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Liver X Receptor Activation Reduces Angiogenesis by Impairing Lipid Raft Localization and Signaling of Vascular Endothelial Growth Factor Receptor-2

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Objective—Liver X receptors (LXRα, LXRβ) are master regulators of cholesterol homeostasis. In the endothelium, perturbations of cell cholesterol have an impact on fundamental processes. We, therefore, assessed the effects of LXR activation on endothelial functions related to angiogenesis in vitro and in vivo.

Methods and Results—LXR agonists (T0901317, GW3965) blunted migration, tubulogenesis, and proliferation of human umbilical vein endothelial cells. By affecting endothelial cholesterol homeostasis, LXR activation impaired the compartmentation of vascular endothelial growth factor receptor-2 in lipid rafts/caveolae and led to defective phosphorylation and downstream signaling of vascular endothelial growth factor receptor-2 upon vascular endothelial growth factor-A stimulation. Consistently, the antiangiogenic actions of LXR agonists could be prevented by coadministration of exogenous cholesterol. LXR agonists reduced endothelial sprouting from wild-type but not from LXRα$^{-/-}$/LXRβ$^{-/-}$ knockout aortas and blunted the vascularization of implanted angioreactors in vivo. Furthermore, T0901317 reduced the growth of Lewis lung carcinoma grafts in mice by impairing angiogenesis.

Conclusion—Pharmacological activation of endothelial LXRs reduces angiogenesis by restraining cholesterol-dependent vascular endothelial growth factor receptor-2 compartmentation and signaling. Thus, administration of LXR agonists could exert therapeutic effects in pathological conditions characterized by uncontrolled angiogenesis. **(*Arterioscler Thromb Vasc Biol*. 2012;32:2280-2288.)**

Key Words: angiogenesis ■ cholesterol ■ liver X receptor ■ vascular endothelial growth factor ■ lipid rafts

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Liver X receptors α and β (LXRα/NR1H3, LXRβ/NR1H2) are retinoid X receptor α (RXRα/NR2B1) heterodimers belonging to the nuclear hormone receptor superfamily. Through coordinate transcriptional actions, LXRs orchestrate cellular and systemic cholesterol homeostasis.[1] LXRs reduce cholesterol absorption, inhibit cholesterol synthesis, and favor cholesterol output via reverse transport and bile secretion. Endogenous LXR agonists, such as 22(R)-, 24-, and 25-hydroxycholesterol, are formed upon cholesterol loading of cells and mediate this physiological feedback loop.[2] Furthermore, LXR activation with synthetic agonists (eg, T0901317, GW3965) exerts beneficial effects in atherosclerosis.[3–6]

LXRs affect cell biology well beyond cholesterol metabolism. For instance, the antiatherosclerotic properties of LXR agonists also involve anti-inflammatory actions.[7] Furthermore, LXR agonists can restrain the proliferation of several cell types by affecting cell cycle control and prosurvival pathways.[8–14] The precise mechanisms linking these complex effects to cholesterol homeostasis are largely unknown. One possibility is that LXR-dependent cholesterol mobilization may affect the structure and dynamics of specific membrane and vesicular compartments. Indeed, T0901317 has been shown to reduce the size and associated signaling of lipid rafts, cholesterol-enriched domains of the plasma membrane constituting fundamental signaling hubs for multiple transduction pathways.[13,15]

Administration of LXR agonists can impair multicellular processes that involve angiogenesis, such as liver regeneration and tumor growth.[9,13,16] We and others have reported that endothelial cells express functional LXRs, with LXRβ representing the most important LXR isoform in this cell type.[17–19] Endothelial cells are particularly sensitive to cholesterol levels, given their wide membrane surface and richness in lipid rafts/caveolae. In line with this view, impairment of cholesterol homeostasis with itraconazole or cyclodextrin has been previously shown to affect vascular endothelial growth factor receptor-2 (VEGFR2) signaling in endothelial cells.[20,21] Taken together, these findings imply that the tissue actions of LXR agonists may also involve so far unappreciated antiangiogenic effects.

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Herein, we show that the pharmacological activation of endothelial LXRs is antiangiogenic as a result of synergistic reductions in cell migration, tubulogenesis, and proliferation. The antiangiogenic effects of LXR agonists involve endothelial cholesterol depletion and impaired VEGFR2 compartmentation and signaling. These findings highlight the potential for LXR-targeted therapeutic interventions in conditions of pathological angiogenesis.

Methods

A full description of experimental methods is provided in the online-only Data Supplement.

Gene Expression Analysis

RNA was extracted using affinity columns (Qiagen, Hilden, Germany) or TRIzol (Invitrogen, Carlsbad, CA). Microarray gene expression profiling was performed with HG-U133 Plus 2.0 microarrays (Affymetrix).[19] For quantitative reverse transcription real-time polymerase chain reaction, mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification system with 18S rRNA as the housekeeping gene. Gene silencing was performed with short hairpin RNA (shRNA) Mission RNA interference vectors (Sigma, St. Louis, MO) against LXRβ (Target Set NM_007121) or scramble shRNA.

Cell Cholesterol

Lipids were extracted using chloroform:isopropanol: tergitol-type NP-40 (7:11:0.1). After homogenization and spinning, the organic phase was dried and suspended in a reaction mix containing cholesterol oxidase and a colorimetric probe (Biovision, Milpitas, CA). Absorbance at 570 nm was finally measured in a microplate reader.

Immunofluorescence

Human umbilical vein endothelial cells (HUVECs) were fixed with 4% paraformaldehye, blocked, and incubated with anti–caveolin-1 (Santa Cruz, Santa Cruz, CA) and anti-VEGFR2 (R&D systems, Minneapolis, MN) antibodies, followed by incubation with secondary antibodies (Molecular Probes, Invitrogen). Nuclei were counterstained with DAPI (Molecular Probes, Invitrogen). Images were captured with a Leica AF6000 workstation equipped with a total internal reflection fluorescence module and analyzed with ImageJ software. Tumor sections were incubated with anti-ABCA1 (Novus Biological, Littleton, CO) or anti-CD31 (BD Biosciences Franklin Lakes, NJ) antibodies. Images were acquired by using a Leica TCS SP2 AOBS confocal microscope and analyzed with Leica Confocal Software.

Cell Fractionation

For fractionation, cell lysates were adjusted to 45% sucrose by the addition of 90% sucrose and placed into ultracentrifugation tubes. A 5% to 35% sucrose discontinuous gradient was formed above, and samples were centrifuged at 246×10^3 g for 16 hours at 4°C in a SW-55Ti rotor (Beckman, Brea, CA). Ten fractions were collected from the top of each gradient.

Migration and Tubulogenesis Assays

HUVEC migration was assayed in a Boyden's chamber with a gelatin-coated polycarbonate membrane. The lower compartment of the chamber was filled with endothelial basal medium-2 (Lonza, Basel, Switzerland) containing vascular endothelial growth factor-A (VEGF-A; 10 ng/mL). HUVECs were serum starved overnight in the presence of the indicated compounds and then added to the upper compartment of the chamber. After 5 hours of incubation, the membrane was fixed and stained for microscopic analysis.

