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

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.
Copyright © 2009 by The Endocrine Society
doi: 10.1210/me.2008-0453 Received December 5, 2008. Accepted July 8, 2009.
First Published Online July 16, 2009

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.

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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 (Ptch), 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 Ptch 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 *Drosophila* 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. LXRs 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-binding sites in their promoters (34, 35). Such genes include inflammatory cytokines and chemokines such as IL-6, IL-1β, monocyte chemotactic protein-1, and matrix metalloproteinase 9, as well as enzymes involved in generation of bioactive molecules such as inducible nitric oxide synthase and cyclooxygenase 2 (34, 35). Furthermore, LXR appears to play a role in growth and progression of various tumors including breast and prostate, certain types of which occur as a result of aberrant Hh signaling (36–39). As such, LXRs may serve as therapeutic targets for various disorders including atherosclerosis, diabetes, Alzheimer's, and cancer (33, 40–42).

In the present report, we demonstrate the previously unrecognized interaction between LXR and Hh signaling, whereby LXR activation by pharmacological ligands in pluripotent mesenchymal cells [marrow stromal cells (MSCs)], which are capable of differentiating into various cell types including osteoblasts and adipocytes (43), causes significant inhibition of Shh-induced signaling. This inhibition also translates into inhibition of Shh-induced osteogenic differentiation of MSC, an important finding that presents yet another step in regulation of Hh-induced lineage-specific differentiation of MSC, as well as having implications in bone homeostastis. Moreover, we also demonstrate that LXR activation inhibits Hh signaling in calvaria organ cultures *ex vivo*, and in mouse tissues *in vivo*.

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 LXRs 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 Ptch1 and Gli1 after 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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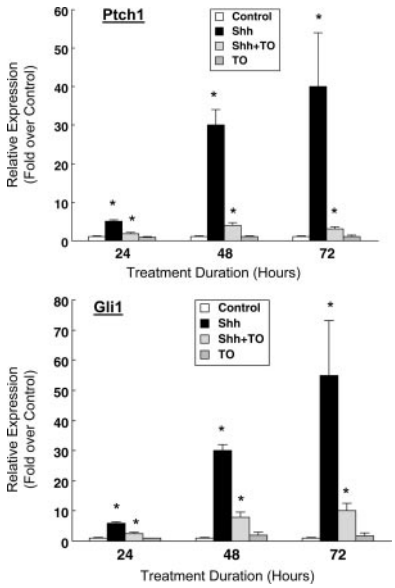

FIG. 1. Effect of TO on Shh-induced Ptch1 and Gli1 expression in marrow stromal cells. M2-10B4 cells were treated with control vehicle (C) or 200 ng/ml Shh, alone or in combination with 2 μM TO. After 24, 48, and 72 h, RNA was isolated and analyzed for Ptch1 and Gli1 mRNA expression by Q-RT-PCR. Results from a representative experiment are reported as the mean of triplicate determinations ± SD (*, $P < 0.001$ for C *vs.* Shh and for Shh *vs.* Shh + TO at all time points for both Ptch1 and Gli1).

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

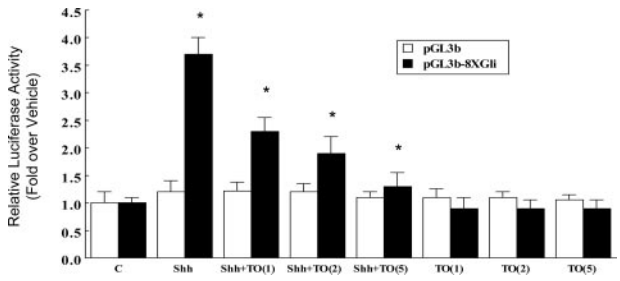

FIG. 2. Effect of LXR ligand TO on Gli-reporter activity. M2-10B4 cells transfected with a Gli-luciferase reporter construct (pGL3b-8XGli) or the empty reporter construct without Gli response elements (pGL3b) were treated with Shh (200 ng/ml), alone or in combination with TO (μM). Gli-luciferase reporter activity was tested after 48 h of treatment. Results from a representative experiment are shown as the mean of quadruplicate determinations ± SD and expressed as fold induction over control vehicle-treated cells (*, $P < 0.001$ for C *vs.* Shh and for Shh *vs.* Shh plus all concentrations of TO in pGL3b–8XGli cells). C, Control.

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 Ptch1 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 Ptch1 and Gli1 expression (Fig. 5, A and B).

Furthermore, we examined the inhibitory effect of TO in mouse embryonic fibroblasts (MEFs) derived from LXR$\alpha\beta$ 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 Ptch1 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).

