Negative Regulation of Hedgehog Signaling by Liver X Receptors

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Hedgehog (Hh) signaling is indispensable in embryonic development, and its dysregulated activity results in severe developmental disorders as shown by genetic models of naturally occurring mutations in animal and human pathologies. Hh signaling also functions in postembryonic development and adult tissue homeostasis, and its aberrant activity causes various human cancers. Better understanding of molecular regulators of Hh signaling is of fundamental importance in finding new strategies for pathway modulation. Here, we identify liver X receptors (LXRs), members of the nuclear hormone receptor family, as previously unrecognized negative regulators of Hh signaling. Activation of LXR by specific pharmacological ligands, TO901317 and GW3965, inhibited the responses of pluripotent bone marrow stromal cells and calvaria organ cultures to sonic Hh, resulting in the inhibition of expression of Hh-target genes, Gli1 and Patched1, and Gli-dependent transcriptional activity. Moreover, LXR ligands inhibited sonic Hh-induced differentiation of bone marrow stromal cells into osteoblasts. Elimination of LXRs by small interfering RNA inhibited ligand-induced inhibition of Hh target gene expression. Furthermore, LXR ligand did not inhibit Hh responsiveness in mouse embryonic fibroblasts that do not express LXRs, whereas introduction of LXR into these cells reestablished the inhibitory effects. Daily oral administration of TO901317 to mice after 3 d significantly inhibited baseline Hh target-gene expression in liver, lung, and spleen. Given the importance of modulating Hh signaling in various physiological and pathological settings, our findings suggest that pharmacological targeting of LXRs may be a novel strategy for Hh pathway modulation. (Molecular Endocrinology 23: 1532–1543, 2009)

Hedgehog (Hh) molecules play key roles in a variety of processes including tissue patterning, mitogenesis, morphogenesis, cellular differentiation, stem cell physiology, embryonic development, cancer, and cardiovascular disease (1–7). In mammals, three members of the Hh family of proteins have been identified, namely sonic Hh (Shh), indian Hh, and desert Hh (known to be mainly present in neuronal tissues and gonadal cells). In addition to its role in embryonic development, Hh signaling plays a crucial role in postnatal development and maintenance of tissue/organ integrity and function (8–14). Studies using genetically engineered mice have demonstrated that Hh signaling is critical during skeletogenesis and vasculogenesis, as well as in development of osteoblasts, chondrocytes, and endothelial cells in vitro and in vivo (15–18). Aberrant Hh signaling has been implicated in various cancers including hereditary forms of medulloblastoma, basal cell carcinoma, and prostate, breast, colon, and lung

Abbreviations: ALP, Alkaline phosphatase; BSP, bone sialoprotein; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; GW, GW3965; Hh, hedgehog; LXR, liver X receptor; LXRE, LXR response element; MEF, mouse embryonic fibroblast; MSC, bone marrow stromal cell; OCN, osteocalcin; PM, purmorphamine; Ptch, Patched; Q-RT-PCR, quantitative RT-PCR; Shh, sonic Hh; siRNA, small interfering RNA; Smo, Smoothened; TO, TO901317.
cancers, whereas reduced or interrupted Hh pathway activity can cause severe developmental defects in mice and humans (1, 4, 19). Given these roles in various physiological and pathological conditions, a better understanding of molecular regulators of Hh signaling is of fundamental importance. In addition, modulation of Hh signaling through novel mechanisms may be beneficial in targeting various human disorders (20).

Hh signaling involves a complex network of factors that includes plasma membrane proteins, kinases, phosphatases, and factors that facilitate the shuttling and distribution of Hh molecules (21–23). Production of Hh proteins from a subset of producing/signaling cells involves synthesis, autoprocessing, and lipid modification (24, 25). In the absence of Hh proteins, Patched (Ptc), present on the plasma membrane of the responding cells, keeps Hh signaling in a silent mode by preventing the activity of another plasma membrane-associated signal transducer molecule, Smoothened (Smo). In the presence of Hh, the inhibition of Smo by Ptc is alleviated, and Smo transduces the signal that regulates the transcription of Hh target genes. This transcriptional regulation in part involves the Ci/Gli transcription factors that enter the nucleus from the cytoplasm after a very intricate interaction between the members of a complex of accessory molecules, including Fused, suppressor of Fused (Sufu), and Rab23 that regulate localization and stability of Gli (26–28). Many, but clearly not all, regulators of Hh pathway signaling and their functions are conserved between Droso phila and vertebrates, and there is still much to be learned about the intracellular and extracellular regulators of this critical signaling network.