Tubulogenesis assays were performed in solidified basement membrane matrix (Matrigel; BD Biosciences). After incubation with the indicated compounds, HUVECs were seeded and overlaid with EGM-2 medium (Lonza) containing the indicated compounds. After 8 hours, tubular structures were microscopically examined and photographed (Leica, Wetzlar, Germany) for subsequent processing.

Cell Viability and Cell Cycle

Cell viability was determined using the tetrazole assay (Roche, Penzberg, Germany). For apoptosis, annexin-positive HUVECs were detected using the Annexin V-PE apoptosis kit (Merck, Whitehouse Station, NJ). HUVEC proliferation rate and cell cycle were evaluated using the Click-iT EdU flow cytometry assay kit (Invitrogen) and propidium iodide staining, as described.[22]

Animal Studies

Wild-type mice were 8- to 12-week-old female C57BL/6. The generation of $LXR\alpha^{-/-}/LXR\beta^{-/-}$ mice has been previously described.[23,24] Animal procedures were approved by the local ethics committee.

Aortic Ring Angiogenic Assay

Thoracic aortas were removed from 8- to 12-week-old mice and incubated in serum-free medium with treatment compounds.[25] Rings were transferred to Matrigel-coated culture dishes and covered with endothelial growth medium containing the indicated compounds. Tubular structures were examined with an inverted-phase contrast microscope and photographed (Leica) for subsequent processing.

In Vivo Neoangiogenesis

In vivo neoangiogenesis was performed using a modified directed in vivo angiogenesis assay (Trevigen, Gaithersburg, MD). On day 14, mice were euthanized, and angioreactors were dissected. The vascularized basement membrane extract of each angioreactor was recovered and digested using CellSperse solution (Trevigen). Vessel-derived cells were pelleted, suspended in PBS, and counted by fluorescence-activated cell sorter analysis.

Tumor Grafts

For Lewis lung carcinoma (LLC-1) grafts, 10^6 cells were injected subcutaneously into the back of 8- to 12-week-old female C57BL/6 mice. After 1 week, mice were randomized into a treatment group (T0901317 in 0.5% carboxymethyl cellulose–0.25% Tween 20, 20 mg/kg IP QD) and a control group (same amount of vehicle). Tumor growth was checked daily by caliper measurement. Mice were euthanized after 1 week of treatment.

Data Analysis

Quantification of tubular structures was performed with WinRhizo Pro software (Regent Instruments, Quebec City, QC, Canada).[26] Quantification of immunofluorescence microphotographs was performed with ImageJ software (National Institutes of Health, Bethesda, MD). Data are presented as average±SEM. Statistical significance was tested with unpaired Student *t* test or 1-way ANOVA with post hoc analysis. The number of corresponding experimental replicates is provided in each figure legend.

Results

LXR Activation Reduces HUVEC Cholesterol

Human endothelial cells express functional LXRs.[19] Gene expression profiling by microarray (Figure IA in the online-only Data Supplement) and real-time polymerase chain reaction (Figure 1A–1E) indicated that treatment of HUVECs with the synthetic LXR agonist T0901317 modulated the mRNA expression of key genes involved in cholesterol trafficking: ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G

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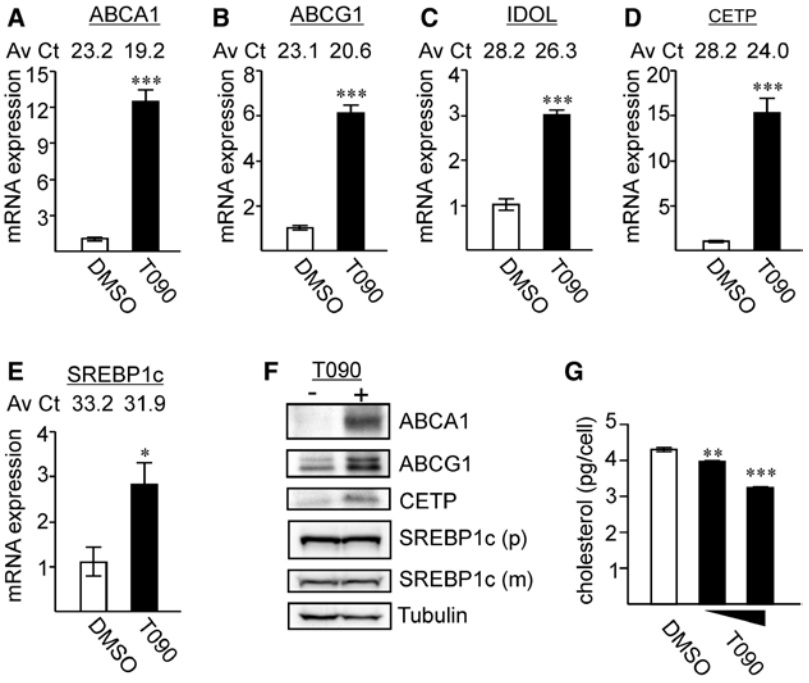

Figure 1. Liver X receptor (LXR) activation reduces endothelial cholesterol. **A** to **E**, Relative mRNA expression of LXR target genes ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1), inducible degrader of the low-density lipoprotein receptor (IDOL), cholesteryl ester transfer protein (CETP), and sterol regulatory element binding transcription factor 1c (SREBP1c) in human umbilical vein endothelial cells (HUVECs) treated for 18 hours with T0901317 (T090, 1 μmol/L) or vehicle (dimethyl sulfoxide [DMSO]). **P*<0.05, ****P*<0.001, n=4. **F**, Protein levels of ABCA1, ABCG1, CETP, and SREBP1c (p=precursor, m=mature) in HUVECs treated for 18 hours with T0901317 (1 μmol/L) or vehicle (DMSO). Representative blots of 3 experiments. **G**, Cell cholesterol (pg/cell) in HUVECs treated for 24 hours with T0901317 (1, 10 μmol/L) or vehicle (DMSO). ***P*<0.01, ****P*<0.001 vs DMSO, n=4.

member 1 (ABCG1), inducible degrader of the low-density lipoprotein receptor, cholesteryl ester transfer protein, and sterol regulatory element binding transcription factor 1c. The upregulation of these genes was blunted by shRNA-mediated silencing of LXRβ (Figures IB, IC, and II in the online-only Data Supplement), thus confirming that ABCA1, ABCG1, inducible degrader of the low-density lipoprotein receptor, cholesteryl ester transfer protein, and sterol regulatory element binding transcription factor 1c are bona fide LXR targets also in endothelial cells.

In line with transcriptional data, T0901317 caused a robust protein induction of cholesterol transporters ABCA1 and ABCG1 (Figure 1F), which mediate cholesterol efflux and reverse cholesterol transport.[27,28] Instead, cholesteryl ester transfer protein and sterol regulatory element binding transcription factor 1c protein levels were only marginally affected by T0901317 in HUVECs. Accordingly, T0901317 reduced HUVEC cholesterol content dose-dependently (Figure 1G).

