BIOCHEMICAL PHARMACOLOGY 77 (2009) 186–195

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Suppression of β-catenin signaling by liver X receptor ligands

Shigeyuki Uno [a], *Kaori Endo* [a], *Yangsik Jeong* [b], *Katsuyoshi Kawana* [a], *Hiroyuki Miyachi* [c], *Yuichi Hashimoto* [c], *Makoto Makishima* [a,*]

[a] *Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine, 30-1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173-8610, Japan*

[b] *Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA*

[c] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan*

ARTICLE INFO

Article history:
Received 8 August 2008
Accepted 3 October 2008

Keywords:
Liver X receptor
β-Catenin
Transcription
Colon cancer
Nuclear receptor
Proliferation

ABSTRACT

The nuclear receptors liver X receptor (LXR) α and LXRβ serve as oxysterol receptors and play an important role in the regulation of lipid metabolism. We investigated the potential effects of LXRs on pathways of colon carcinogenesis and found that LXR activation suppresses the transactivation activity of β-catenin, a key molecule in Wnt signaling. LXRα and LXRβ inhibited β-catenin transactivation of T cell factor-mediated transcription in a ligand-dependent manner. LXR activation suppressed an oncogenic β-catenin, which has phosphorylation site mutations, and did not change β-catenin protein expression in cells. In contrast, β-catenin enhanced LXR transactivation activity. Nuclear LXRs and β-catenin were coimmunoprecipitated in colon cancer HCT116 cells, and *in vitro* experiments showed that LXRs bind directly to the Armadillo repeat region of β-catenin in a ligand-independent manner. LXR ligand decreased mRNA expression of β-catenin targets, MYC, MMP7 and BMP4, and recruited LXRs to MYC and MMP7 promoters. Transfection of a dominant negative LXR to HCT116 cells and experiments using LXR-null cells showed the involvement of cellular LXRs in β-catenin suppression and proliferation inhibition. The results show lipid-sensing receptor LXRs regulate the β-catenin activity and cellular proliferation.

1. Introduction

Nuclear receptors have been shown to regulate lipid metabolism as sensors of the metabolic environment [1,2]. Nuclear receptors are ligand-inducible transcription factors that are involved in many biological processes, including cell growth and differentiation, embryonic development, and metabolic homeostasis. The transcriptional activity of nuclear receptors is modulated by ligands such as steroids, retinoids, and other lipid soluble compounds. Upon ligand binding, nuclear receptors undergo a conformational change that results in the dissociation of corepressors and recruitment of coactivators [3]. Cofactor interactions allow nuclear receptors to modulate the transcription of specific target genes. The liver X receptors (LXRs) are oxysterol-activated nuclear receptors which regulate the expression of genes involved in cholesterol and triglyceride metabolism [4]. LXRα (NR1H3) is localized to the liver, adipose tissue, intestine, kidney, and macrophage, while LXRβ (NR1H2) is ubiquitously expressed. LXRs regulate intestinal absorption and biliary excretion of cholesterol by inducing the expression of target genes such as the ATP-binding cassette (ABC) transporters ABCA1, ABCG5, and ABCG8. Conversion of cholesterol to bile acids in the rodent liver is positively regulated by LXRα. LXRs stimulate reverse cholesterol transport from peripheral tissues and exhibit antiatherogenic activity. LXR activation also stimulates lipogenesis in liver by inducing lipogenic genes, including sterol regulatory element-binding protein-1c, fatty acid

* *Corresponding author*. Tel.: +81 3 3972 8199; fax: +81 3 3972 8199.
E-mail address: maxima@med.nihon-u.ac.jp (M. Makishima).

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.10.007

synthase and stearyl CoA desaturase-1. Cholesterol and fatty acid metabolism is not only associated with nutritional homeostasis but also with cell growth and cancer pathogenesis [5,6]. As lipid-sensing receptors, LXRs are suggested to regulate cell growth mechanisms.

β-Catenin is a key mediator of the Wnt pathway, which plays a critical role in embryogenesis and oncogenesis [7,8]. In the absence of Wnt signaling, β-catenin is phosphorylated by a multiprotein complex composed of the tumor suppressor adenomatous polyposis coli (APC), Axin2 and glycogen synthase kinase-3β (GSK3β), and is subsequently degraded by the proteasome. Activation of Wnt signaling inhibits β-catenin degradation, resulting in its accumulation and nuclear localization. The nuclear β-catenin protein complexes with the T cell factor (TCF) family of transcription factors, leading to expression of the target genes, such as MYC, Cyclin D1 and matrix metallopeptidase 7 (MMP7) [8,9]. Abnormal intracellular accumulation of β-catenin due to genetic alterations in genes such APC and β-catenin has been observed in colon cancers [7]. Maintenance of nutritional absorptive capacity requires constant cellular renewal through Wnt-cascade controlled proliferation of the intestinal epithelium [10]. The unique cell-turnover rate of the intestinal epithelium may contribute to a high susceptibility to malignant transformation. Although several molecular mechanisms of colon carcinogenesis have been established [11], the molecular link between dietary/endogenous lipid signaling and cellular proliferation pathways in intestinal mucosal cells remains unknown. In this study, we investigated the effects of the lipid-sensing nuclear receptor LXRs in colon cancer cells and found that LXR activation suppressed β-catenin transactivation by direct interaction and inhibited cellular proliferation.

2. Materials and methods

2.1. Chemical compounds

T0901317 was purchased from Cayman Chemical Company (Ann Arbor, MI) and GW3965 was synthesized in our laboratory [12].

2.2. Plasmids

Fragments of human LXRα (GenBank accession number NM_005693) and LXRβ (GenBank accession number NM_007121) were inserted into pCMX vector to make pCMX-LXRα and pCMX-LXRβ, respectively [13]. Full-length and mutant (S33A, S37A, T41A, S45A) β-catenin cDNAs (GenBank accession number NM_007614) were inserted into pCMX vector to make pCMX-β-catenin (wild-type; WT) and pCMX-β-catenin (mutant; MT), respectively [14]. TCF-responsive TOPGLOW (Millipore, Billerica, MA) was utilized to evaluate the activity of β-catenin. FOPGLOW (Millipore), which has mutation in the TCF-binding element (TBE), was used as a control for TOPGLOW. The ABCA1 promoter-luciferase reporter pGL3-ABCA1 was constructed by inserting 1030 base pairs of human ABCA1 promoter (GenBank accession number AF258623) to the luciferase reporter pGL3 (Promega Corporation, Madison, WI). pGEX vector (GE Healthcare, Chalfont St. Giles, United Kingdom) was utilized to generate glutathione S-transferase (GST) fusions [13], and the amino acid 1–436 fragment of LXRα was cloned into the pCMV-Myc (Takara Bio Inc., Otsu, Japan) make pCMV-Myc-LXRα-dAF2.