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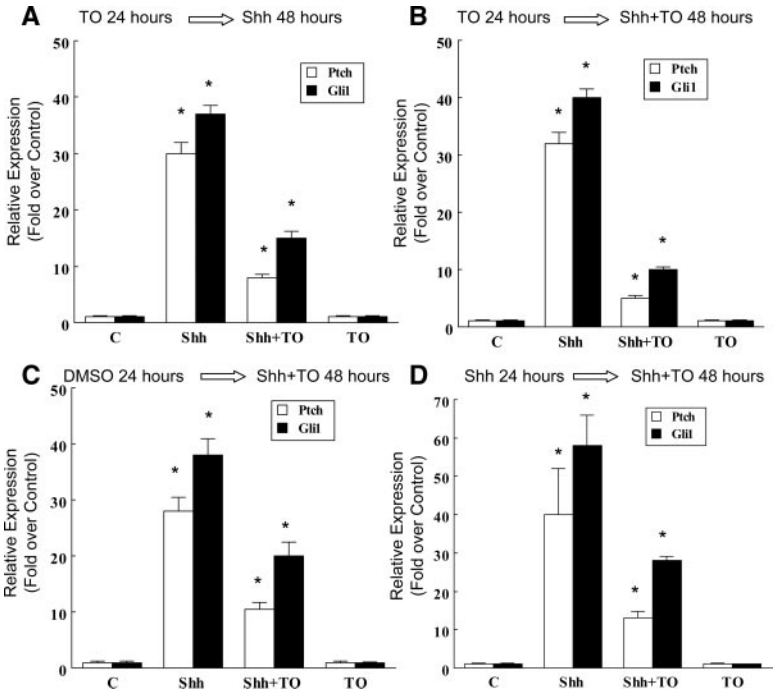

FIG. 3. Effect of pretreatment, cotreatment, and posttreatment with TO on Shh-induced Patched1 (Ptch) and Gli1 expression in marrow stromal cells. M2-10B4 cells were treated with control vehicle (C) or TO (2 μM) and Shh (200 ng/ml), alone or in combination, in the following manner: panel A, pretreated with TO for 24 h, followed by removal of TO and addition of Shh for 48 h without TO; panel B, pretreated with TO for 24 h followed by the addition of Shh + TO for 48 h; panel C, cotreated with Shh and TO for 48 h; or panel D, first treated with Shh for 24 h and subsequently with Shh + TO for 48 h. RNA was isolated and analyzed for Ptch1 and Gli1 mRNA expression by Q-RT-PCR. Results from a representative experiment are reported as the mean of triplicate determinations ± SD (*, $P < 0.001$ for C *vs.* Shh and for Shh *vs.* Shh + TO for both Ptch1 and Gli1, and for all the various treatment modalities described). DMSO, Dimethylsulfoxide.

Effect of LXR activation on molecular components of Hh signaling

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 cilium (51–53), we examined whether TO inhibits the formation/integrity of primary cilium in M2 cells. Cells were treated for 48 h with TO and then stained for primary cilium-associated acetylated α-tubulin and analyzed by fluorescence microscopy. In contrast with chloral hydrate, which has been shown to disrupt primary cilium 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 cilium 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).

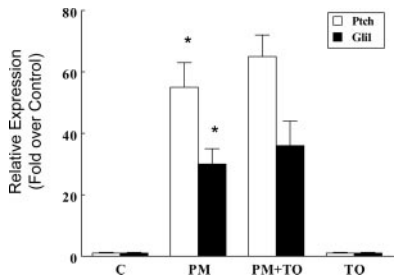

FIG. 4. Effect of LXR ligand TO on PM-induced Patched1 (Ptch) and Gli1 expression in marrow stromal cells. M2-10B4 cells were treated with control vehicle (C), 1 μM PM, or 2 μM TO, alone or in combination. After 48 h, mRNA was extracted and analyzed for Ptch1 and Gli1 expression by Q-RT-PCR. Data from a representative experiment are reported as the mean of triplicate determinations ± SD (*, $P < 0.001$ for C *vs.* PM for both Ptch1 and Gli1).