LXR Activation Inhibits Endothelial Migration and Tubulogenesis

Modifications in endothelial cholesterol balance and trafficking have been shown to affect key biological processes.[21,29,30] To assess the impact of LXR activation on endothelial angiogenesis, we first examined the effect of different LXR agonists on VEGF-A–induced migration of HUVECs. LXR agonists GW3965 and T0901317 reduced migration dose-dependently compared with vehicle (Figure 2A). To rule out off-target effects of LXR-activating compounds, HUVEC migration was also evaluated in the background of LXR silencing. Although T0901317 reduced the migration of HUVECs infected with scramble shRNA, T0901317 did not affect the migration of LXRβ-silenced HUVECs (Figure 2B). HUVECs treated with GW3965 or T0901317 also showed a significant impairment in Matrigel tubulogenesis. GW3965 and T0901317 reduced the average tubule length by 47±14% and 55±10% ($P<0.05$) (Figure 2C) and the fork number by 63±9% and 68±11% ($P<0.05$) (Figure IIIA in the online-only Data Supplement), respectively, compared with vehicle. A similar effect was also produced by natural LXR agonist 22-hydroxycholesterol (Figure IIIB in the online-only Data Supplement). Tubulogenesis was unchanged by T0901317 when HUVECs were coincubated with exogenous cell-soluble cholesterol, thus suggesting that LXR activation impairs in vitro angiogenesis by affecting cell cholesterol homeostasis (Figure 2C; Figure IIIA in the online-only Data Supplement). Furthermore, T0901317 reduced tubulogenesis of HUVECs infected with scramble shRNA but not with LXRβ-targeting shRNA (Figure 2D). Taken together, these findings suggested that inhibition of endothelial migration and morphogenesis by LXR agonists is mediated by LXR-specific effects on cell cholesterol homeostasis.

LXR Activation Inhibits Endothelial Proliferation Without Affecting Apoptosis

Besides motility, angiogenesis involves endothelial proliferation. As LXR agonists have been shown to reduce cell cycle progression in non-endothelial cells,[8–11,14] we assessed the effect of LXR agonists on HUVEC proliferation. GW3965 and T0901317 reduced HUVEC proliferation dose-dependently (Figure 3A). A similar effect was also produced by natural LXR agonist 22-hydroxycholesterol (Figure IIIC in the online-only Data Supplement). T0901317 reduced the protein levels of cyclins A and D1 and increased the protein levels of cyclin-dependent kinase inhibitor p27/Kip-1 (Figure 3B), indicating that LXR activation specifically perturbs cell cycle progression, as previously reported in other cell types.[14,31,32] However, T0901317 did not cause apoptosis (Figure 3C). Incubation of HUVECs with T0901317 specifically restrained

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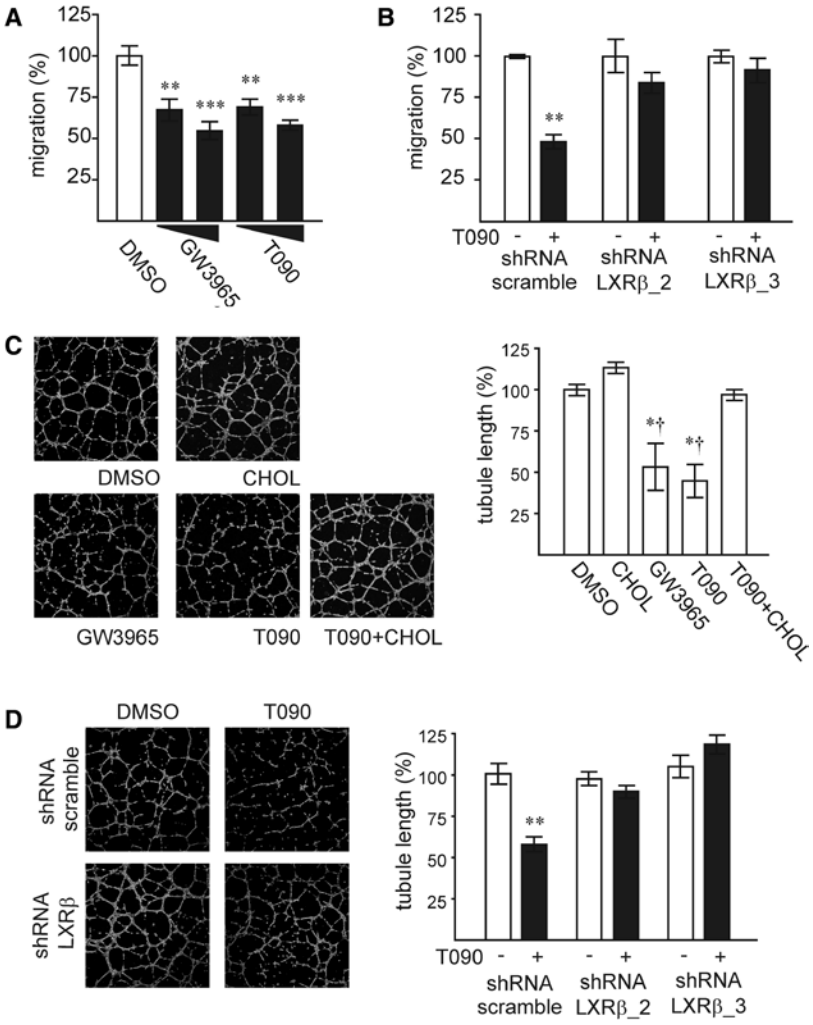

Figure 2. Liver X receptor (LXR) activation impairs endothelial migration and tubulogenesis. **A**, Vascular endothelial growth factor-A (VEGF-A)–induced migration of human umbilical vein endothelial cells (HUVECs) treated for 18 hours with GW3965 (1 or 10 μmol/L), T0901317 (T090, 1 or 10 μmol/L), or vehicle (dimethyl sulfoxide [DMSO]). $^{**}P<0.01$, $^{***}P<0.001$ vs DMSO, n=4. **B**, VEGF-A–induced migration of LXRβ-silenced (shRNA-LXRβ_2/3) or control HUVECs (shRNA scramble) treated for 18 hours with T0901317 (10 μmol/L) or vehicle (DMSO). $^{**}P<0.01$ vs DMSO, n=4. **C**, Matrigel tubulogenesis of HUVECs treated for 18 hours with the indicated compounds: vehicle (DMSO), cholesterol (CHOL, 20 μmol/L), GW3965 (10 μmol/L), or T0901317 (10 μmol/L). Left: Representative images of endothelial tubules. Right: Quantification of relative tubule length per digital image, expressed as percentage of control (DMSO). $^{*}P<0.05$ vs DMSO, †$P<0.05$ vs T090+CHOL, n=4. **D**, Matrigel tubulogenesis of LXRβ-silenced (shRNA-LXRβ_2/3) or control HUVECs (shRNA scramble) treated as in **B**. Left: Representative images of endothelial tubules. Right: Quantification of relative tubule length per digital image, expressed as percentage of DMSO-treated shRNA scramble. $^{**}P<0.01$ vs DMSO, n=4.

the G_1–S cell cycle transition, as assessed by propidium iodide staining (Figure 3D), and reduced the DNA incorporation of a thymidine analog (Figure 3E). The effect of T0901317 on G_1–S transition and DNA synthesis was relieved by coincubation of HUVECs with exogenous cell-soluble cholesterol (Figure 3D and 3E), thus indicating that LXR activation impairs endothelial cell cycle and proliferation by affecting cell cholesterol homeostasis.