2.3. Cell culture and transfection assay

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Human colon carcinoma HCT116 and SW480 cells were cultured in DMEM containing 10% FBS. Mouse embryo fibroblasts (MEFs) were isolated from wild-type and Lxrαβ$^{-/-}$ 14-day-postcoitus mouse embryos [15,16], and were cultured in DMEM supplemented with 10% FBS. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center (Dallas, TX). Transfections in HEK293 cells were performed by the calcium phosphate coprecipitation assay as described previously [13]. Eight hours after transfection, compounds were added. Cells were harvested after 16–24 h and were assayed for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). For most experiments in HEK293 cells, DNA transfection experiments used 50 ng of reporter plasmid, 10 ng of pCMX-β-galactosidase and 15 ng of each expression plasmid per well of a 96-well plate. HCT116 cell was transfected 150 ng of reporter plasmid and 10 ng of pCMX-β-galactosidase by Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) and SW480 cell transfections used 100 ng of reporter plasmid and 50 ng of pCMX-β-galactosidase in combination with pCMX-Myc plasmid by Fugene HD (Roche Applied Science, Indianapolis, IN). Luciferase data were normalized to the internal β-galactosidase control and represent the mean ± S.D. of triplicate assays.

2.4. Coimmunoprecipitation and immunoblotting

Nuclear extracts were prepared as described previously [17], and were subjected to immunoblotting or coimmunoprecipitation. Coimmunoprecipitations were performed by using Nuclear Complex Co-IP Kit (Active Motif, Carlsbad, CA). The primary antibodies used were anti-β-catenin antibody (Millipore), anti-LXRα antibody (Perseus Proteomics Inc., Tokyo, Japan), and anti-LXRβ antibody (Perseus Proteomics Inc.), and antibody-binding proteins were precipitated with Protein A (Roche Applied Science). The proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane, probed with anti-β-catenin antibody, anti-LXRα antibody, anti-LXRβ antibody, or anti-Lamin B antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and visualized with an alkaline phosphatase conjugate substrate system (Bio-Rad Laboratories, Inc., Hercules, CA).

2.5. GST pull-down assays

GST-fusion proteins were expressed in BL21 DE3 cells (Promega Corporation), and purified using Glutathione Sepharose 4B (GE Healthcare). [^{35}S]-labeled proteins were

generated using the TNT Quick Coupled Transcription/Translation System (Promega Corporation). GST pull-down assays were performed as reported previously [13]. About 5 μg of GST chimera protein was bound to Glutathione Sepharose 4B and equilibrated in binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40, 2 mg/ml bovine serum albumin). Bound GST proteins were incubated with 10 μl of reticulocyte lysate containing [^{35}S]-labeled proteins and were treated with or without T0901317 for 16 h at 4 °C. After binding, beads were washed four times with washing buffer (20 mM Hepes, pH 7.7, 50 mM KCl, 20% glycerol, 0.1% Nonidet P-40) and then once with binding buffer, resuspended in SDS sample buffer, and loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, bound isotope-labeled proteins were visualized with the BAS2000 system (Fujifilm Corporation, Tokyo, Japan).

2.6. Quantitative real-time RT-PCR analysis

Total RNAs from samples were prepared with RNAgents Total RNA Isolation system (Promega Corporation) or RNA STAT-60 (Tel-Test, Inc., Friendswood, TX), and cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation). Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using power SYBR Green PCR Master Mix (Applied Biosystems) [13] or using an ABI 7900HT Sequence Detection System (Applied Biosystems) as previously described [15]. Primers for human genes were as follows: ABCA1 (GenBank accession number NM_005502), 5′-AAT CCT GAC CGG GTT GTT CCC-3′ and 5′-CCG CCT TCA CGT GCT TCT CA-3′; MYC (GenBank accession number NM_002467), 5′-CCC TCC ACT CGG AAG GAC TAT-3′ and 5′-CAC GCA GGG CAA AAA AGC T-3′; Cyclin D1 (GenBank accession number NM_053056), 5′-TGC CAA CCT CCT CAA CGA C-3′ and 5′-GGC TCT TTT TCA CGG GCT C-3′; MMP7 (GenBank accession number NM_002423), 5′-TGT TAA ACT CCC GCG TCA TAG A-3′ and 5′-GCC TTT GAC ACT AAT CGA TCC AC-3′; bone morphogenetic protein 4 (BMP4) (GenBank accession number NM_130851), 5′-GAA GAA TAA GAA CTG CCG GCG-3′ and 5′-GCA TGG TTG GTT GAG TTG AGG T-3′; glyceraldehyde-3-phosphate dehydrogenase (GenBank Accession number NM_002046), 5′-ACA TCG CTC AGA CAC CAT GG-3′ and 5′-GTA GTT GAG GTC AAT GAA GGG-3′. Primers for mouse genes were as follows: Bmp4 (GenBank accession number NM_007554), 5′-GCC GAG CCA ACA CTG TGA-3′ and 5′-TGG TCC CTG GGA TGT TCT C-3′. Primers for mouse Lxrα, Lxrβ and 18S were reported previously [15,16]. The RNA values were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase for human genes and to that of 18S for mouse genes.

2.7. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using Acetyl-Histone H4 ChIP Assay Kit (Millipore) and anti-LXRα antibody or anti-LXRβ antibody (Perseus Proteomics Inc.). DNA was purified with MonoFas DNA Purification Kit (GL Sciences, Inc. Japan, Tokyo, Japan). PCR was performed by using GoTaq Master Mix (Promega Corporation) with the following primers: MYC (GenBank accession number NT_008046) TBE1, 5′-TCC ACT TGC CCC TTT TAG GA-3′ and 5′-TGA GAT GTG TCT GCC TGT TC-3′; TBE2; 5′-GCG CCC ATT AAT ACC CTT CT-3′ and 5′-GCA TCC TTG TCC TGT GAG TA-3′; MMP7 (GenBank accession number NW_925173), 5′-GAT ACC TAT GAG AGC AGT CA-3′ and 5′-CTG CTA GTG ACT GCA GAA AT-3′. Primers for ABCA1 and Cyclin D1 were prepared as previously reported [18–20].

2.8. Cell proliferation and caspase assays

HCT116 cells and MEFs were seeded at 1000 cells/well and 2000 cells/well, respectively, in a 96-well plate one day before ligand addition. Cell proliferation was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay [21]. Four hours after addition of MTT (Nacalai Tesque, Inc., Kyoto, Japan), the culture medium was discarded, and cells were dissolved in dimethyl sulfoxide. Specific absorbance was measured with a microplate reader (Molecular Devices). Caspase 3 and 7 activities were measured using a Caspase-Glo 3/7 Assay kit (Promega Corporation). Enzyme activity data were normalized to the cell numbers.

