena BJ, Indranie C, Altaf M, Steele VE, Rao CV. β-Ionone inhibits colonic aberrant crypt foci in rats, suppresses cell growth and induces retinoid X receptor α in human colon cancer cells. Mol Cancer Ther 2008;7:181–90.
17. Vogel VG, Costantino JP, Wickerham DL. Effect of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. JAMA 2006;295:2727–41.
18. Raghow S, Hooshdaran MZ, Katiyar S, Steiner MS. Toremifene prevents prostate cancer in the transgenic adenocarcinoma of mouse prostate model. Cancer Res 2002;62:1370–6.
19. Jordan VC, Morrow M. Tamoxifen, raloxifene, and the prevention of breast cancer. Endocr Rev 1999; 20:253–78.
20. Xu X, Thomas ML. Estrogen receptor-mediated direct simulation of colon cancer cell growth *in vitro*. Mol Cell Endocrinol 1994;105:197–201.
21. Wong NACS, Malcomson RDG, Jodrell ID, et al. ER-β isoform expression in colorectal carcinoma: an *in vivo* and *in vitro* study of clinicopathological and molecular correlates. J Pathol 2005;207:53–60.
22. Campbell-Thompson M, Lynch IJ, Bhardwaj B. Expression of estrogen receptor (ER) subtypes and ER-β isoforms in colon cancer. Cancer Res 2001;61:632–40.
23. Fiorelli G, Picariello L, Martineti V, Tonelli F, Brandi ML. Functional estrogen receptor β in colon cancer cell lines. Biophys Res Commun 1999;261:521–7.
24. Jensen EV, Cheng G, Palmieri C, et al. Estrogen receptors and proliferation markers in primary and recurrent breast cancer. Proc Natl Acad Sci U S A 2001;4:4.
25. Krista AP, Thompson LU. Ligand-induced regulation of ERα and ERβ is indicative of human breast cancer cell proliferation. Breast Cancer Res Treat 2003;81:209–21.
26. Rao CV, Wang C-X, Simi B, et al. Enhancement of experimental colon cancer by genistein. Cancer Res 1997;57:3717–22.
27. Speirs V, Malone C, Walton DS, Kerin MJ, Atkin SL. Increased expression of estrogen receptor β mRNA in tamoxifen-resistant breast cancer patients. Cancer Res 1999;59:5421–4.
28. Konstantinopoulos PA, Kominea AV, Oros G, et al. Oestrogen receptor β (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. Eur J Cancer 2003;39:1251–8.
29. Witte D, Chirala M, Younes A, Li Y, Younes M. Estrogen receptor β is expressed in human colorectal adenocarcinoma. Hum Pathol 2001;32:940–44.
30. Wada-Hiraike O, Imamov O, Hiraike H, et al. Role of estrogen receptor β in colonic epithelium. Proc Natl Acad Sci U S A 2006;103:2959–64.
31. Giroux V, Lemay F, Bernatchez G, Yolaine R, Carrier JC. Estrogen receptor β deficiency enhances small intestinal tumorigenesis in $Apc^{Min/+}$ mice. Int J Cancer 2008;123:303–11.
32. Cho NL, Javid SH, Carothers AM, Redston M, Beragnolli MM. Estrogen receptors α and β are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. Cancer Res 2007;67:2366–72.
33. Rao CV, Desai D, Simi B, Kulkarni N, et al. Inhibitory effect of caffeic acid esters on azoxymethane-induced biochemical changes and aberrant crypt foci formation in rat colon. Cancer Res 1993;53:4182–8.
34. Rao CV, Simi B, Reddy BS. Inhibition by dietary

curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. Carcinogenesis 1993;14:2219–25.

35. Rao CV, Yoshinobu H, Cooma I, Reddy BS. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. Cancer Res 2001;61:1927–33.

36. Wakeling AE, Valcaccia B. Antiestrogenic and antitumor activities of a series of non-steroidal antiestrogens. J Endocrinol 1983;99:455–64.

37. Yi KI, Kim B-C, Seong Do H, et al. Raloxifene, a mixed estrogen agonist/antagonist, induces apoptosis in androgen-independent human prostate cancer cell lines. Cancer Res 2002;62:5365–9.

38. Mclellan EA, Bird RP. Aberrant crypts: potential preneoplastic lesions in the murine colon. Cancer Res 1988;48:6187–92.

39. Mclellan E, Medline A, Bird RP. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci-putative preneoplastic lesions. Cancer Res 1991;51:5270–4.

40. Pretlow TP, O'Riordan MA, Pretlow TG, Stellato TA. Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. J Cell Biochem 1992; 166:55–62.

41. Wargovich MJ, Chen CD, Jimenez A, et al. Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. Cancer Epidemiol Biomarkers Prev 1996;5: 355–60.

42. Chambliss KL, Yuhanna IS, Anderson RGW, et al. ER-β has nongenomic action in caveolae. Mol Endocrinol 2002;16:938–46.

43. Patlolla JM, Swamy MV, Raju J, Rao CV. Overexpression of caveolin-1 in experimental colon adenocarcinomas and human colon cancer cell lines. Oncol Rep 2004;11:957–63.

44. Emanuela E, Anna I, Giuseppina MR, et al. Raloxifene, a selective estrogen receptor modulator, reduces carrageenan-induced acute inflammation in normal and ovariectomized rats. Endocrinology 2005;146:3301–8.

45. Dowsett M, Bundred NJ, Decensi A, et al. Effect of raloxifene on breast cancer cell Ki67 and apoptosis. Cancer Epidemiol Biomarkers Prev 2001;10: 961–6.

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Naveena B. Janakiram, Vernon E. Steele and Chinthalapally V. Rao

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