LXR Activation Reduces Signaling of VEGFR2

The observed effects of LXR agonists in endothelial cells suggested the involvement of LXR in the angiogenic signaling. As previously reported in macrophages, T0901317 upregulated the mRNA expression of VEGF-A also in HUVECs (Figure IVA in the online-only Data Supplement).[33] However, the protein levels of VEGF-A were unchanged by T0901317 in HUVECs (Figure IVB in the online-only Data Supplement). The mRNA and protein expressions of VEGFR2, the pivotal VEGF receptor controlling angiogenesis,[34] were not affected by T0901317 (Figure IVC in the online-only Data Supplement and Figure 4A). However, treatment of HUVECs with T0901317 blunted the functional activation of VEGFR2 by VEGF-A, as demonstrated by reduced phosphorylation of VEGFR2 on Tyr1175 and by reduced phosphorylation of phospholipase Cγ (PLCγ), a key mediator of VEGFR2-dependent angiogenic responses (Figure 4A).[34] VEGF-A–dependent phosphorylation of VEGFR2 and PLCγ was unchanged by T0901317 when HUVECs were coincubated with exogenous cell-soluble cholesterol (Figure 4B and 4C), suggesting that LXR activation restrains VEGFR2 signaling by affecting cell cholesterol homeostasis. In line with this finding, the inhibitory effect of T0901317 on VEGF-A/VEGFR2-driven chemotaxis was specifically attenuated by the addition of exogenous cell-soluble cholesterol (Figure IVD in the online-only Data Supplement). On the contrary, T0901317 treatment did not affect the migration of HUVECs stimulated by fibronectin in a haptotaxis assay (Figure IVE in the online-only Data Supplement).

LXR Activation Reduces Compartmentation of VEGFR2 in Lipid Rafts/Caveolae

Cholesterol depletion by T0901317 has been associated with a decrease in lipid raft size and signaling.[13] We thus tested the hypothesis that T0901317 may affect VEGFR2 signaling by impairing its compartmentation in lipid rafts/caveolae. We analyzed the cell surface distribution of caveolar rafts, visualized by caveolin-1 staining, and of VEGFR2, by total internal

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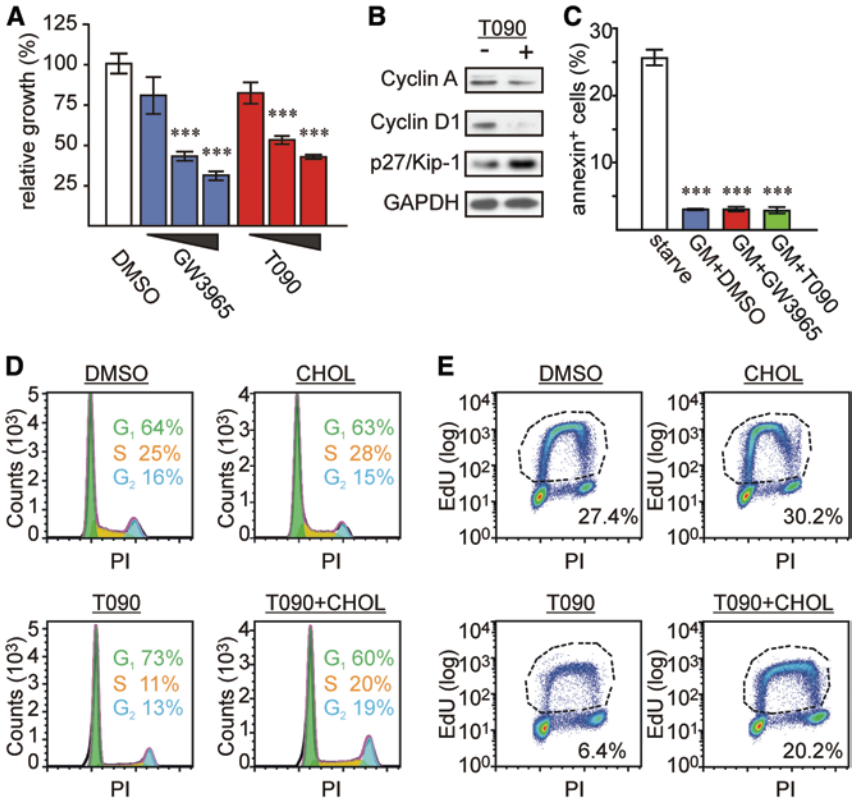

Figure 3. Liver X receptor (LXR) activation impairs endothelial proliferation without affecting apoptosis. **A**, Cell growth of human umbilical vein endothelial cells (HUVECs) treated for 48 hours with vehicle (dimethyl sulfoxide [DMSO]), GW3965 (1, 5, 10 μmol/L), or T0901317 (T090, 1, 5, 10 μmol/L). Cell growth was assessed by tetrazole. ***P<0.001 vs DMSO, n=6. **B**, Protein levels of cyclin A, cyclin D1, and p27/Kip-1 in HUVECs treated for 24 hours with DMSO or T0901317 (10 μmol/L). Representative blots of 3 experiments. **C**, Apoptosis of HUVECs treated for 24 hours with the indicated compounds: vehicle (DMSO), GW3965 (10 μmol/L), T0901317 (10 μmol/L). Starve: endothelial basal medium-2 medium, GM: EGM-2 medium. ***P<0.01 vs DMSO, n=6. **D**, Cell cycle distribution of HUVECs treated for 24 hours with vehicle (DMSO), cholesterol (CHOL, 20 μmol/L), and T0901317 (10 μmol/L). DNA content was determined by propidium iodide staining and assessed by fluorescence-activated cell sorter analysis (representative experiment, n=3). **E**, Proliferation rate of HUVECs treated as in **D**. DNA incorporation of the thymidine fluorescent analog 5-ethynyl-2′-deoxyuridine (EdU) was detected by flow cytometry. The percentage of proliferating cells is indicated (representative experiment, n=3).

reflection fluorescence microscopy. Treatment of HUVECs with T0901317 reduced the caveolin-1–positive area by 53±8% (P<0.05) (Figure 4D and 4E), indicating a depletion in endothelial lipid rafts/caveolae. Furthermore, the amount of VEGFR2 localized in caveolar structures was reduced by 54±7% (P<0.05) (Figure 4D and 4F). The effect of T0901317 on caveolin-1 and VEGFR2 compartmentation was blunted by the addition of exogenous cell-soluble cholesterol (Figure 4E and 4F). Membrane fractionation experiments confirmed that T0901317 displaced VEGFR2 from the buoyant flotillin-2–positive and caveolin-1–positive fractions (1–3), representing endothelial lipid rafts/caveolae. Instead, the amount of VEGFR2 in the heavier, binding immunoglobulin protein–positive fractions, representing the endoplasmic reticulum, was not affected (Figure 4G and 4H). Coincubation of HUVECs with exogenous cell-soluble cholesterol attenuated the T0901317-dependent depletion of VEGFR2 in lipid rafts/caveolae (Figure 4I), indicating that LXR activation modifies VEGFR2 membrane compartmentation by affecting cell cholesterol homeostasis.