3. Results

3.1. Inhibition of β-catenin-dependent transcriptional activation by LXRs

To examine the possibility of a functional interaction between LXRs and β-catenin, we transiently transfected HEK293 cells with an LXR expression vector, a β-catenin expression vector and a TCF-responsive luciferase reporter. As reported previously [22], exogenous expression of wild-type β-catenin activated the TCF-responsive TOPGLOW reporter (Fig. 1A). The effects of LXRα and LXRβ on β-catenin transactivation were examined by cotransfection in the absence or presence of a ligand. Cotransfection of LXRα slightly increased the β-catenin-induced reporter activity, while LXRβ decreased it. Cotransfection of LXRα and treatment with the LXR ligand T0901317 decreased β-catenin-induced TOPGLOW activity in a concentration-dependent manner. T0901317-liganded LXRβ inhibited β-catenin transactivation more effectively than LXRα. Since β-catenin is rapidly degraded after phosphorylation by the APC-Axin2-GSK3β complex and HEK293 cells highly express endogenous APC [22], a phosphorylation site mutant β-catenin was introduced into HEK293 cells. The β-catenin mutant was 10-fold more effective than wild-type β-catenin in inducing TOPGLOW activity in HEK293 cells. Cotransfection of LXRα and LXRβ did not change mutant β-catenin-induced reporter activity, and addition of T0901317 decreased transactivation of mutant β-catenin (Fig. 1B), indicating that LXRα and LXRβ inhibit β-catenin activity by a mechanism that is independent of β-catenin phosphorylation on mutated sites. To examine the effect of LXR ligand on transcriptional activity of endogenous β-catenin in colon cancer cells and to rule out non-specific effects of overexpression of LXR, we transfected HCT116 cells, which have degradation-resistant β-catenin mutation [22], with a TOPGLOW reporter and evaluated endogenous β-catenin activity by comparing luciferase values with the control FOPGLOW reporter. TOPGLOW was strongly activated in HCT116 cells,

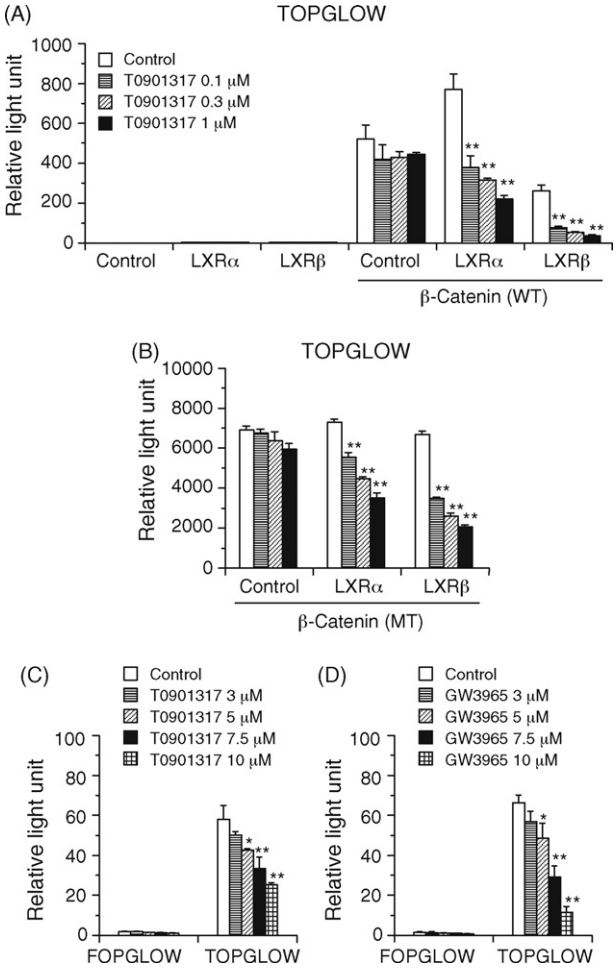

Fig. 1 – Inhibition of β-catenin transactivation activity by LXR activation. (A) Effect of ligand-stimulated LXRα and LXRβ on wild-type β-catenin activation of a TCF-responsive reporter. HEK293 cells were transfected with control CMX vector, CMX-LXRα, or CMX-LXRβ in the absence or presence of CMX-β-catenin (wild-type; WT), and TOPGLOW, and were treated with 0, 0.1, 0.3, or 1 μM T0901317. (B) Inhibition of mutant β-catenin activity by LXRα and LXRβ activation. HEK293 cells were transfected with control CMX vector, CMX-LXRα, or CMX-LXRβ, in combination with β-catenin (mutant; MT), and TOPGLOW, and were treated with T0901317 as in (A). (C) Effect of T0901317 on endogenous β-catenin activity in HCT116 cells. Cells were transfected with FOPGLOW or TOPGLOW reporter, and were treated with 0, 3, 5, 7.5, or 10 μM T0901317. (D) Effect of GW3965 on endogenous β-catenin activity in HCT116 cells. Cells were transfected with FOPGLOW or TOPGLOW reporter, and were treated with 0, 3, 5, 7.5, or 10 μM GW3965. All values represent means ± S.D. of triplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with vehicle control.

while FOPGLOW was not induced (Fig. 1C), indicating that HCT116 cells contain active β-catenin proteins as reported [22]. Treatment of HCT116 cells with the LXR ligand T0901317 repressed TOPGLOW activity. Since T0901317 was reported to have LXR-independent effects, including activation of pregnane X receptor [23], we examined the effect of another LXR ligand, GW3965, on β-catenin activity in HCT116 cells. GW3965 also inhibited TOPGLOW activity in HCT116 cells (Fig. 1D). These findings indicate that endogenous LXR activation suppresses β-catenin activity in HCT116 cells. Thus, the LXR activation suppresses exogenous and endogenous β-catenin transactivation.

The possibility of reciprocal regulation of LXR-induced transactivation by β-catenin was examined. LXRα, stimulated with the ligand T0901317, induced a luciferase reporter containing the LXR-binding element from the rat Cyp7a promoter and cotransfection of wild-type β-catenin enhanced LXRα transcriptional activity (Fig. 2A). Mutant β-catenin on LXRα transactivation was less effective than wild-type β-catenin. As reported previously [24], ligand-dependent transactivation of LXRβ was weaker than that of LXRα (Fig. 2B). Wild-type β-catenin also enhanced the LXRβ transactivation, while mutant β-catenin was less effective. Therefore, β-catenin augments LXR transactivation and wild-type β-catenin is more effective than phosphorylation-resistant β-catenin mutant.