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-

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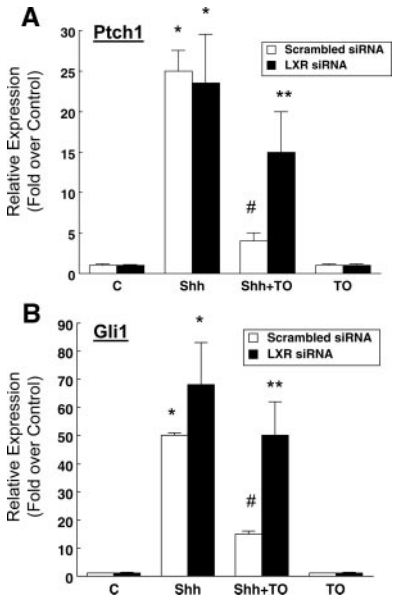

FIG. 5. Effect of LXR siRNA on Ptch1 and Gli1 mRNA expression in marrow stromal cells. M2-10B4 cells were transfected with scrambled or LXRα and LXRβ siRNAs as described in *Materials and Methods*. Cells were treated 48 h after transfection with control vehicle (C), 200 ng/ml Shh, or 1 μM TO, alone or in combination. After 48 h, the expression of Ptch1 (A) and Gli1 (B) mRNA was analyzed by Q-RT-PCR. Results from a representative experiment are reported as the mean of triplicate determination ± SD and expressed as fold induction over control untreated cells (*, $P < 0.001$ for C *vs.* Shh for scrambled and LXR siRNA for Ptch1 and Gli1 expression; #, $P < 0.001$ for Shh *vs.* Shh + TO for scrambled siRNA for Ptch1 and Gli1 expression; **, $P < 0.05$ for Shh *vs.* Shh + TO for LXR siRNA for Ptch1 and Gli1 expression).

genesis (58–60). Results showed that TO inhibited the Shh-induced expression of Ptch1 and Gli1 *ex vivo*, further confirming the inhibitory effects of LXR activation on Hh signaling (Fig. 9). TO (5 μM) consistently caused at least a significant 4-fold increase in ABCA1 expression confirming LXR activation in the calvaria organ cultures (data not shown).

LXR activation inhibits Hh target gene expression *in vivo*

Hh signaling has been implicated in postembryonic tissue maintenance and regeneration in various organs including the liver, lung, and spleen (8–14, 61–63). To examine the potential effect of LXR activation on Hh signaling *in vivo*, we examined the expression of Ptch1 and Gli1 in RNA isolated from liver, lung, and spleen of mice after 3 d 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 was confirmed by a robust and significant TO-induced expression of LXR target genes ABCA1, ABCG1, and SREBP1c in the lung and spleen, and SREBP1c and ABCG1 in the liver compared with mice treated with control vehicle (data not shown). We found that TO administration 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 induced the expression of ABC transporters in all cells tested, and it is plausible that transport

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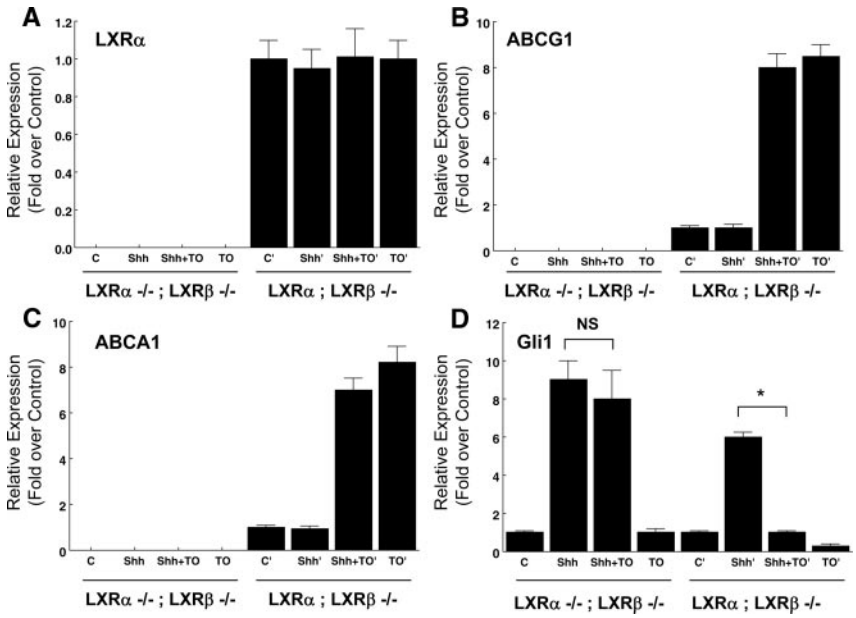

FIG. 6. LXR activation does not inhibit Hh signaling in LXR null MEFs. LXR$\alpha-/-$; LXR$\beta-/-$ MEFs were infected with pBabe-Hyg empty vector or pBabe-mLXRα and selected with hydromycin as described in *Materials and Methods*. LXRα expressing cells (LXRα; LXR$\beta-/-$) and control double-null cells (LXR$\alpha-/-$; LXR$\beta-/-$) 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 LXRα 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, $P = 0.1564$).