Liver X receptors α and β (LXRα and LXRβ) are nuclear hormone receptors that, upon activation, regulate the expression of target genes in various physiological pathways (29–31). Perhaps the most well-studied property of LXR is its ability to regulate intracellular lipid and sterol metabolism by regulating the genes the products of which are key members of the cholesterol biosynthetic pathway and lipid homeostasis (29–32). LXRs also regulate reverse cholesterol transport from peripheral tissues to the liver mainly by increasing the expression of members of the ABC superfamily of membrane transporters (32, 33). Among most studied members are ABCA1 and ABCG1, which mediate sterol efflux from various cell types. LXR were thought to be orphan nuclear receptors until it was found that specific oxysterols act as their physiological ligands (29–31). Although most studies have revolved around LXR’s ability to regulate cholesterol homeostasis, more recent reports demonstrate its ability to regulate inflammatory responses through indirect trans-repression of genes that do not have LXR-bind-

Results

LXR activation inhibits Hh signaling

In previous studies, we and others have demonstrated the role of Hh pathway in mediating the lineage-specific differentiation of pluripotent bone MSCs into bone-forming osteoblasts and inhibition of their differentiation into adipocytes (44–46). Furthermore, in previous studies we reported the presence of LXR in MSCs as well as the inhibition of osteoblast differentiation marker, alkaline phosphatase (ALP) activity, upon treatment of these cells with LXR ligands including TO901317 (TO) (43). These findings prompted us to examine whether LXR activation interferes with Hh signaling and ultimately Hh-induced osteogenic differentiation of MSCs. Treatment of M2-10B4 (M2) MSCs with TO induced the mRNA expression of LXR target genes ABCA1 and ABCG1 approximately 20- and 2-fold, respectively, after 48 h of treatment (data not shown). TO inhibited the expression of Shh-induced target genes Ptc1 and Gli1 approximately 24, 48, and 72 h of treatment (Fig. 1) in a dose-dependent manner (supplemental Fig. 1 published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). Similar results were ob-

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tained using a structurally distinct LXR ligand, GW3965 (GW, supplemental Fig. 2). In addition, Shh-induced Gli transcriptional activity, measured using an 8×Gli luciferase reporter (44), showed dose-dependent inhibition by TO (Fig. 2). In contrast, LXR activation did not inhibit bone morphogenetic protein 2 signaling in MSCs as assessed by the lack of any effects of TO on phosphorylation of Smad/1/5/8 proteins and mRNA expression of bone morphogenetic protein 2-target genes Id1 and Msx2 (data not shown). This suggests that the inhibitory effects of LXR are not due to a generalized inhibition of all cellular responses.

To further investigate the kinetics of the inhibitory effects of LXR activation on Shh-induced signaling, M2 cells were treated in the following manner: 1) pretreated with TO for 24 h, followed by removal of TO and addition of Shh for 48 h without TO; 2) pretreated with TO for 24 h followed by the addition of Shh + TO for 48 h; 3) cotreated with Shh + TO for 48 h; and 4) first treated with Shh for 24 h and subsequently with Shh + TO for 48 h. Quantitative RT-PCR (Q-RT-PCR) analysis of RNA extracted at the end of the experiment demonstrated that TO inhibited Shh-induced expression of Ptc1 and Gli1 under all the above treatment conditions (Fig. 3).

In contrast to its ability to inhibit Shh-induced Hh target gene expression, TO was unable to inhibit responses of M2 cells to purmorphamine (PM), a ligand that activates Hh signaling through direct binding to Smo (47) (Fig. 4).

**Effect of LXR elimination on inhibition of Shh signaling by TO**

To further confirm that the effects of TO on Shh signaling are mediated through LXR activation and not other mechanism(s), first we employed small interfering RNA (siRNA) to inhibit both LXRα and LXRβ expression in M2 cells. This approach consistently inhibited the mRNA expression for LXRs by more than 80% (supplemental Fig. 3, A and B) and inhibited TO-induced ABCA1 expression (supplemental Fig. 3C). Presence of Shh had no effect on TO-induced ABCA1 expression in the presence or absence of LXR siRNA (supplemental Fig. 3C). LXR siRNA significantly reversed the inhibitory effect of TO on Shh-induced Ptc1 and Gli1 expression (Fig. 5, A and B).