3.2. *Direct interaction of LXRs and β-catenin*

Expression of LXR and β-catenin proteins and their interaction were examined in HCT116 colon cancer cells. Ligand activation of retinoid X receptor and peroxisome proliferator-activated receptor γ was reported to repress β-catenin-mediated transcription by enhancing β-catenin degradation in a GSK3β-independent manner [25,26]. We examined the effect of LXR ligand on nuclear expression of β-catenin protein in HCT116 cells. T0901317 treatment did not decrease nuclear β-catenin expression (Fig. 3A). LXR activation did not change β-catenin protein levels in HEK293 cells (data not shown), which express wild-type β-catenin [22]. The protein levels of nuclear LXRα and LXRβ were not changed by T0901317 treatment (Fig. 3A). Next, *in vivo* interaction between β-catenin and LXRs was examined. Nuclear extracts from HCT116 cells were immunoprecipitated with anti-β-catenin and then subjected to immunoblotting with anti-LXRα and anti-LXRβ antibodies. LXRα and LXRβ proteins were observed in β-catenin immunoprecipitate (Fig. 3B). Immunoprecipitation with anti-LXRβ antibody revealed the formation of β-catenin-LXRβ complex. However, β-catenin was not detected in the immunoprecipitate with anti-LXRα antibody. Interaction of anti-LXRα antibody with LXRα may disturb the formation of stable complex of β-catenin and LXRα. Thus, β-catenin interacts with LXRα and LXRβ in the cells.

We performed a GST pull-down assay to examine the *in vitro* interaction of LXRs and β-catenin. We generated a β-catenin GST-fusion protein and evaluated the interaction between GST-β-catenin and isotope-labeled LXRs in the absence or presence of the LXR ligand T0901317. LXRα bound to wild-type and mutant β-catenin proteins in the presence or absence of ligand while the interaction of LXRα with the coactivator steroid receptor coactivator-1 was ligand dependent (Fig. 4A). To identify the region of β-catenin that interacts with LXR, we generated β-catenin GST-fusion proteins corresponding to the N-terminal region (amino acid fragment

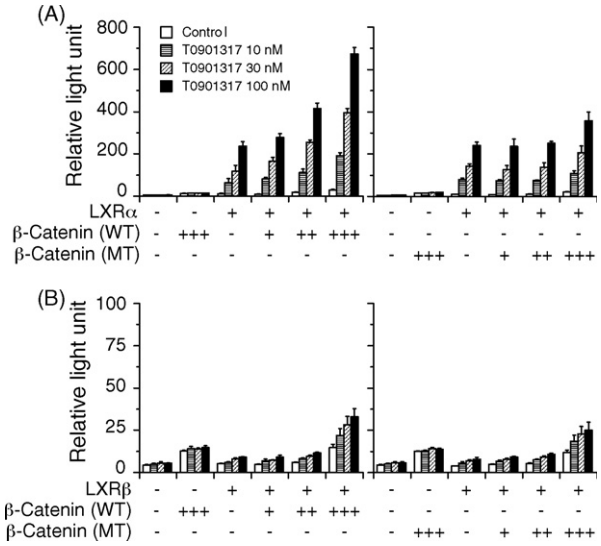

Fig. 2 – Enhancement of LXR transactivation activity by β-catenin. (A) Effect of β-catenin on LXRα transactivation. (B) Effect of β-catenin on LXRβ transactivation. HEK293 cells were transfected with 1 ng/well of control CMX vector (−), CMX-LXRα, or CMX-LXRβ in the absence (−) or presence of 1 ng/well (+), 10 ng/well (++), or 90 ng/well (+++) of β-catenin (wild-type; WT, or mutant; MT), and (rCYP7A-DR4)x3-tk-LUC, and treated with 0, 10, 30, or 100 nM T0901317.

2–130), Armadillo repeats (131–680) and C-terminal region (681–781). The β-catenin N-terminal region contains serine/threonine residues that affect protein stability, the central Armadillo repeats mediate interactions between β-catenin and its binding partners, including Axin2, APC, TCFs, and E-cadherin, and the C-terminal region contains potent transcriptional activation elements, which bind to the TATA-box binding protein [27]. Isotope-labeled LXRα interacted with the β-catenin Armadillo repeats and not with N-terminal and C-terminal regions (Fig. 4B). LXRβ also bound to the Armadillo repeat-containing protein. Next, we examined the interaction between GST-fused LXRα and an isotope-labeled Armadillo repeat region of β-catenin in the absence or presence of T0901317. The Armadillo repeat fragment bound to GST-LXRα in a ligand-independent manner (Fig. 4C). These findings indicate that LXRα and LXRβ bind directly to β-catenin through the Armadillo repeats.

3.3. *Suppression of β-catenin activity by LXR ligand in colon cancer cells*

We examined the effects of LXR ligand on the expression of endogenous β-catenin target genes in colon cancer cells. T0901317 decreased the expression of MYC, MMP7 and BMP4 in HCT116 cells, while it induced the LXR target gene ABCA1 (Fig. 5A). Next, we performed ChIP assay to examine the recruitment of LXRs to these promoters. Immunoprecipitation on HCT116 cell lysate with an anti-LXRα antibody or an anti-LXRβ antibody revealed the weak recruitment of LXRα and LXRβ to the ABCA1 promoter, and addition of T0901317 enhanced their association (Fig. 5B). LXRβ was recruited to both TBE1 and TBE2 in MYC promoter in the absence of ligand and T0901317 treatment increased the LXRβ recruitment to the TBEs. LXRα was recruited to the MYC promoter TBE1 and TBE2 in a ligand-dependent manner. LXRα and LXRβ were not recruited to TBEs in Cyclin D1 promoter even in the presence of