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 response to exogenously added Shh-N, intact production, transport, and distribution of endogenous Hh proteins are important during development and perhaps postembryonic tissue maintenance (22). We speculate that LXR activation and increased sterol removal from cells might also affect posttranslational processing and sterol modification of Hh molecule in Hh-producing cells, thereby causing impaired transport and distribution of Hh molecules (22) and Hh-regulated processes. The specific molecular mechanism(s) by which 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 preconditions 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.

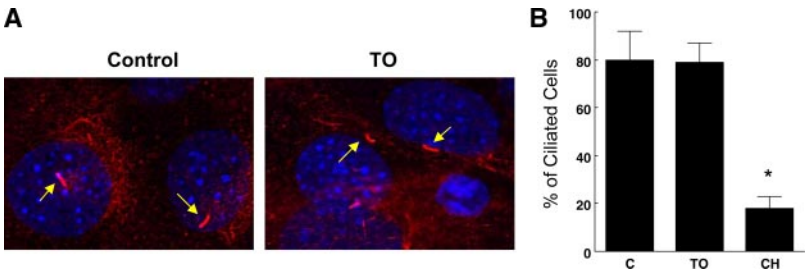

FIG. 7. Effects of LXR ligand TO and chloral hydrate on primary cilium in MSCs. M2-10B4 cells at confluence were treated with 2 μM TO or 4 mM chloral hydrate (CH) for 48 h. Cells were then fixed in 4% paraformaldehyde and processed for immunostaining with antibody to acetylated α-tubulin, which is concentrated 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.

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 transcription repressors, competing for coactivators, or inhibiting other transcriptional

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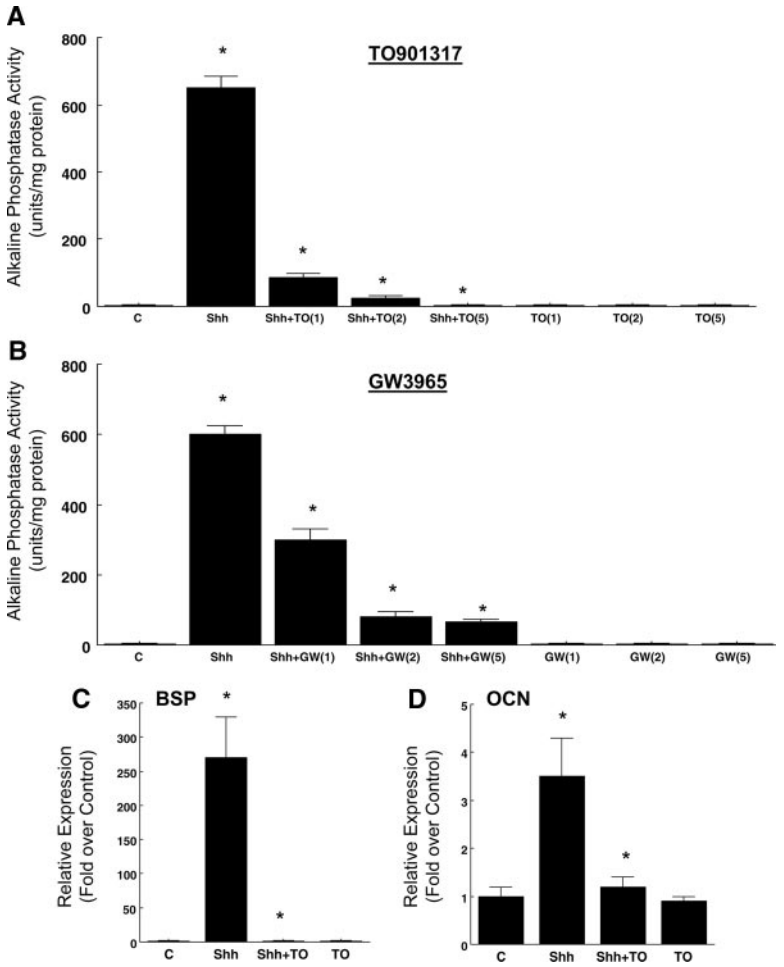

FIG. 8. Effect of LXR ligands, TO and GW3965, on Shh-induced osteogenic differentiation markers in M2-10B4 MSCs. Panels A and B, M2-10B4 cells were treated with control vehicle (C) or 200 ng/ml Shh, alone or in combination with increasing doses of TO or GW ligands (μM). After 3 d, ALP activity assay was performed on whole-cell lysates. Results from a representative experiment are reported as the mean of quadruplicate determinations ± SD (*, $P < 0.001$ for C *vs.* Shh and for Shh *vs.* Shh plus all concentrations of TO and GW). Panels C and D, M2-10B4 cells were treated with control vehicle (C), 200 ng/ml Shh, or 2 μM TO, alone or in combination. After 6 d, RNA was isolated and analyzed for BSP and OCN mRNA expression by Q-RT-PCR. Results from a representative experiment are reported as the mean of triplicate determinations ± SD (*, $P < 0.001$ for C *vs.* Shh and for Shh *vs.* Shh + TO for both BSP and OCN).