Furthermore, we examined the inhibitory effect of TO in mouse embryonic fibroblasts (MEFs) derived from LXRαβ null mice, which do not express either isotype of LXR, and do not express ABCA1 or ABCG1 mRNA in response to TO (Fig. 6, A–C). Treatment of MEFs with Shh caused a significant induction in Gli1 expression; however, TO was unable to inhibit this response (Fig. 6D). Upon retroviral introduction of LXRα into LXR null MEFs, TO was able to exert its inhibitory effect on Shh-induced Gli1 expression (Fig. 6D). Similar to reports from other cell types including neuronal cells (48, 49), Shh-induced Ptc1 expression in null MEFs was minimal (~1.3-fold; data not shown). Interestingly, expression of LXRα in null MEFs reduced the response of these cells to Shh compared with MEFs devoid of LXR (Fig. 6D).
We investigated the potential effect of TO-induced LXR activation on the expression of positive and negative regulators of Hh signaling. Treatment of M2 cells for 24 and 48 h with 2 μM TO showed no significant changes in the baseline mRNA expression for Gli1, Gli2, Gli3, and Smo, which are deemed positive regulators of the Hh pathway (21), and whose expression levels may have been reduced by TO (data not shown). TO also caused no significant change in the mRNA expression of Ptch1 or Sufu, which are negative regulators of Hh pathway activity (50) the levels of expression of which may have been induced by TO as a mechanism of inhibiting responses to Shh (data not shown).

Because recent studies have demonstrated that Hh signaling is mediated at the level of primary cilia (51–53), we examined whether TO inhibits the formation/integrity of primary cilia in M2 cells. Cells were treated for 48 h with TO and then stained for primary cillum-associated acetylated α-tubulin and analyzed by fluorescence microscopy. In contrast with chloral hydrate, which has been shown to disrupt primary cillum and Hh signaling in various cell types (54, 55) including in M2 cells (data not shown), TO treatment had no apparent effects on the shape of primary cillum or the percentage of ciliated cells (Fig. 7, A and B).

In addition, Q-RT-PCR and Western blot analysis of M2 cells treated with TO for 48 h did not show any change in mRNA and protein expression for Polaris, respectively (data not shown). Polaris is a member of the intraflagellar transport (Ift) proteins, the disruption of which in other cell types was found to inhibit Hh signaling (51, 56, 57).

LXR activation inhibits Shh-induced osteogenic differentiation of bone MSCs

We and others have previously reported that Shh induces the osteogenic differentiation of pluripotent mesenchymal cells, including MSCs (44, 45). Treatment of M2 cells with TO or GW caused a dose-dependent inhibition of Shh-induced osteogenic differentiation as measured by its effects on differentiation markers ALP activity, and bone sialoprotein (BSP) and osteocalcin (OCN) mRNA expression (Fig. 8, A–D).

LXR activation inhibits Hh signaling in calvaria organ cultures ex vivo

To further validate the inhibitory effects of LXR activation on Hh signaling, we examined the effects of TO on Shh-induced target gene expression in mouse calvaria organ cultures ex vivo. Calvaria organ cultures derived from mouse pups contain a population of immature preosteoblasts that differentiate and mature ex vivo and have been previously demonstrated to be excellent indicators of the in vivo biological effects of various regulators of osteo-
mice after 3 days of daily administration of TO (40 mg/kg) by oral gavage in 8-wk-old male C57BL/6 mice. Q-RT-PCR analysis showed the baseline expression of Ptch1 and Gli1 in all tissues examined, and LXR activation in each tissue caused a significant inhibition of Ptch1 and Gli1 in the liver and spleen of mice and a significant inhibition of Ptch1 in the lung compared with mice treated with control vehicle (Fig. 10).

**Discussion**

Given the multitude of physiological and pathological processes that are regulated by Hh signaling, gaining greater insights into its molecular regulation is of fundamental importance to its therapeutic targeting. Cross talk between Hh signaling and nuclear hormone receptors including the steroid hormone receptors (64), retinoic acid receptor (65), and peroxisome proliferator-activated receptors (66) among others have been reported in the past. The present report provides the first line of evidence for negative regulatory effects of LXR on Hh signaling.