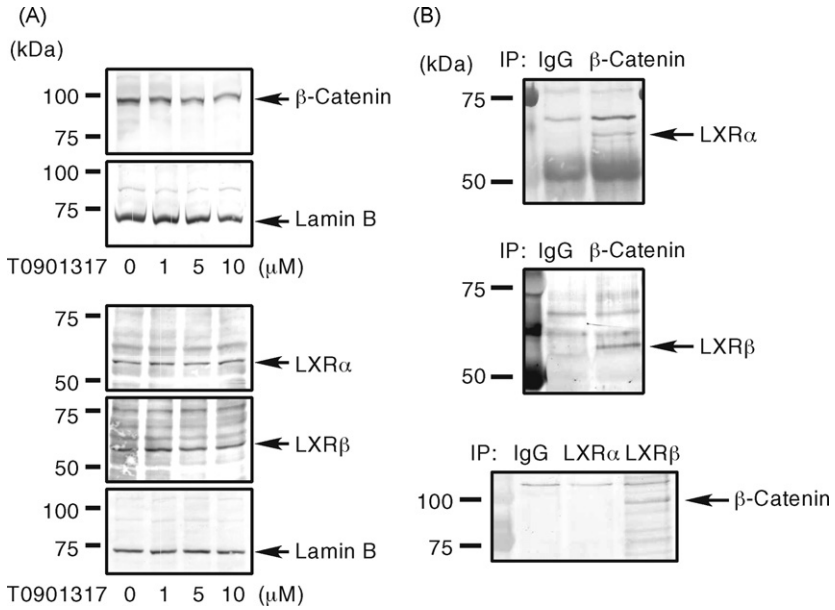

Fig. 3 – Expression and interaction of β-catenin and LXRs in colon cancer cells. (A) Nuclear expression of β-catenin, LXRα, LXRβ, and Lamin B in HCT116 cells. Cells were treated with 0, 1, 5, or 10 μM T0901317 for 24 h. After preparation of nuclear extracts, 20 μg was subjected to SDS-PAGE for LXRα and LXRβ, and 10 μg for β-catenin and lamin B. (B) Interaction of LXRs and β-catenin in HCT116 cells. Immunoprecipitation (IP) was performed on 500 μg nuclear extracts by using control IgG, anti-β-catenin antibody, anti-LXRα-antibody, or anti-LXRβ-antibody, followed by Western blot analysis with antibodies indicated in each case.

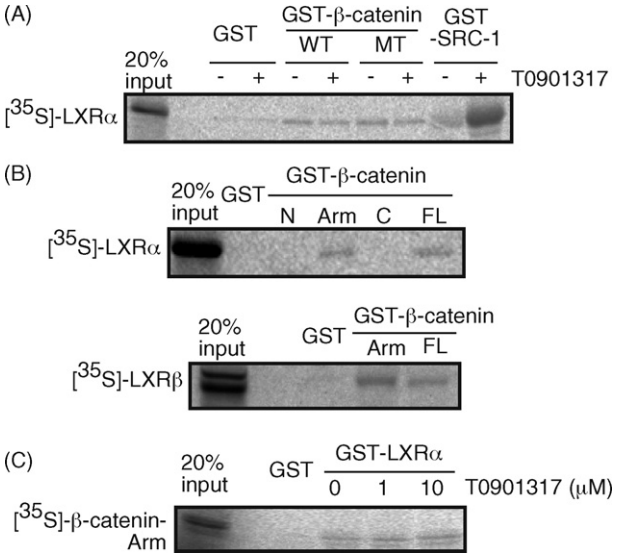

Fig. 4 – LXRs and β-catenin interact directly *in vitro*. (A) Direct binding of LXRα to β-catenin protein in a ligand-independent manner. Interaction of LXRα with steroid receptor coactivator-1 (SRC-1) was strongly induced by the addition of ligand. GST pull-down assays were performed to assess interactions of GST-fusion proteins and [^{35}S]-labeled LXRα protein in the absence or presence of 10 μM T0901317. (B) LXRα and LXRβ proteins bound to the Armadillo repeat domain of β-catenin. Interaction of LXRα protein with the N-terminal region (N-term; amino acid 2–130), Armadillo repeats (Arm; 131–680), C-terminal region (C-term; 681–781), and full-length (FL) of β-catenin was examined in GST pull-downs. Interaction of LXRβ protein with Armadillo repeats and full-length of β-catenin was also examined. (C) GST-LXRα fusion protein interacted with [^{35}S]-labeled β-catenin Armadillo repeat domain in a ligand-independent manner. GST pull-down assays were performed to assess interaction of GST-LXRα and [^{35}S]-labeled β-catenin Armadillo repeats (Arm) in the presence of 0, 1, or 10 μM T0901317.

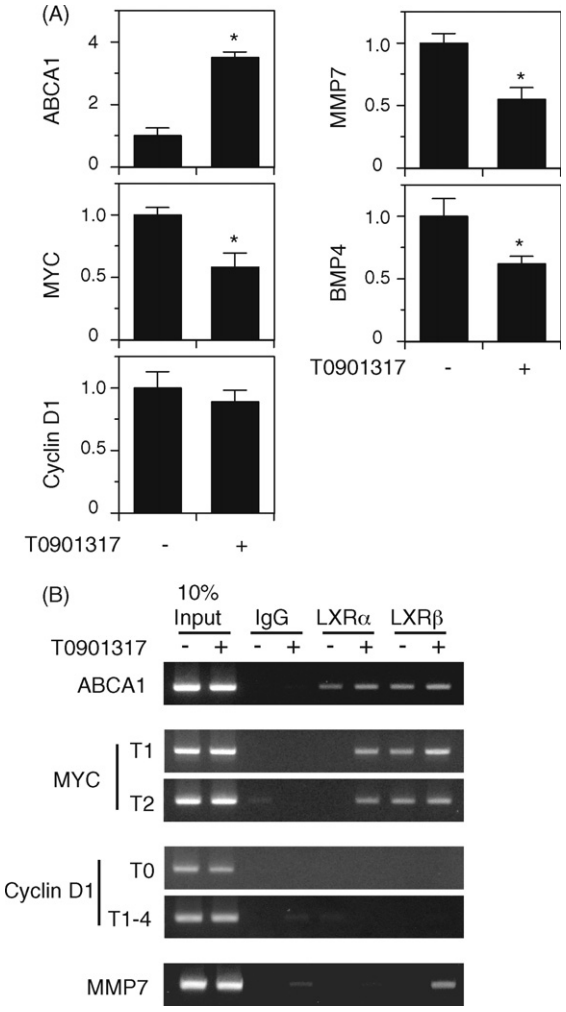

Fig. 5 – Suppression of β-catenin activity and recruitment of LXR to β-catenin target genes in colon cancer cells. (A) Effect of LXR ligand on mRNA expression in HCT116 cells. Quantitative real-time RT-PCR was performed for ABCA1, MYC, Cyclin D1, MMP7, and BMP4 mRNA expression in HCT116 cells treated without or with 1 μM T0901317 for 24 h. All values represent means ± S.D. of triplicate assays. *$p < 0.05$ compared with vehicle control. (B) ChIP analysis of LXRs in HCT116 cells. Cells were treated without or with 5 μM T0901317 for 30 min and then subjected to ChIP analysis using control, anti-LXRα, or anti-LXRβ antibody. PCR was performed to detect ABCA1 promoter region including LXR-binding element, MYC promoter regions including TBE1 (T1) and TBE2 (T2), Cyclin D1 promoter regions including TBE0 (T0) and TBE1-4 (T1-4), and MMP7 promoter region including TBE.

ligand. While LXRα was not coimmunoprecipitated with MMP7 promoter, LXRβ was recruited to this promoter in the presence of ligand. The recruitment of LXRs to ABC1, MYC, and MMP7 promoters is consistent with ligand-induced changes of these gene expressions shown in Fig. 5A. Distinct pattern of LXRα and LXRβ in the promoter complexes may be due to efficiency of antibodies, expression of these receptors, binding affinity to β-catenin, or ligand-independent activation of LXRβ [28].