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 LXR-binding sites [LXR response elements (LXREs); data not shown]. Therefore we speculate that similar to inflammatory genes, inhibition of Hh-target gene expression by LXR may involve trans-repression, in addition to cellular changes that may limit Hh signaling directly as proposed above.

In addition, our data show that the expression of osteogenic genes in response to Shh was inhibited by LXR activation. Such inhibition may be 1) through interference with Shh-induced signaling that, in turn, induces the expression of osteogenic genes, and/or 2) through direct inhibition of osteogenic genes regardless of the inducer molecule. It is noteworthy that preliminary examination of the 5-kb region upstream of transcription start site for mouse ALP, BSP, and OCN showed no apparent LXREs in ALP and BSP and only one putative LXRE in OCN genes (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 LXR$\beta-/-$ 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 LXR$\beta-/-$ 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 Ptch1 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

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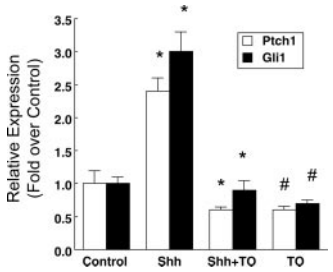

FIG. 9. Effect of LXR ligand, TO, on *ex vivo* Hh signaling. Calvaria from mouse pups were extracted and incubated for 24 h with growth medium. Next, cultures were treated with control vehicle, 400 ng/ml Shh, or 5 μM TO, alone or in combination. RNA from organ cultures was extracted after 48 h of treatment and analyzed for Ptch1 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 Ptch1 and Gli1; #, $P < 0.05$ for control *vs.* TO for Ptch1 and Gli1).

role in embryonic development as demonstrated by the severe defects that occur when Hh signaling is impaired. Furthermore, LXRs and LXR target genes have been identified in developing embryos as early as embryonic d 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 LXR$\alpha\beta$ 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 Ptch, 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).

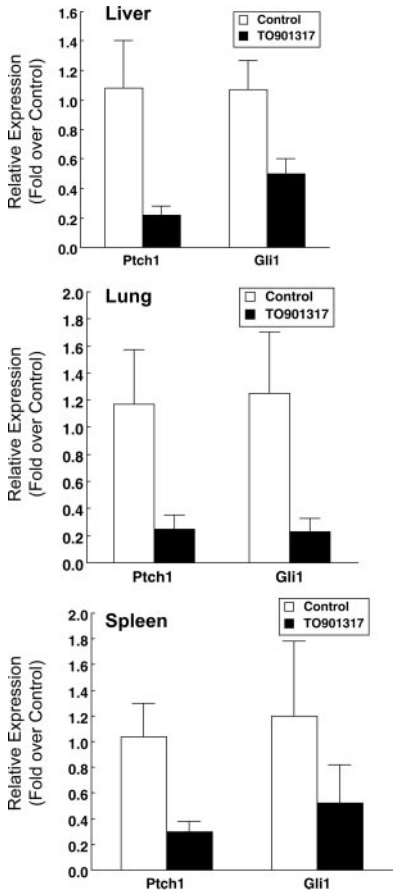

FIG. 10. Effect of LXR ligand, TO, on *in vivo* Hh signaling. Mice were gavaged daily for 3 d with TO (40 mg/kg/d) or vehicle (0.5% methylcellulose). On the third day, mice were euthanized and RNA from liver, lung, and spleen was extracted and analyzed by Q-RT-PCR for Hh target genes, Ptch1 and Gli1, 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).

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-

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cause LXRs are thought to be potential drug targets for various indications by promoting antiinflammatory, cholesterol homeostatic, and glucose tolerance effects, we propose that activating LXRs 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 chloral 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 $\Delta\Delta$Ct 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% confluency 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. Nonspecific 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 599 goat-anti-mouse or Alexa 488 goat-antirabbit secondary antibody (1:250, Invitrogen) for 60 min at room temperature. After rinsing with 0.1% Tween/PBS, cell nuclei were counterstained with 320 nM 4′,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-

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Rad protein assay, 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 CO_2 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, Ptch1, 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.

Acknowledgments

We thank Dr. Xioayan Liao for expert technical assistance.