LXRs are expressed in various tissues during embryonic and postembryonic stages of life, and their role in tissue physiology, inflammation, and lipid metabolism has been demonstrated (29–31). We speculate that another one of LXR’s significant and yet unrecognized physiological roles may involve regulation of Hh signaling. Data presented here definitively show that pharmacological activation of LXR by small molecule ligands inhibits the responses of Hh-responsive cells to Shh, and that in the absence of LXR these ligands do not exert their inhibitory effects. However, interestingly, LXR activation was not effective in inhibiting Hh responsiveness when the activation of Hh pathway was caused through direct interaction of PM with Smo rather than that achieved by Shh, which binds to Ptch (21). These differential effects of LXR activation on Hh pathway suggest the targeting of an LXR-regulated molecular event that modulates Hh signaling through direct regulation of Ptch and/or a Ptch-regulated secondary event that occurs upstream of Smo. We did not find any significant effects of TO on mRNA expression for Ptch; therefore, any effect on Ptch would most likely be at the level of protein expression or subcellular localization, perhaps to primary cilium (67). Our initial studies of the effect of LXR activation on primary cilium and one of the Ift proteins, Polaris, did not show any significant changes in TO-treated cells. However, we cannot rule out that other features of the primary cilium, such as its molecular integrity and composition, that affect its ability to transduce signals may not be altered by LXR activation. Alternatively, it has been suggested that Ptch may somehow regulate the interaction of a small molecule, such as an oxysterol, with Smo (68, 69), which could be the target of LXR activation. It is noteworthy that LXR activation induce the expression of ABC transporters in all cells tested, and it is plausible that transport
and of a lipophilic molecule or sterol by these transporters away from Smo might negatively regulate Hh signaling. Although this possibility is yet to be tested, the previous observation that sterol depletion of cells hampers their response to Hh proteins is supportive of that hypothesis (70). Moreover, although our studies were performed in the absence of TO, it is possible that TO might reverse Shh-induced signaling and/or dampen Hh responses. This suggests that LXR activation negatively regulates Hh signaling remains to be elucidated in future studies.

The present observations also demonstrate that pretreatment of cells with TO, and its removal before treatment with Shh, had significant inhibitory effects on Hh target gene expression. This suggests that LXR activation precondition the cells to dampen their response to Shh, perhaps through expression of inhibitory molecules or other cellular changes that remain intact even after the LXR ligand is removed. For example, we found that TO-induced ABCA1 expression in M2 cells remains elevated for at least 72 h after removal of the ligand from the cultures (data not shown). Therefore, if ABCA1 plays a regulatory role in limiting Hh signaling as speculated in the previous paragraph, its persistent expression may be one mechanism for preconditioning of cells upon TO treatment. In addition, treatment of cells with TO subsequent to exposure of cells to Shh still showed inhibitory effects on Shh-induced gene expression. This suggests that LXR activation can reverse Shh-induced signaling and/or limit its magnitude by causing cellular changes as described above.

Transcriptional repression of inflammatory genes by LXR has been reported in the past and occurs through trans-repression (35). Such genes, including IL-6, IL-1β, cyclooxygenase 2, and matrix metalloproteinase 9 among others, do not possess LXR binding sites in their proximal promoters (35), suggesting an indirect mechanism of transcriptional repression. Such indirect mechanisms may involve inducing the expression of repression repressors, competing for coactivators, or inhibiting other transcriptional

FIG. 6. LXR activation does not inhibit Hh signaling in LXR null MEFs. LXRα−/−; LXRβ−/− MEFs were infected with pBabe-Hyg empty vector or pBabe-mLXRα and selected with hygromycin as described in Materials and Methods. LXRα expressing cells (LXRα; LXRβ−/−) and control double-null cells (LXRα−/−; LXRβ−/−) at confluence were treated with control vehicle (C), 200 ng/ml Shh, or 2 μM TO, alone or in combination. After 48 h, mRNA was isolated and analyzed for LXRA expression (panel A) and expression of LXR target genes ABCG1 (panel B) and ABCA1 (panel C) to demonstrate that introduction of mLXRα in double-null MEFs restores the regulation of LXR target genes by TO. Analysis of expression of Gli1 (panel D) in MEFs was also performed in response to Shh and in the presence or absence of TO. Results from a representative of three separate experiments are shown as mean ± SD and expressed as fold induction over control vehicle-treated cells (*, P < 0.05; NS, not significant, P = 0.1564).