3.4. *LXR-dependency of β-catenin suppression*

The involvement of endogenous LXR in ligand-dependent inhibition of β-catenin activity was examined by using LXRα-dAF2, an LXRα deletion mutant lacking the activation function domain 2 (AF2). Upon ligand binding, nuclear receptors undergo a conformational change that induces AF2-dependent recruitment of coactivators [3]. LXRα-dAF2 lost ligand-dependent transactivation activity and inhibited the function of intact LXRα and LXRβ by a dominant negative effect. The luciferase activity on ABCA1 promoter was increased by T0901317 and this activity was inhibited by transfection of LXRα-dAF2 (Fig. 6A), indicating that LXRα-dAF2 acts as a dominant negative receptor against endogenous LXRs. The LXRα-dAF2 was introduced with the β-catenin-responsive

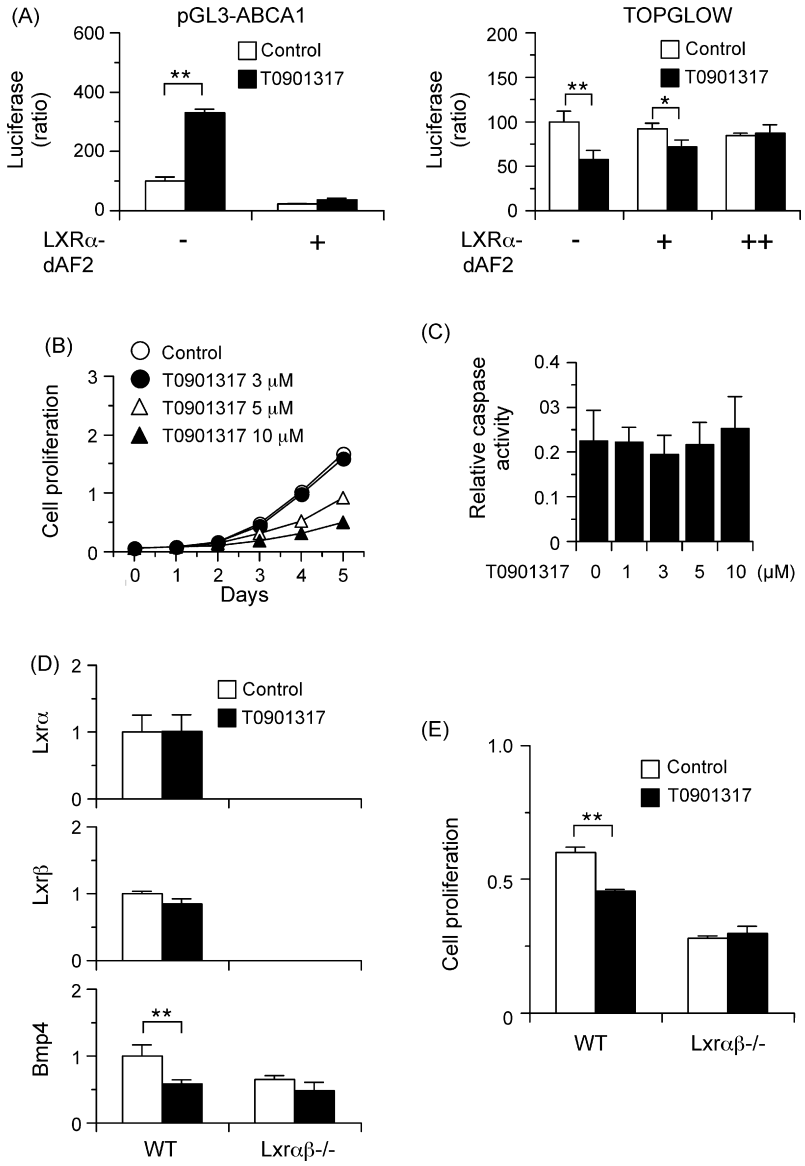

Fig. 6 – LXRs are required for β-catenin suppression and proliferation inhibition. (A) Dominant negative effect of LXRα-dAF2 on ligand-dependent activation of ABCA1 promoter and suppression of β-catenin transactivation. SW480 cells were transfected with CMV-Myc or CMV-Myc-LXRα-dAF2 in the combination with pGL3-ABCA1, and were treated with vehicle control or 5 μM T0901317. HCT116 cells were transfected with 0 (−), 50 (+), or 100 ng (++) of CMV-Myc-LXRα-dAF2 in the combination with TOPGLOW, and were treated with vehicle control or 5 μM T09211317. (B) Effect of T0901317 on growth inhibition of HCT116 cells. Cells were treated with 0, 3, 5, or 10 μM T0901317, and cell number was evaluated with the MTT assay. (C) T0901317 does not induce apoptosis in HCT116 cells. Cells were treated with 0, 1, 3, 5, or 10 μM T0901317 for 48 h, and caspase 3/7 activities were measured. (D) mRNA expression of Lxrα, Lxrβ, and Bmp4 in MEFs. MEFs from wild-type mice (WT) or Lxrαβ$^{-/-}$ mice were treated with vehicle control or 1 μM T0901317 for 24 h. (E) LXR ligand suppresses cell proliferation of wild-type MEFs but not of Lxrαβ$^{-/-}$ MEFs. Cells were treated with vehicle control or 1 μM T0901317 for 7 days, and cell number was evaluated with MTT assay. All values represent means ± S.D. of triplicate assays. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with vehicle control.

TOPGLOW reporter in HCT116 cells. The LXRα-dAF2 abolished the inhibitory activity of T0901317 on TOPGLOW concentration dependently. T0901317 treatment inhibited the proliferation of HCT116 cells in a concentration- and time-dependent manner (Fig. 6B). T0901317 did not increase caspase 3/7 activities in HCT116 cells (Fig. 6C). These findings indicate that the LXR ligand inhibits endogenous β-catenin activity in colon cancer cells in an LXR-dependent manner and cell proliferation without inducing apoptosis.

We next examined the effect of T0901317 on mRNA expression of β-catenin target genes (Bmp4, Myc, Cyclin D1, and Mmp7) in MEFs from wild-type mice. Mmp7 mRNA was not detected and the expression of Myc and Cyclin D1 was not changed after treatment with T0901317 (data not shown).