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This work was supported by in part by National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health Grant RO1 AR050426, and American Heart Association Grant-In-Aid 0755119Y (to F.P.).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Mullor JL, Sánchez P, Ruiz i Altaba A** 2002 Pathways and consequences: Hedgehog signaling in human disease. Trends Cell Biol 12:562–569
2. **Ehlen HWA, Buelens LA, Vortkamp A** 2006 Hedgehog signaling in skeletal development. Birth Defects Res 78:267–279
3. **Bale AE** 2002 Hedgehog signaling and human disease. Annu Rev Genomics Hum Genet 3:47–65
4. **Rubin LL, de Sauvage FJ** 2006 Targeting the Hedgehog pathway in cancer. Nat Rev Drug Discov 5:1026–1033
5. **Bijlsma MF, Peppelenbosch MP, Spek CA** 2006 Hedgehog morphogen in cardiovascular disease. Circulation 114:1985–1991
6. **Robert B, Lallemand Y** 2006 Anteroposterior patterning in the limb and digit specification: contribution of mouse genetics. Dev Dynam 235:2337–2352
7. **Hill RE, Heaney SJ, Lettice LA** 2003 Sonic hedgehog: restricted expression and limb dysmorphologies. J Anat 202:13–20
8. **Maeda Y, Nakamura E, Nguyen MT, Suva LJ, Swain FL, Razzaque MS, Mackem S, Lanske B** 2007 Indian hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trabecular bone. Proc Natl Acad Sci USA 104:6382–6387
9. **Miyaji T, Nakase T, Iwasaki M, Kuriyama K, Tamai N, Higuchi C, Myoui A, Tomita T, Yoshikawa H** 2003 Expression and distribution of transcripts for sonic hedgehog in the early phase of fracture repair. Histochem Cell Biol 119:233–237
10. **Podlasek CA, Meroz CL, Korolis H, Tang Y, McKenna KE, McVary KT** 2005 Sonic hedgehog, the penis and erectile dysfunction: a review of sonic hedgehog signaling in the penis. Curr Pharm Des 11:4011–4027
11. **Akazawa C, Tsuzuki H, Nakamura Y, Sasaki Y, Ohsaki K, Nakamura S, Arakawa Y, Kohsaka S** 2004 The upregulated expression of sonic hedgehog in motor neurons after rat facial nerve axotomy. J Neurosci 24:7923–7930
12. **Pola R, Ling LE, Aprahamian TR, Barban E, Bosch-Marce M, Curry C, Corbley M, Kearney M, Isner JM, Losordo DW** 2003 Postnatal recapitulation of embryonic hedgehog pathway in response to skeletal muscle ischemia. Circulation 108:479–485
13. **Dimmler A, Brabletz T, Hlubek F, Häfner M, Rau T, Kirchner T, Faller G** 2003 Transcription of sonic hedgehog, a potential factor for gastric morphogenesis and gastric mucosa maintenance, is upregulated in acidic conditions. Lab Invest 83:1829–1837
14. **Saika S, Muragaki Y, Okada Y, Miyamoto T, Ohnishi Y, Ooshima A, Kao WW** 2004 Sonic hedgehog expression and role in healing corneal epithelium. Invest Opthalmol Vis Sci 45:2577–2585
15. **St-Jacques B, Hammerschmidt M, McMahon, AP** 1999 Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 13: 2072–2086
16. **Long F, Chung UI, Ohba S, McMahon J, Kronenberg HM, McMahon AP** 2004 Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. Development 131:1309–1318
17. **Lawson ND, Vogel AM, Weinstein BM** 2002 Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 3:127–136
18. **Byrd N, Grabel L** 2004 Hedgehog signaling in murine vasculogenesis and angiogenesis. Trends Cardiovasc Med 14:308–313
19. **Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA** 1996 Cyclopia and defective axial patterning in mice lacking Sonic Hedgehog gene function. Nature 383:407–413
20. **Chen JK, Taipale J, Young KE, Maiti T, Beachy PA** 2002 Small molecule modulation of Smoothened activity. Proc Natl Acad Sci USA 99:14071–14076
21. **Lum L, Beachy PA** 2004 The Hedgehog response network: sensors, switches, and routers. Science 304:1755–1759
22. **Bijlsma MF, Spek CA, Peppelenbosch MP** 2004 Hedgehog: an unusual signal transducer. Bioessays 26:387–394
23. **Riobó NA, Lu K, Ai X, Haines GM, Emerson Jr CP** 2006 Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc Natl Acad Sci USA 103:4505–4510
24. **Wendler F, Franch-Marro X, Vincent JP** 2006 How does cholesterol affect the way Hedgehog works? Development 133:3055–3061
25. **Porter JA, Young KE, Beachy PA** 1996 Cholesterol modification of hedgehog signaling proteins in animal development. Science 274: 255–259
26. **Huangfu D, Anderson KV** 2006 Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from *Drosophila* to vertebrates. Development 133:3–14
27. **Kogerman P, Grimm T, Kogerman L, Krause D, Undén AB, Sandstedt B, Toftgård R, Zaphiropoulos PG** 1999 Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat Cell Biol 1:312–319
28. **Kalderon D** 2005 Hedgehog signaling: an arrestin connection? Curr Biol 15:R175–R178
29. **Edwards PA, Kennedy MA, Mak PA** 2002 LXRs; oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. Vasc Pharm 38:249–256
30. **Kalaany NY, Mangelsdorf DJ** 2006 LXRS and FXR: the yin and yang of cholesterol and fat metabolism. Annu Rev Physiol 68:159–191