LXR Activation Inhibits Endothelial Sprouting and In Vivo Neoangiogenesis

The impact of LXR agonists on angiogenesis was next assayed ex vivo and in vivo. Incubation of mouse aortic rings with GW3965 or T0901317 reduced ex vivo endothelial sprouting by 89±2% and 73±10%, respectively, compared with vehicle (P<0.05). However, endothelial sprouting was unchanged by T0901317 when aortic rings were coincubated with cholesterol, indicating that LXR activation impairs endothelial sprouting through cholesterol depletion (Figure 5A). T0901317 reduced endothelial sprouting when applied to aortic rings obtained from wild-type mice but not to rings obtained from mice deficient for LXRs (LXR$\alpha^{-/-}$/LXR$\beta^{-/-}$) (Figure 5B), thus confirming that the antiangiogenic effect of T0901317 is LXR-specific. In vivo neoangiogenesis was next assessed through the subcutaneous implantation in mice of angioreactors containing VEGF-A and fibroblast growth factor–enriched extracellular matrix. Addition of T0901317 to the extracellular matrix critically reduced neoangiogenesis by 86±7% compared with vehicle (P<0.05) (Figure 5C), as assessed by red blood cell content in the angioreactors 2 weeks after implantation. Taken together, these data indicated that LXR agonists can inhibit angiogenesis in mouse tissues.

LXR Activation Inhibits Tumor Angiogenesis

Neoangiogenesis is strictly required for tumor growth. To highlight the effect of LXR activation on cancer angiogenesis, we used LLC-1 tumor grafts. Contrary to HUVECs, LLC-1 cells did not regulate the protein levels of cholesterol transporters ABCA1 and ABCG1 upon treatment with T0901317 (Figure 6A). Also, the levels of low-density lipoprotein receptor (LDLR) were unchanged by T0901317 in LLC-1 cells. Of note, T0901317 did not modify the proliferation (Figure VA and VB in the online-only Data Supplement), p27/Kip-1 expression (Figure VC in the online-only Data Supplement), and apoptosis (Figure VD in the online-only Data Supplement) of LLC-1 cells in vitro. LLC-1 cells were thus injected into the flank of wild-type mice, where they gave rise to palpable tumors within 7 days. Starting on day 7 after injection, mice were treated with T0901317 or vehicle daily (20 mg/kg IP) for 1 week. Administration of T0901317 significantly reduced

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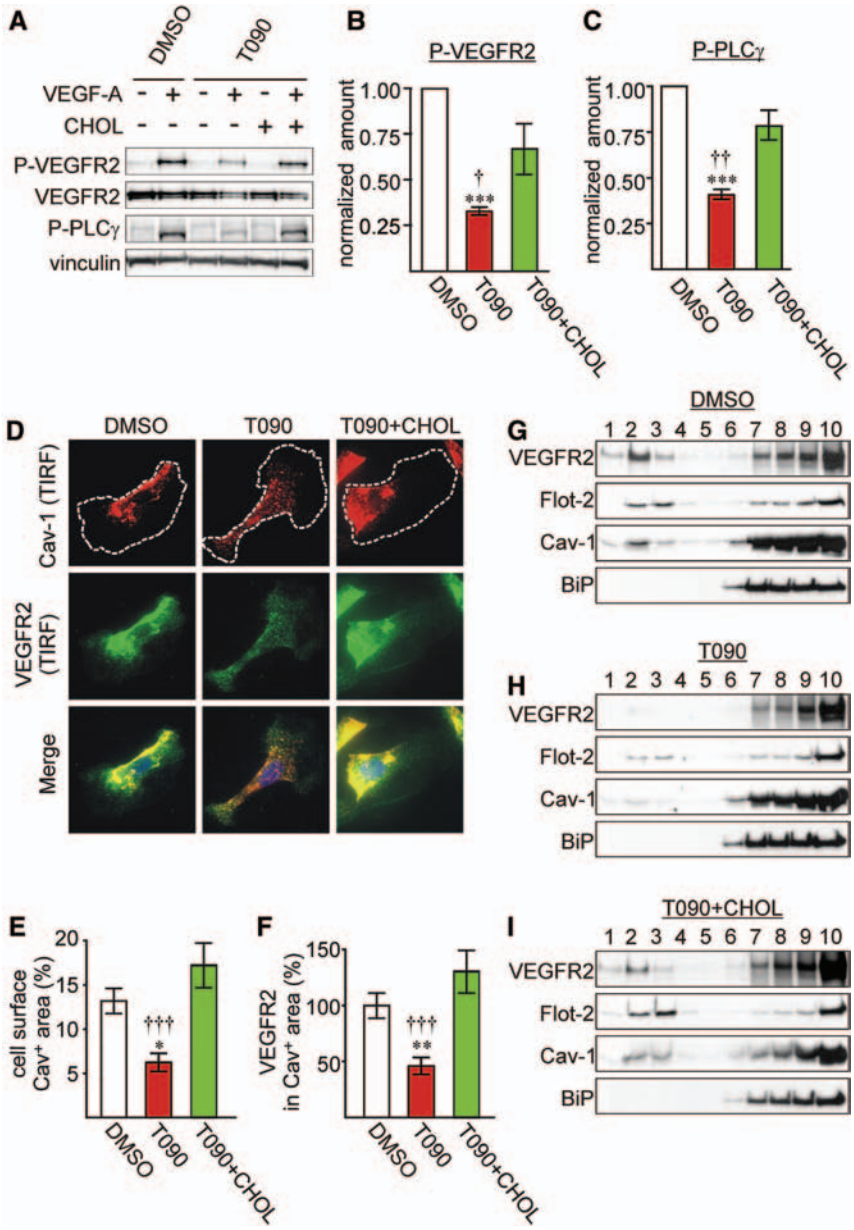

Figure 4. Liver X receptor (LXR) activation impairs vascular endothelial growth factor receptor-2 (VEGFR2) signaling and compartmentation in lipid rafts/caveolae. **A**, Protein levels of phospho-VEGFR2 (p-VEGFR2), total VEGFR2, and phospho-phospholipase Cγ (P-PLCγ) in human umbilical vein endothelial cells (HUVECs) treated with VEGF-A (30 ng/mL, 5 minutes) after incubation for 18 hours with vehicle (dimethyl sulfoxide [DMSO]), T0901317 (T090, 10 µmol/L), and cholesterol (CHOL, 20 µmol/L). Representative blots of 4 experiments. **B**, Quantification of P-VEGFR2 in HUVECs treated as in **A**. ***P<0.001 vs DMSO, †P<0.05 vs T090+CHOL, n=4. **C**, Quantification of P-PLCγ in HUVECs treated as in **A**. ***P<0.001 vs DMSO, ††P<0.01 vs T090+CHOL, n=3. **D**, Immunofluorescence staining of HUVECs treated for 18 hours with vehicle (DMSO), T0901317 (10 µmol/L), and cholesterol (20 µmol/L). Representative images of total internal reflection fluorescence (TIRF): red (caveolin-1), green (VEGRF2), blue (DAPI), yellow (merge caveolin-1/VEGFR2). **E**, Quantification of cell surface caveolin-1–positive area. *P<0.05 vs DMSO, †††P<0.001 vs T090+CHOL, n=20. **F**, Quantification of cell surface VEGFR2 in caveolin-1–positive areas. **P<0.01 vs DMSO, †††P<0.001 vs T090+CHOL, n=20. **G** to **I**, Distribution of VEGFR2, flotillin-2 (Flot-2), caveolin-1 (Cav-1), and binding immunoglobulin protein (BiP) in the protein fractions obtained from HUVECs (ultracentrifugation on a discontinuous sucrose gradient) treated as in **D**. The lipid raft compartment is represented in the lighter fractions (1–3), whereas the endoplasmic reticulum peaks in the heavier fractions (7–10). Representative blots of 3 experiments.