FIG. 7. Effects of LXR ligand TO and chloral hydrate on primary cilium in MSCs. M2-10B4 cells at confluence were treated with 2 μM TO or 4 mM chloral hydrate (CH) for 48 h. Cells were then fixed in 4% paraformaldehyde and processed for immunostaining with antibody to acetylated α-tubulin, which is concentrated in primary cilium and DAPI for staining nuclei. Staining of primary cilium was examined under a fluorescent microscope with a magnification of ×100 (A; arrows). B, Ciliated cells were counted in six fields per well, in triplicate wells per treatment, and total number of cells in each field was determined by counting DAPI-stained nuclei. Percent of ciliated cells was calculated for each field and averaged for each treatment. Results from a representative experiment are reported as the mean ± SD (*, P < 0.001 for C vs. CH). C, Control.
activators, such as nuclear factor-κB in the case of inflammatory gene repression (35). Using Genomatix software, preliminary examination of the 5-kb region upstream of transcription start site for mouse Ptch1 and Gli1, both of which are repressed by TO, did not reveal any putative LXREs (LXR response elements; data not shown). Therefore, inhibition of some osteogenic genes by LXR may be direct whereas others may involve an indirect trans-repression mechanism similar to that reported for inflammatory genes. LXR-induced inhibition of osteogenic genes that mediate osteoblast differentiation and activity may be important in regulation of bone formation and bone homeostasis. Evidence for the negative regulation of osteoblasts by LXR was also presented in a recent publication where it was found that in LXRβ−/− mice there is an increased mRNA expression of the master regulator of osteogenesis, Runx2 transcription factor, and osteogenic genes such as osteopontin, OCN, BSP, ALP, and type I collagen (71). In addition, there was an increase in serum levels of OCN and ALP in LXRβ−/− mice suggesting increased osteoblast activity (71). These findings suggest the presence of more active osteoblasts in the absence of LXRβ, and an inhibitory effect of LXR on osteogenesis.

Finally, our data also demonstrate that systemic administration of TO in mice inhibits the baseline expression of Hh target genes Ptc1 and Gli1 in liver, lung, and spleen, although inhibition of Gli1 in the spleen did not reach statistical significance. Inhibition of Hh target genes by TO was correlated with the induced expression of LXR target genes as expected. This finding further supports the potential role of Hh signaling in adult tissue physiology and implicates the potential role of endogenous and exogenous factors that regulate LXR signaling in modulation of Hh signaling-regulated processes during embryonic development and postembryonic tissue homeostasis. Therefore, future investigations of LXR-Hh interactions in vivo and in various physiological and pathological settings would be of great importance.

The observation that LXR activation negatively regulates Hh signaling may have a number of implications in physiology and pathology. Hh signaling plays a major
role in embryonic development as demonstrated by the severe defects that occur when Hh signaling is impaired. Furthermore, LXRα and LXRβ target genes have been identified in developing embryos as early as embryonic day 11.5 (72). Therefore, it is conceivable that cross talk between Hh and LXR signaling is important for the proper regulation of embryonic development. However, because we are unaware of any reports of defects in embryonic development of LXRα/β double-knockout mice, it is not clear whether Hh-LXR cross talk is of physiological importance during embryonic development. We are also unaware of any reports regarding the potential adverse effects of LXR hyperactivity on embryonic development, which might be caused through perturbation of Hh signaling. This apparent absence of developmental defects in LXR null mice, in the face of the expected increase in Hh signaling, may be due to extremely tight compensatory regulation of Hh signaling during embryonic development given its critical role in various developmental processes. However, in postembryonic development and tissue maintenance, LXR-Hh cross talk may be of greater significance. Aberrant Hh signaling results in the formation of various tumors, among which medulloblastoma and prostate tumors have been well studied (37–39, 48, 73). It has been shown that defects in negative regulators of Hh signaling, namely Ptc1, Sufu, REN, and pituitary adenylate cyclase-activating polypeptide, cause or enhance the up-regulation of Hh signaling and formation of medulloblastoma (48, 74, 75). Administration of cyclopamine or small molecule antagonists of Hh signaling has been found effective in inhibiting cell proliferation and medulloblastoma in mice (73). Similarly, aberrant Hh signaling also contributes to prostate cancer, and a number of studies have demonstrated that interference with Hh signaling inhibits tumorigenesis in the prostate (37–39).