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31. **Edwards PA, Kast HR, Anisfeld AM** 2002 BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. J Lipid Res 43:2–12
32. **Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH, Rader DJ** 2006 Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. Circulation 113:90–97
33. **Joseph SB, McKillingin 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** 2002 Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proc Natl Acad Sci USA 99:7604–7609
34. **Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P** 2003 Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat Med 9:213–219
35. **Zelcer N, Tontonoz P** 2006 Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest 116:607–614
36. **Gong H, Guo P, Zhai Y, Zhou J, Uppal H, Jarzynka MJ, Song WC, Cheng SY, Xie W** 2007 Estrogen deprivation and inhibition of breast cancer growth *in vivo* through activation of the orphan nuclear receptor liver X receptor. Mol Endocrinol 21:1781-1790
37. **Chuu CP, Hiipakka RA, Kokontis JM, Fukuchi J, Chen RY, Liao S** 2006 Inhibition of tumor growth and progression of LNCaP prostate cancer cells in athymic mice by androgen and liver X receptor agonist. Cancer Res 66:6482–6486
38. **Chuu CP, Kokontis JM, Hiipakka RA, Liao S** 2007 Modulation of liver X receptor signaling as novel therapy for prostate cancer. J Biomed Sci 14:543–553
39. **Chuu CP, Chen RY, Hiipakka RA, Kokontis JM, Warner KV, Xiang J, Liao S** 2007 The liver X receptor agonist T0901317 acts as androgen receptor antagonist in human prostate cancer cells. Biochem Biophys Res Commun 357:341–346
40. **Rader DJ** 2007 Liver X receptor and farnesoid X receptor as therapeutic targets. Am J Cardiol 100:15N–19N
41. **Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, Castrillo A, Wilpitz DC, Mangelsdorf DJ, Collins JL, Saez E, Tontonoz P** 2003 Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proc Natl Acad Sci USA 100:5419–5424
42. **Zelcer N, Khanlou N, Clare R, Jiang Q, Reed-Geaghan EG, Landreth GE, Vinters HV, Tontonoz P** 2007 Attenuation of neuroinflammation and Alzheimer's disease pathology by liver X receptors. Proc Natl Acad Sci USA 104:10601–10606
43. **Kha HT, Basseri B, Shouhed D, Richardson J, Tetradis S, Hahn TJ, Parhami F** 2004 Oxysterols regulate differentiation of mesenchymal stem cells: pro-bone and anti-fat. J Bone Miner Res 19:830–840
44. **Dwyer JR, Sever N, Carlson M, Nelson SF, Beachy PA, Parhami F** 2007 Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. J Biol Chem 282:8959–8968
45. **Spinella-Jaegle S, Rawadi G, Kawai S, Gallea S, Faucheu C, Mollat P, Courtois B, Berguad B, Ramez V, Blanchet AM, Adelmant G, Baron R, Roman-Roman S** 2001 Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J Cell Sci 114:2085–2094
46. **Kim WK, Meliton V, Amantea CM, Hahn TJ, Parhami F** 2007 20(S)-hydroxycholesterol inhibits PPARγ expression and adipogenic differentiation of bone marrow stromal cells through a hedgehog-dependent mechanism. J Bone Miner Res 22:1711–1719
47. **Sinha S, Chen JK** 2006 Purmorphamine activates the Hedgehog pathway by targeting Smoothened. Nat Chem Biol 2:29–30
48. **Lelievre V, Seksenyan A, Nobuta H, Yong WH, Chhith S, Niewiadomski P, Cohen JR, Dong H, Flores A, Liau LM, Kornblum HI, Scott MP, Waschek JA** 2008 Disruption of the PACAP gene promotes medulloblastoma in ptc1 mutant mice. Dev Biol 313:359–370
49. **Dahmane N, Sánchez P, Gitton Y, Palma V, Sun T, Beyna M, Weiner H, Ruiz i Altaba A** 2001 The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development 128:5201–5212
50. **Di Marcotullio L, Ferretti E, De Smaele E, Screpanti I, Gulino A** 2006 Suppressors of hedgehog signaling: linking aberrant development of neural progenitors and tumorigenesis. Mol Neurobiol 34:193–204
51. **Huangfu D, Anderson KV** 2005 Cilia and Hedgehog responsiveness in the mouse. Proc Natl Acad Sci USA 102:11325–11330
52. **Christensen ST, Ott CM** 2007 Cell signaling. A ciliary signaling switch. Science 317:330–331
53. **Caspary T, Larkins CE, Anderson KV** 2007 The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell 12:767–778
54. **Praetorius HA, Spring KR** 2003 Bending the primary cilium opens Ca2+-sensitive intermediate-conductance K+ channels in MDCK cells. J Membr Biol 191:69–76
55. **Malone AM, Anderson CT, Tummala P, Kwon RY, Johnson TR, Stearns T, Jacobs CR** 2007 Primary cilia mediate mechanosensing in bone cells by calcium-independent mechanism. Proc Natl Acad Sci USA [Erratum (2008) 105:825] 104:13325–13330
56. **Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV** 2003 Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature 426:83–87
57. **Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK** 2005 Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 1:480–488
58. **Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G** 1999 Stimulation of bone formation in vitro and in rodents by statins. Science 286:1946–1949
59. **Traianedes K, Dallas MR, Garret IR, Mundy GR, Bonewald LF** 1998 5-Lipoxygenase metabolites inhibit bone formation *in vitro*. Endocrinology 139:3178–3184
60. **Yano H, Ohya K, Amagasa T** 1994 Effects of insulin on in vitro bone formation in fetal rat parietal bone. Endocr J 41:293–300
61. **Omenetti A, Diehl AM** 2008 The adventures of sonic hedgehog in development and repair. II. Sonic hedgehog and liver development, inflammation, and cancer. Am J Physiol Gastrointest Liver Physiol 294:G595–G598
62. **Li Y, Zhang H, Choi SC, Litingtung Y, Chiang C** 2004 Sonic hedgehog signaling regulates Gli3 processing, mesenchymal proliferation, and differentiation during mouse lung organogenesis. Dev Biol 270:214–231
63. **Perry JM, Harandi OF, Porayette P, Hegde S, Kannan AK, Paulson RF** 2009 Maintenance of BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. Blood 113:911–918
64. **Jetten AM, Joo JH** 2006 Retinoid-related orphan receptors (RORs): roles in cellular differentiation and development. Adv Dev Biol 16:313–355
65. **Niederreither K, Dollé P** 2008 Retinoic acid in development towards an integrated view. Nat Rev Genet 9:541–553
66. **Varnat F, Heggeler BB, Grisel P, Boucard N, Corthésy-Theulaz I, Wahli W, Desvergne B** 2006 PPARβ/δ regulates paneth cell differentiation via controlling the hedgehog signaling pathway. Gastroenterology 131:538–553
67. **Rohatgi R, Milenkovic L, Scott MP** 2007 Patched1 regulates hedgehog signaling at the primary cilium. Science 317:372–376
68. **Chen JK, Taipale J, Cooper MK, Beachy PA** 2002 Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 16:2743–2748