tumor growth by 62±20% on day 7 compared with vehicle (P<0.05) (Figure 6B). Body weight remained similar in treated and control animals (data not shown). Administration of T0901317 upregulated ABCA1 mRNA within the tumor mass (Figure VE in the online-only Data Supplement). In particular, T0901317 significantly increased the protein expression of ABCA1 selectively within tumor endothelial cells (Figure 6C). Of note, T0901317 treatment reduced the endothelial density of LLC-1 tumors by 50±3% compared with vehicle (P<0.01), as assessed by CD31 immunostaining of tumor sections (Figure 6D). Taken together, these findings indicated that pharmacological targeting of LXRs can significantly reduce tumor angiogenesis.

Discussion

We provide evidence that LXR activation can restrain angiogenesis. The antiangiogenic effects of LXR agonists are strictly connected to their impact on endothelial cholesterol homeostasis, because coincubation of HUVECs or aortic rings with exogenous cholesterol largely neutralized the actions of LXR agonists on endothelial tubulogenesis, sprouting, and proliferation. These results are in line with previous reports that drugs leading to cholesterol deprivation or impaired cholesterol trafficking, such as β-cyclodextrin, itraconazole, or high-dose statins, can negatively affect angiogenesis.[29,30,35] In endothelial cells, T0901317 lowered cell cholesterol by regulating the expression of multiple targets. In particular, LXR activation increased the protein levels of cholesterol transporters ABCA1 and ABCG1, which promote cholesterol output toward apolipoprotein A–containing lipoproteins, as well as transendothelial high-density lipoprotein traffic.[28,36] T0901317 also increased the mRNA expression of inducible degrader of the low-density lipoprotein receptor, which targets LDLR to ubiquitin-mediated

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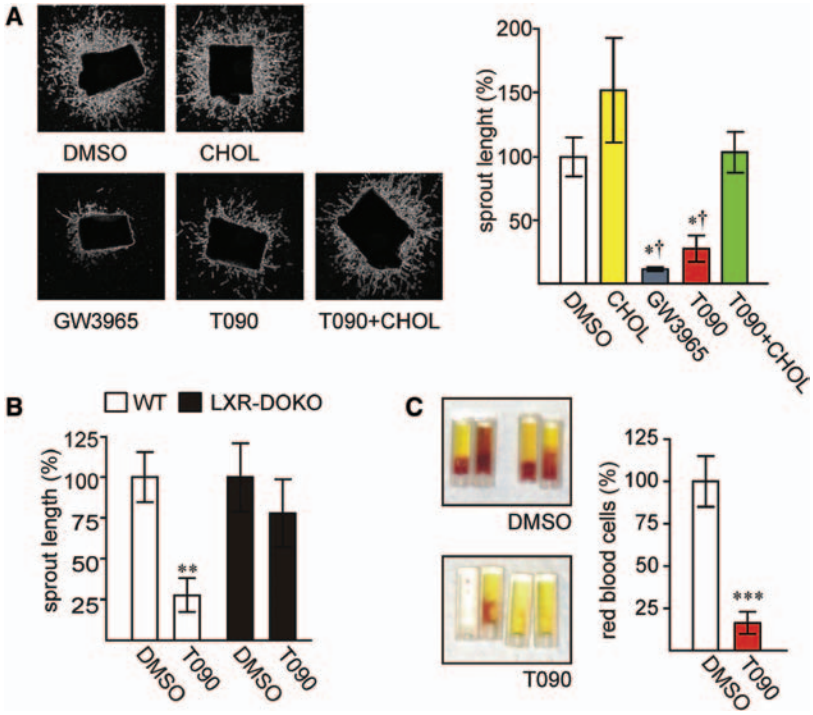

Figure 5. Liver X receptor (LXR) activation impairs angiogenesis in mouse tissues. **A**, Endothelial sprouting from mouse aortic rings incubated with the indicated compounds for 5 days: vehicle (dimethyl sulfoxide [DMSO]), GW3965 (10 μmol/L), T0901317 (T090, 10 μmol/L), cholesterol (CHOL, 20 μmol/L). Left: Representative images of sprouting. **P*<0.05 vs DMSO, †*P*<0.05 vs T090+CHOL, n=4. **B**, Endothelial sprouting from wild-type (WT) and LXRα$^{-/-}$/LXRβ$^{-/-}$ (LXR-DOKO) aortic rings incubated with vehicle (DMSO) or T0901317 (10 μmol/L). ***P*<0.01 vs DMSO, n=4. **C**, Neoangiogenesis in angioreactors implanted subcutaneously in wild-type mice for 2 weeks. Left: Representative images of explanted angioreactors filled with basement membrane extract containing vascular endothelial growth factor-A and fibroblast growth factor-2 plus vehicle (DMSO) or T0901317 (10 μmol/L). ****P*<0.001, n=14.

degradation.[37] As the expression of LDLR is negligible in HUVECs, the effect of LXRs on endothelial cholesterol homeostasis is, therefore, essentially mediated by ABCA1 and ABCG1, at least in vitro. We previously reported that incubation of endothelial cells with natural LXR agonist 22(R)-hydroxycholesterol downregulates the expression of several genes controlling cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase, mevalonate kinase, and squalene epoxidase.[19] Hence, natural oxysterol LXR ligands may further impact on endothelial cholesterol homeostasis through synergic effects on cholesterol synthesis and traffic. Nonetheless, we cannot exclude that antiagiogenic effects of LXRs may also relate to so far unappreciated perturbations of the nonsterol branch of the mevalonate pathway. For instance, LXR agonists may modify the synthesis of isoprenoids and affect protein prenylation, which is required for proper compartmentation and function of key proteins involved in angiogenesis (eg, small GTPases).

Cholesterol is essential to maintain a normal structure and function of cellular membranes. Furthermore, cholesterol-rich microdomains of the plasma membrane called lipid rafts/caveolae function as preferential sites for membrane receptor clustering and signaling.[15] LXR activation has been previously shown to reduce lipid raft size and restrain lipid raft–associated signaling in a cancer cell line.[13] We now provide evidence that in endothelial cells, treatment with LXR agonist T0901317 specifically impairs biological signals stemming from lipid raft/caveolar domains that are critical for angiogenesis. In particular, LXR activation (1) impaired VEGFR2 phosphorylation and downstream signaling to PLCγ, and (2) blunted the compartmentation of VEGFR2 in lipid rafts/caveolae. Both effects appear to be mediated by the LXR-dependent perturbation of cholesterol homeostasis. These findings are in line with previous data showing that changes in endothelial cholesterol affect VEGFR2 signaling, most likely as a result of complex interactions of the receptor and its signaling platform within lipid rafts/caveolae.[20,21] Nonetheless, the molecular circuitry linking cholesterol balance to angiogenesis is only beginning to emerge. In our study, LXR activation significantly reduced caveolin-rich regions of the plasma membrane. As knockout of caveolin-1 inhibits angiogenesis by impairing VEGFR2 compartmentation and by causing a detrimental deregulation of endothelial NO synthase, LXR activation may restrain angiogenesis by altering the physiological conditions of caveolin-1 expression and internalization, and possibly endothelial NO synthase activity.[38,39] Furthermore, as lipid rafts/caveolae are membrane microdomains harboring multiple signaling platforms, we cannot exclude that the effects of LXR activation may extend to other relevant receptors and pathways, such as those of fibroblast growth factor or transforming growth factor-β.