Treatment of MEFs with T0901317 repressed Bmp4 expression by 41%, while it did not change Lxrα or Lxrβ expression (Fig. 6D). This ligand-dependent suppression of Bmp4 expression was abolished in MEFs from Lxrαβ$^{-/-}$ mice. The interaction between LXRs and β-catenin is suggested to be involved in reduced expression of Bmp4 in MEFs. T0901317 inhibited the proliferation of wild-type MEFs, but not of Lxrαβ$^{-/-}$ cells (Fig. 6E). Thus, LXRs mediate the effect of T0901317 on Bmp4 expression and proliferation inhibition in MEFs.

4. Discussion

In this study we have shown that LXR bound directly to β-catenin and suppressed its transactivation activity in a ligand-dependent manner. β-Catenin is a key component of Wnt-mediated regulation of multiple cellular functions in embryogenesis and tumorigenesis, such as in colon cancer [7,8]. Ligand-activated LXR inhibited the transcriptional activity of wild-type β-catenin and a β-catenin mutant that is resistant to GSK3β phosphorylation and subsequent degradation. LXR activation did not change β-catenin protein levels in HCT116 cells, which have β-catenin mutation, and in HEK293 cells, which express intact β-catenin [22]. These findings indicate that the effect of LXR on β-catenin is not due to an alternation of β-catenin phosphorylation or degradation. While the *in vitro* GST pull-down assays showed that the interaction of LXRs and β-catenin was ligand-independent, LXRs repressed β-catenin-induced gene expression in a ligand-dependent manner. β-Catenin participates in a multiprotein complex of TCF coactivators including TATA-binding protein, CBP/p300, and additional poorly characterized proteins [27]. LXR ligand activation is suggested to induce an allosteric conformational change in the β-catenin transcription factor complex. Wild-type β-catenin effectively enhanced LXR transactivation activity. SIRT1, an NAD^+-dependent protein deacetylase, has been shown to interact with LXR proteins and promote deacetylation and subsequent ubiquitination, resulting in enhanced transactivation [29]. SIRT1 also deacetylates β-catenin but suppresses its transactivation activity [30]. Dynamic modification of an LXR/β-catenin protein complex, such as deacetylation, may influence the transactivation activities of LXR and β-catenin.

LXR ligand decreased the expression of MYC, MMP4 and BMP7, but not Cyclin D1 in HCT116 cells. Cyclin D1 has been reported to a target of the β-catenin/TCF complex and several TBEs have been identified in the Cyclin D1 promoter [19,20,31]. However, conditional inactivation of APC *in vivo* has shown that Cyclin D1 does not play a role in intestinal tumor initiation [32]. On the other hand, MYC is an important β-catenin target in colon carcinogenesis induced by APC inactivation [33]. A study using small interfering RNA directed against β-catenin has shown that β-catenin reduction decreased MYC expression in HCT116 cells [34]. MMP7 is another β-catenin target, which is overexpressed in about 80% of human colorectal cancers, and contains TBEs in the promoter [9,35]. Decreased expression of MYC, MMP7 and BMP4 induced by LXR ligand was associated with recruitment of LXRα and/or LXRβ on their promoters including TBEs, indicating that LXR activation suppresses β-catenin signaling by forming a complex with β-catenin/TCF on promoters. LXR ligand decreased the expression of BMP4 (Bmp4) in HCT116 cells and in MEFs. TBEs has been identified in mouse Bmp4 gene [36]. When the oncogenic allele of β-catenin is deleted in HCT116 cells, BMP4 expression is almost extinguished [37]. Conditional ablations of β-catenin in mouse embryonic endoderm, ectoderm and mesoderm are associated with decreased expression of Bmp4 [38–40]. These findings suggest that LXR ligand-induced repression of Bmp4 in MEFs is mediated by interaction of LXRs with β-catenin. Lung-specific deletion of β-catenin results in decreased Bmp4 expression, but not associated with epithelial cell proliferation [36]. BMP4 inhibits proliferation and promotes differentiation of lung fibroblast [41]. LXR activation may suppress the proliferation of MEFs by a Bmp4-independent mechanism.

Environmental factors, including diet, physical activity and smoking, are considered to play an important role in the etiology and pathogenesis of colon cancer [42]. Animal meat contains a large amount of saturated and ω-6 polyunsaturated fatty acids and cholesterol, dietary fats that may be associated with an increased risk of colon cancer. Bile acids, which are the major cholesterol metabolites, have been also implicated in the pathogenesis of colon cancer [43]. In contrast, phytosterols, as well as vegetable fibers, are thought to protect against the development of colon cancer [44]. We previously reported that phytosterol derivatives act as potent LXR agonists in intestinal cells [13]. Several phytosterols have been reported to induce LXR activation and these effects are thought to contribute their hypocholesterolemic action [45,46]. We demonstrate in this study that LXR activation inhibits endogenous β-catenin activity in colon cancer cells and cellular proliferation. These findings suggest that phytosterol intake might suppress colon carcinogenesis through LXR activation and subsequent inhibition of β-catenin activity. Activation of Wnt/β-catenin is also associated with the development of hepatocellular carcinoma and other cancers [47]. Further studies are required to elucidate the role of LXRs in carcinogenesis.

In conclusion, we demonstrated that LXRs regulate the β-catenin activity and cellular proliferation. LXRs may be regulators that link metabolic signaling to cell proliferation pathways.

Acknowledgements

The authors thank Dr. Ken-ichirou Morohashi of the National Institute for Basic Biology (Okazaki, Japan) for providing β-catenin vectors, and Dr. Makoto Shimizu of University of Texas Southwestern Medical Center, Dr. Sachiko Yamada and members of the Makishima, Mangelsdorf and Hashimoto laboratories for technical assistance and helpful comments. K. Kawana was a Research Fellow of the Japan Society for the Promotion of Science. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (Grain-in-Aid for Scientific Research on Priority Areas, 18077005 to M. Makishima), and the Japan Society for the Promotion of Science (K. Kawana), and was partly funded by Kyowa Hakko Co. Ltd., Japan (M. Makishima).