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69. **Rohatgi R, Scott MP** 2007 Patching the gaps in Hedgehog signaling. Nat Cell Biol 9:1005–1009
70. **Cooper MK, Wassif CA, Krakowiak PA, Taipale J, Gong R, Kelley RI, Porter FD, Beachy PA** 2003 A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. Nat Genet 33:508–513
71. **Robertson KM, Norgård M, Windahl SH, Hultenby K, Ohlsson C, Andersson G, Gustafsson JA** 2006 Cholesterol-sensing receptors, liver X receptor α and β, have novel and distinct roles in osteoclast differentiation and activation. J Bone Miner Res 21: 1276–1287
72. **Annicotte JS, Schoonjans K, Auwerx J** 2004 Expression of the liver X receptor α and β in embryonic and adult mice. Anat Rec 277A: 312–316
73. **Berman DM, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA** 2002 Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 297:1559–1561
74. **Lee Y, Kawagoe R, Sasai K, Li Y, Russell HR, Curran T, McKinnon PJ** 2007 Loss of suppressor-of-fused function promotes tumorigenesis. Oncogene 26:6442–6447
75. **Di Marcotullio L, Ferretti E, De Smaele E, Argenti B, Mincione C, Zazzeroni F, Gallo R, Masuelli L, Napolitano M, Maroder M, Modesti A, Giangaspero F, Screpanti I, Alesse E, Gulino A** 2004 REN(KCTD11) is a suppressor of Hedgehog signaling and is deleted in human medulloblastoma. Proc Natl Acad Sci USA 101: 10833–10838
76. **Marathe C, Bradley MN, Hong C, Lopez F, Ruiz de Galarreta CM, Tontonoz P, Castrillo A** 2006 The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. J Biol Chem 281:32197–32206

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