We provide evidence that the antiangiogenic effects of LXR activation can be therapeutically meaningful, because T0901317 reduced both the vascularization and tumor growth of LLC-1 grafts. Of note, the anti-cancer properties of T0901317 were detectable only in vivo, because this drug did not modify the proliferation and apoptosis of LLC-1 cells in vitro. The resistance of LLC-1 cells to LXR activation is in line with previous observations that LXR agonists can impair cell cycle progression and favor apoptosis (eg, by increasing the expression of p27/Kip-1) only in certain cancer cell lines, whereas others are resistant to LXR activation.[9,12,13,40] It has been suggested that the sensitivity to LXR agonists may relate to the cell-specific effects of LXRs on cholesterol homoeostasis.[31] Our findings support this hypothesis, because in LLC-1 cells T0901317 did not modify the expression of both cholesterol-handling

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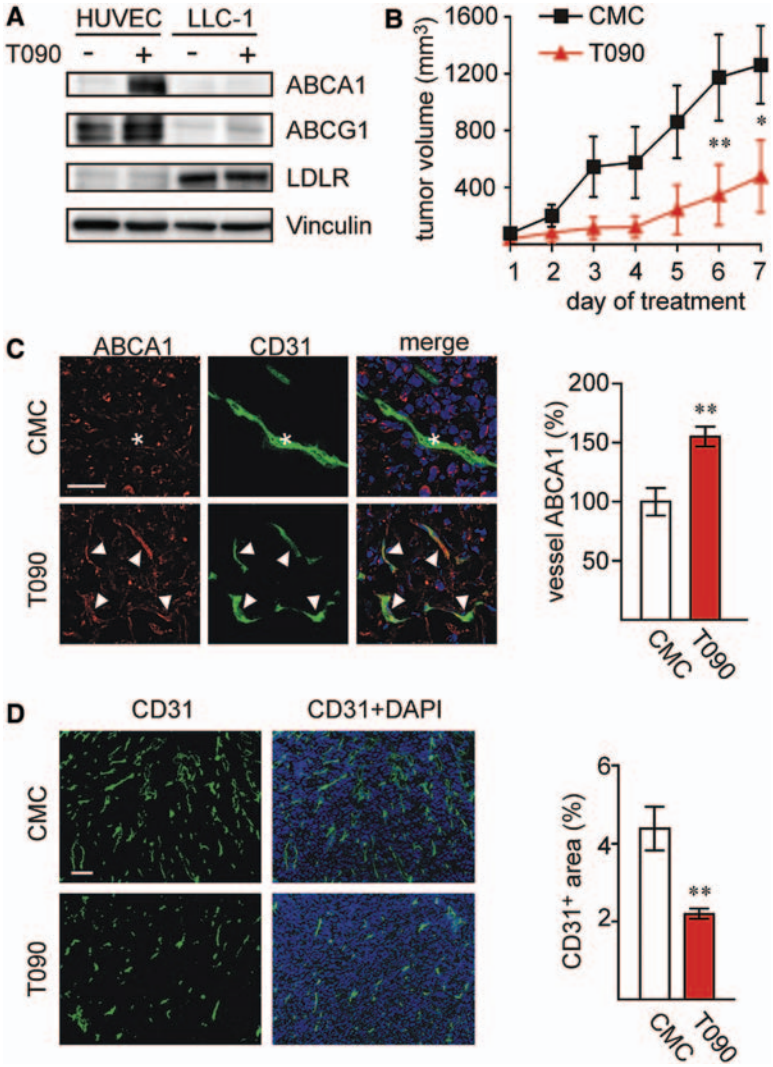

Figure 6. Liver X receptor (LXR) activation reduces tumor angiogenesis. **A**, Protein levels of ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1), and low-density lipoprotein receptor (LDLR) in human umbilical vein endothelial cells (HUVECs) and Lewis lung carcinoma (LLC-1) cells treated for 18 hours with vehicle (dimethyl sulfoxide [DMSO]) or T0901317 (T090, 1 μmol/L). Representative blots of 3 experiments. **B**, Growth curve of LLC-1 tumor grafts in mice treated with vehicle (CMC-Tween 20) or T0901317 (20 mg/kg IP QD) for 1 week. Tumor volume was calculated based on daily caliper measurements. **P*<0.05, ***P*<0.01, n=10 mice per group. **C**, ABCA1/CD31 immunostaining in tumor vessels. Treatments were performed as in **B**. Left: Representative images (red: ABCA1, green: CD31, blue: DAPI), ×40 magnification, scale bar 30 μm. Right: Quantification of ABCA1 fluorescence in CD31+ areas. ***P*<0.01, n=10 per group. **D**, CD31 immunostaining in tumor sections. Treatments were performed as in **B**. Left: Representative images, ×10 magnification, scale bar 100 μm (green: CD31, blue: DAPI). Right: Quantification of CD31 immunofluorescence (% of tumor area). ***P*<0.01, n=10 per group.

proteins (ABCA1, ABCG1, and LDLR) and cell cycle regulator p27/Kip-1.

Taken together, the present findings picture a scenario where LXR agonists can limit cancer growth in vivo by exerting substantial antiangiogenic effects. Nonetheless, it can be foreseen that in certain cancer types, the angiostatic properties of LXR-activating compounds may synergize with direct antiproliferative and pro-apoptotic actions of LXRs on tumor cells. As the systemic administration of LXR-activating drugs is presently limited by side effects, such as liver steatosis and increased LDL-cholesterol, these drugs may find earlier applications as locally delivered therapies.

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Disclosures

None.

References

1. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest*. 2006;116:607–614.
2. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. 1996;383:728–731.
3. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci USA*. 2002;99:7604–7609.
4. Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci USA*. 2002;99:11896–11901.
5. Bischoff ED, Daige CL, Petrowski M, Dedman H, Pattison J, Juliano J, Li AC, Schulman IG. Non-redundant roles for LXRalpha and LXRbeta in atherosclerosis susceptibility in low density lipoprotein receptor knockout mice. *J Lipid Res*. 2010;51:900–906.
6. Lo Sasso G, Murzilli S, Salvatore L, D'Errico I, Petruzzelli M, Conca P, Jiang ZY, Calabresi L, Parini P, Moschetta A. Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. *Cell Metab*. 2010;12:187–193.