REFERENCES

[1] Makishima M. Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. J Pharmacol Sci 2005;97:177–83.
[2] Shulman AI, Mangelsdorf DJ. Retinoid X receptor heterodimers in the metabolic syndrome. N Engl J Med 2005;353:604–15.
[3] Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 2006;20:1405–28.
[4] Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. Mol Endocrinol 2003;17:985–93.
[5] Demierre M-F, Higgins PDR, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer 2005;5:930–42.
[6] Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer 2007;7:763–77.
[7] Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781–810.
[8] Clevers H. Wnt/β-catenin signaling in development and disease. Cell 2006;127:469–80.
[9] Gustavson MD, Crawford HC, Fingleton B, Matrisian LM. Tcf binding sequence and position determines β-catenin and Lef-1 responsiveness of MMP-7 promoters. Mol Carcinog 2004;41:125–39.
[10] Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science 2005;307:1904–9.
[11] Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
[12] Noguchi-Yachide T, Aoyama A, Makishima M, Miyachi H, Hashimoto Y. Liver X receptor antagonists with a phthalimide skeleton derived from thalidomide-related glucosidase inhibitors. Bioorg Med Chem Lett 2007;17:3957–61.
[13] Kaneko E, Matsuda M, Yamada Y, Tachibana Y, Shimomura I, Makishima M. Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. J Biol Chem 2003;278:36091–8.
[14] Mizusaki H, Kawabe K, Mukai T, Ariyoshi E, Kasahara M, Yoshioka H, et al. Dax-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) gene transcription is regulated by Wnt4 in the female developing gonad. Mol Endocrinol 2003;17:507–19.
[15] Kalaany NY, Gauthier KC, Zavacki AM, Mammen PP, Kitazume T, Peterson JA, et al. LXRs regulate the balance between fat storage and oxidation. Cell Metab 2005;1:231–44.
[16] Cummins CL, Volle DH, Zhang Y, McDonald JG, Sion B, Lefrancois-Martinez A-M, et al. Liver X receptors regulate adrenal cholesterol balance. J Clin Invest 2006;116:1902–12.
[17] Inaba Y, Yamamoto K, Yoshimoto N, Matsunawa M, Uno S, Yamada S, et al. Vitamin D_3 derivatives with adamantane or lactone ring side chains are cell type-selective vitamin D receptor modulators. Mol Pharmacol 2007;71:1298–311.
[18] Huuskonen J, Fielding PE, Fielding CJ. Role of p160 coactivator complex in the activation of liver X receptor. Arterioscler Thromb Vasc Biol 2004;24:703–8.
[19] Toualbi K, Guller MC, Mauriz JL, Labalette C, Buendia MA, Mauviel A, et al. Physical and functional cooperation between AP-1 and β-catenin for the regulation of TCF-dependent genes. Oncogene 2006;26:3492–502.
[20] Takayama S, Rogatsky I, Schwarcz LE, Darimont BD. The glucocorticoid receptor represses cyclin D1 by targeting the Tcf-β-catenin complex. J Biol Chem 2006;281:17856–63.
[21] Goto I, Yamamoto-Yamaguchi Y, Honma Y. Enhancement of sensitivity of human lung adenocarcinoma cells to growth-inhibitory activity of interferon a by differentiation-inducing agents. Br J Cancer 1996;74:546–54.
[22] Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 1997;275:1787–90.
[23] Shenoy SD, Spencer TA, Mercer-Haines NA, Alipour M, Gargano MD, Runge-Morris M, et al. CYP3A induction by liver X receptor ligands in primary cultured rat and mouse hepatocytes is mediated by the pregnane X receptor. Drug Metab Dispos 2004;32:66–71.
[24] Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRa. Cell 1998;93:693–704.
[25] Xiao J-H, Ghosn C, Hinchman C, Forbes C, Wang J, Snider N, et al. Adenomatous polyposis coli (APC)-independent regulation of β-catenin degradation via a retinoid X receptor-mediated pathway. J Biol Chem 2003;278:29954–62.
[26] Sharma C, Pradeep A, Wong L, Rana A, Rana B. Peroxisome proliferator-activated receptor γ activation can regulate β-catenin levels via a proteasome-mediated and adenomatous polyposis coli-independent pathway. J Biol Chem 2004;279:35583–94.
[27] Hurlstone A, Clevers H. T-cell factors: turn-ons and turn-offs. EMBO J 2002;21:2303–11.
[28] Wiebel FF, Steffensen KR, Treuter E, Feltkamp D, Gustafsson J-A. Ligand-Independent coregulator recruitment by the triply activatable OR1/retinoid X receptor-α nuclear receptor heterodimer. Mol Endocrinol 1999;13:1105–18.
[29] Li X, Zhang S, Blander G, Tse JG, Krieger M, Guarente L. SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell 2007;28:91–106.
[30] Firestein R, Blander G, Michan S, Oberdoerffer P, Ogino S, Campbell J, et al. The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth. PLoS ONE 2008;3:e2020.
[31] Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
[32] Sansom OJ, Reed KR, van de Wetering M, Muncan V, Winton DJ, Clevers H, et al. Cyclin D1 is not an immediate target of β-catenin following Apc loss in the intestine. J Biol Chem 2005;280:28463–7.
[33] Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, et al. Myc deletion rescues Apc deficiency in the small intestine. Nature 2007;446:676–9.
[34] Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against β-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. Clin Cancer Res 2003;9:1291–300.
[35] Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. β-Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 1999;155:1033–8.
[36] Shu W, Guttentag S, Wang Z, Andl T, Ballard P, Lu MM, et al. Wnt/β-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. Dev Biol 2005;283:226–39.
[37] Kim J-S, Crooks H, Dracheva T, Nishanian TG, Singh B, Jen J, et al. Oncogenic β-catenin is required for bone

morphogenetic protein 4 expression in human cancer cells. Cancer Res 2002;62:2744–8.
[38] Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. β-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 2001;105:533–45.
[39] Barrow JR, Thomas KR, Boussadia-Zahui O, Moore R, Kemler R, Capecchi MR, et al. Ectodermal Wnt3/β-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. Genes Dev 2003;17:394–409.
[40] Hill TP, Taketo MM, Birchmeier W, Hartmann C. Multiple roles of mesenchymal β-catenin during murine limb patterning. Development 2006;133:1219–29.
[41] Jeffery TK, Upton PD, Trembath RC, Morrell NW. BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways. Am J Physiol Lung Cell Mol Physiol 2005;288:L370–8.
[42] Lieberman DA, Prindiville S, Weiss DG, Willett W. Risk factors for advanced colonic neoplasia and hyperplastic polyps in asymptomatic individuals. JAMA 2003;290: 2959–67.
[43] Nagengast FM, Grubben MJ, van Munster IP. Role of bile acids in colorectal carcinogenesis. Eur J Cancer 1995;31A:1067–70.
[44] de Jong A, Plat J, Mensink RP. Metabolic effects of plant sterols and stanols. J Nutr Biochem 2003;14:362–9.
[45] Yang C, Yu L, Li W, Xu F, Cohen JC, Hobbs HH. Disruption of cholesterol homeostasis by plant sterols. J Clin Invest 2004;114:813–22.
[46] Plat J, Nichols JA, Mensink RP. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. J Lipid Res 2005;46:2468–76.
[47] Wong CM, Ng IO. Molecular pathogenesis of hepatocellular carcinoma. Liver Int 2008;28:160–74.