We speculate that activating LXR through pharmacological or gene targeting intervention might be a potential strategy for eradicating tumors that arise from dysregulated Hh signaling. Interestingly, administration of TO or phytosterols that activate LXR in vivo to mouse models of human prostate cancer tumors has been found to inhibit or delay tumor growth and progression (37, 38). Proliferation of various cancer cell lines in vitro was also inhibited by TO, although the concentrations of TO used in those studies were relatively high, and it is not clear whether cytotoxicity of high doses of TO may be, in part, the cause of decreased cell viability and proliferation (39). Furthermore, it would be important to examine whether baseline LXR activity may be compromised in cancers that arise from aberrant Hh signaling.

Altogether, our data clearly demonstrate the negative regulatory effects of LXR on Hh signaling in pluripotent MSCs and embryonic fibroblasts. We speculate that similar effects of LXR on Hh signaling may be found in other cell types in which LXRs are present and functional. Be-

![FIG. 9. Effect of LXR ligand, TO, on ex vivo Hh signaling. Calvaria from mouse pups were extracted and incubated for 24 h with growth medium. Next, cultures were treated with control vehicle, 400 ng/ml Shh, or 5 μM TO, alone or in combination. RNA from organ cultures was extracted after 48 h of treatment and analyzed for Ptc1 and Gli1 mRNA expression by Q-RT-PCR. Results from a representative study are reported as the mean value from triplicate cultures ± SD per condition and expressed as fold induction over untreated control cultures (*, P < 0.001 for control vs. Shh and Shh vs. Shh + TO for both Ptc1 and Gli1; #, P < 0.05 for control vs. TO for Ptc1 and Gli1).](image1)

![FIG. 10. Effect of LXR ligand, TO, on in vivo Hh signaling. Mice were gavaged daily for 3 d with TO (40 mg/kg/d) or vehicle (0.5% methylcellulose). On the third day, mice were euthanized and RNA from liver, lung, and spleen was extracted and analyzed by Q-RT-PCR for Hh target genes, Ptc1 and Gli1, and LXR target genes, ABCA1, ABCG1, and SREBP1c (data not shown). Results are expressed as mean ± SD (*, P < 0.05 for control vs. TO-treated mice; n = 5).](image2)
cause LXRαs are thought to be potential drug targets for various indications by promoting antiinflammatory, cholesterol homeostatic, and glucose tolerance effects, we propose that activating LXRαs may also be effective in targeting Hh signaling and may be used in addition to or in lieu of small molecule inhibitors of Hh pathway. Further studies of the molecular mechanisms underlying the LXR-Hh pathway cross talk and its biological relevance will further test this possibility.

Materials and Methods

Cell culture and reagents

M2-10B4 murine bone MSCs were maintained as previously described (43). Experimental treatments were performed in RPMI 1640 containing 5% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 50 μg/ml ascorbate, and 3 mM β-glycerophosphate. MEFs from LXR double-knockout embryonic d 13.5 embryos were isolated and immortalized with forced expression of large T antigen (pBabe-Puro large T). To generate retroviral overexpression of large T antigen, Phoenix A cells (76) were grown in DMEM with 10% FBS and penicillin/streptomycin and transiently transfected with the large T antigen expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Retroviruses were collected 48 h after transfection and filtered. The LXRα−/−, LXRβ−/− MEFs were infected with virus at 8 μg/ml polybrene overnight and selected with puromycin (2 μg/ml) for 1 wk. Immortalized MEFs were then infected either with pBabe-Hyg control or pBabe-Hyg-mLXRα viruses and selected with hygromycin (800 μg/ml). LXRα expressing cells (LXRα−/−, LXRβ−/−) and control double null cells were used in experiments as described. Recombinant mouse Shh N-terminal peptide was obtained from R&D Systems, Inc. (Minneapolis, MN), TO from Calbiochem (La Jolla, CA), PM from Cayman Chemical (Ann Arbor, MI), and chondral hydrate from Sigma-Aldrich (St. Louis, MO). TO and GW3965 were kind gifts of Timothy Wilson (GlaxoSmithKline, Research Triangle Park, NC).

Animals

Male C57BL/6 mice (8 wk old) were maintained on a standard rodent chow and were gavaged daily for 3 d with either TO (40 mg/kg/d) or vehicle (0.5% methylcellulose), five mice per treatment, before being euthanized, with the last treatment performed on the day of euthanasia. Tissues were immediately frozen in liquid nitrogen and stored in −80 °C until RNA extraction.