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7. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med*. 2003;9:213–219.
8. Blaschke F, Leppanen O, Takata Y, Caglayan E, Liu J, Fishbein MC, Kappert K, Nakayama KI, Collins AR, Fleck E, Hsueh WA, Law RE, Bruemmer D. Liver X receptor agonists suppress vascular smooth muscle cell proliferation and inhibit neointima formation in balloon-injured rat carotid arteries. *Circ Res*. 2004;95:e110–e123.
9. Fukuchi J, Kokontis JM, Hiipakka RA, Chuu CP, Liao S. Antiproliferative effect of liver X receptor agonists on LNCaP human prostate cancer cells. *Cancer Res*. 2004;64:7686–7689.
10. Wente W, Brenner MB, Zitzer H, Gromada J, Efanov AM. Activation of liver X receptors and retinoid X receptors induces growth arrest and apoptosis in insulin-secreting cells. *Endocrinology*. 2007;148:1843–1849.
11. Meng ZX, Nie J, Ling JJ, Sun JX, Zhu YX, Gao L, Lv JH, Zhu DY, Sun YJ, Han X. Activation of liver X receptors inhibits pancreatic islet beta cell proliferation through cell cycle arrest. *Diabetologia*. 2009;52:125–135.
12. Chuu CP, Lin HP. Antiproliferative effect of LXR agonists T0901317 and 22®-hydroxycholesterol on multiple human cancer cell lines. *Anticancer Res*. 2010;30:3643–3648.
13. Pommier AJ, Alves G, Viennois E, Bernard S, Communal Y, Sion B, Marceau G, Damon C, Mouzat K, Caira F, Baron S, Lobaccaro JM. Liver X Receptor activation downregulates AKT survival signaling in lipid rafts and induces apoptosis of prostate cancer cells. *Oncogene*. 2010;29:2712–2723.
14. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell*. 2008;134:97–111.
15. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. *Science*. 2010;327:46–50.
16. Lo Sasso G, Celli N, Caboni M, Murzilli S, Salvatore L, Morgano A, Vacca M, Pagliani T, Parini P, Moschetta A. Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. *Hepatology*. 2009;51:1334–1344.
17. Liao H, Langmann T, Schmitz G, Zhu Y. Native LDL upregulation of ATP-binding cassette transporter-1 in human vascular endothelial cells. *Arterioscler Thromb Vasc Biol*. 2002;22:127–132.
18. Norata GD, Ongari M, Uboldi P, Pellegatta F, Catapano AL. Liver X receptor and retinoic X receptor agonists modulate the expression of genes involved in lipid metabolism in human endothelial cells. *Int J Mol Med*. 2005;16:717–722.
19. Morello F, Saglio E, Noghero A, Schiavone D, Williams TA, Verhovez A, Bussolino F, Veglio F, Mulatero P. LXR-activating oxysterols induce the expression of inflammatory markers in endothelial cells through LXR-independent mechanisms. *Atherosclerosis*. 2009;207:38–44.
20. Labrecque L, Royal I, Surprenant DS, Patterson C, Gingras D, Béliveau R. Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. *Mol Biol Cell*. 2003;14:334–347.
21. Liao WX, Feng L, Zhang H, Zheng J, Moore TR, Chen DB. Compartmentalizing VEGF-induced ERK2/1 signaling in placental artery endothelial cell caveolae: a paradoxical role of caveolin-1 in placental angiogenesis *in vitro*. *Mol Endocrinol*. 2009;23:1428–1444.
22. Noghero A, Arese M, Bussolino F, Gualandris A. Mature endothelium and neurons are simultaneously derived from embryonic stem cells by 2D *in vitro* culture system. *J Cell Mol Med*. 2011;15:2200–2215.
23. Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell*. 1998;93:693–704.
24. Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science*. 2000;289:1524–1529.
25. Primo L, Seano G, Roca C, Maione F, Gagliardi PA, Sessa R, Martinelli M, Giraudo E, di Blasio L, Bussolino F. Increased expression of alpha6 integrin in endothelial cells unveils a proangiogenic role for basement membrane. *Cancer Res*. 2010;70:5759–5769.
26. Cascone I, Napione L, Maniero F, Serini G, Bussolino F. Stable interaction between alpha5beta1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. *J Cell Biol*. 2005;170:993–1004.
27. Vaisman BL, Demosky SJ, Stonik JA, Ghias M, Knapper CL, Sampson ML, Dai C, Levine SJ, Remaley AT. Endothelial expression of human ABCA1 in mice increases plasma HDL cholesterol and reduces diet-induced atherosclerosis. *J Lipid Res*. 2012;53:158–167.
28. Rohrer L, Ohnsorg PM, Lehner M, Landolt F, Rinninger F, von Eckardstein A. High-density lipoprotein transport through aortic endothelial cells involves scavenger receptor BI and ATP-binding cassette transporter G1. *Circ Res*. 2009;104:1142–1150.
29. Xu J, Dang Y, Ren YR, Liu JO. Cholesterol trafficking is required for mTOR activation in endothelial cells. *Proc Natl Acad Sci USA*. 2010;107:4764–4769.
30. Vincent L, Chen W, Hong L, Mirshahi F, Mishal Z, Mirshahi-Khorassani T, Vannier JP, Soria J, Soria C. Inhibition of endothelial cell migration by cerivastatin, an HMG-CoA reductase inhibitor: contribution to its anti-angiogenic effect. *FEBS Lett*. 2001;495:159–166.
31. Kim KH, Lee GY, Kim JI, Ham M, Won Lee J, Kim JB. Inhibitory effect of LXR activation on cell proliferation and cell cycle progression through lipogenic activity. *J Lipid Res*. 2010;51:3425–3433.
32. Vedin LL, Lewandowski SA, Parini P, Gustafsson JA, Steffensen KR. The oxysterol receptor LXR inhibits proliferation of human breast cancer cells. *Carcinogenesis*. 2009;30:575–579.
33. Walczak R, Joseph SB, Laffitte BA, Castrillo A, Pei L, Tontonoz P. Transcription of the vascular endothelial growth factor gene in macrophages is regulated by liver X receptors. *J Biol Chem*. 2004;279:9905–9911.
34. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7:359–371.
35. Vincent L, Soria C, Mirshahi F, Opolon P, Mishal Z, Vannier JP, Soria J, Hong L. Cerivastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme a reductase, inhibits endothelial cell proliferation induced by angiogenic factors *in vitro* and angiogenesis in *in vivo* models. *Arterioscler Thromb Vasc Biol*. 2002;22:623–629.
36. Panzenboeck U, Balazs Z, Sovic A, Hrzenjak A, Levak-Frank S, Wintersperger A, Malle E, Sattler W. ABCA1 and scavenger receptor class B, type I, are modulators of reverse sterol transport at an *in vitro* blood-brain barrier constituted of porcine brain capillary endothelial cells. *J Biol Chem*. 2002;277:42781–42789.
37. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science*. 2009;325:100–104.
38. Sonveaux P, Martinive P, DeWever J, Batova Z, Daneau G, Pelat M, Ghisdal P, Grégoire V, Dessy C, Balligand JL, Feron O. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. *Circ Res*. 2004;95:154–161.
39. Morais C, Ebrahem Q, Anand-Apte B, Parat MO. Altered angiogenesis in caveolin-1 gene-deficient mice is restored by ablation of endothelial nitric oxide synthase. *Am J Pathol*. 2012;180:1702–1714.
40. Mehrotra A, Kaul D, Joshi K. LXR-α selectively reprogrammes cancer cells to enter into apoptosis. *Mol Cell Biochem*. 2011;349:41–55.

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