ALP activity assay

Colorimetric ALP activity assay on whole-cell extracts was performed as previously described (43).

Quantitative real-time PCR

Total RNA from cells or tissues was extracted with the RNA isolation kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. RNA was DNase treated using DNA-free kit from Ambion, Inc. (Austin, TX). RNA (3 μg) was reverse transcribed using reverse transcriptase from Stratagene to make single-stranded cDNA. The cDNAs were mixed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) for Q-RT-PCR assay using a Bio-Rad I-cycler IQ quantitative thermocycler. All PCR samples were prepared in triplicate wells of a 96-well plate. After 40 cycles of PCR, melt curves were examined to ensure primer specificity. Fold changes in gene expression were calculated using the ΔΔCt method (44). Sequences of primers used are reported in supplemental Table 1. PCR products for all primer sets were sequenced by UCLA sequencing core facility to verify the identity of the products.

Transient transfection and Gli-dependent reporter assay

Cells at 70% confluence in 24-well plates were transiently transfected with Gli-dependent firefly luciferase and Renilla luciferase vectors as previously described (44). Total DNA per well did not exceed 500 ng, and FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) was used at a ratio of 3:1 (reagent-DNA). Cells were treated for 48 h before luciferase activity was assessed using the Dual Luciferase Reporter Assay System (Promega Corp., Madison, WI) according to the manufacturer’s instructions. Experiments were performed in triplicate, and error bars indicate 1 SD.

LXR-α and LXR-β siRNA transfection

Both LXR-α and LXR-β siRNAs (ON-TARGETplus SMARTpool; catalog nos. L-040649-01-0010 and L-042839-00-0010) were obtained from Dharmacon (Lafayette, CO). To knock down LXRs, M2 cells at 80% confluence in 24- or six-well plates were transfected with siRNA using DharmaFECT transfection reagent (Dharmacon) to a final concentration of 25 nM of each siRNA. Knockdown of target genes was monitored at the mRNA level by Q-RT-PCR and further assessed by level of LXR target gene expression when cells were treated with LXR ligands.

Immunocytochemistry

M2-10B4 cells cultured on chamber slides were fixed with 4% paraformaldehyde in PBS at room temperature for 7 min. Non-specific binding was blocked with 5% normal goat serum in 0.1% Tween/PBS at room temperature for 60 min. Cells were then incubated with mouse-antiacetylated α-tubulin (1:500, Sigma) prepared in blocking solution for 60 min at room temperature, followed by incubation with Alexa 594 goat-anti mouse or Alexa 488 goat-anti rabbit secondary antibody (1:250, Invitrogen) for 60 min at room temperature. After rinsing with 0.1% Tween/PBS, cell nuclei were counterstained with 3,6-diamidino-2-phenylindole (DAPI) solution (Molecular Probes, Inc., Eugene, OR) in PBS for 5 min according to the manufacturer’s instructions. Staining of primary cilium was examined under a regular fluorescent microscope with a magnification of ×100. Ciliated cells were counted in six fields per well, in triplicate wells per treatment, and total number of cells in each field was determined by counting DAPI-stained nuclei. Percentage of ciliated cells was calculated for each field and averaged for each treatment.

Western blotting

After treatments, cells were lysed in lysis buffer (50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM HEPES, 0.1% Triton X-100), protein concentrations were determined using the Bio-
Rad proteain assy, and SDS-PAGE was performed as previously described (43), probing for native and phosphorylated Smad 1/5/8 proteins using antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Cell Signaling Technology (Danvers, MA), respectively. Antibody to Polaris was a kind gift of Dr. Bradley Yoder from University of Alabama (Tuscaloosa, AL).

Calvaria organ cultures

Calvaria from 7-d-old CD1 mouse pups were excised and cultured in DMEM containing 10% heat-inactivated FBS in a CO2 incubator at 37 C overnight. Next day, the cultures were treated in DMEM containing 5% FBS, 50 µg/ml ascorbate, and 3 mM β-glycerophosphate with control vehicle, Shh, or TO, alone or in combination, for 48 h (four calvaria per treatment condition). The calvaria were then homogenized and the RNA extracted as described above for Q-RT-PCR analysis of Gli1, Ptc1, and ABCA1 mRNA expression.

Statistical analyses

Computer-assisted statistical analyses were performed using the StatView 4.5 program. All P values were calculated using ANOVA and Fisher’s projected least significance test. A value of P < 0.05 was considered significant.

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