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Letter

Ouabagenin, an aglycone of cardiotonic steroid ouabain, functions as LXR ligand but avoids the increase in the SREBP-1 by inducing Krüppel-like factor 15

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ABSTRACT — Liver X receptor (LXR)-alpha and LXR-beta are nuclear receptors activated by oxysterols. They exhibit differential expression patterns and may perform different functional roles. Here we show that LXR-alpha and LXR-beta mutually regulate the expression levels of their counter parts in the normal hepatocyte-derived cell line Fa2N-4. In addition, we demonstrate that ouabagenin (OBG), which was identified as a naturally occurring LXR ligand without causing hepatic steatosis, dramatically increases the expression of LXR-alpha in Fa2N-4 cells that overexpress LXR-beta. However, the expression level of sterol response element binding protein 1c (SREBP-1c), a known target of LXR-alpha, remains marginal in OBG-treated Fa2N-4 cells, in which LXR-alpha expression is upregulated by LXR-beta. Furthermore, we show that OBG stimulates the expression of Krüppel-like factor 15 (KLF15) that is known to form a repressive complex with LXR/RXR and corepressor RIP140, thereby reducing SREBP-1c expression. Thus, we propose a novel mechanism that OBG avoids the increase in the expression of SREBP-1c through the upregulation of KLF15.

Key words: Ouabagenin, Liver X receptor, SREBP-1c, Krüppel-like factor 15

INTRODUCTION

Liver X receptor (LXR)-alpha and LXR-beta, nuclear receptors activated by oxysterols (Janowski *et al*., 1996), belong to a family of ligand-activated transcription factors with a key role in cholesterol synthesis and transport, glucose homeostasis, and modulation of inflammatory and immune responses (Wang *et al*., 2008; Cha and Repa, 2007; Zelcer and Tontonoz, 2006). LXRs form a heterodimer with retinoid X receptor (RXR)-alpha. Upon ligand binding to the LXRs/RXR-alpha complex on the target gene promoter, steroid receptor co-activator (SRC) 1 is recruited to the complex, stimulating transcription of the target gene (Gan *et al*., 2001).

It is known that LXR-alpha and LXR-beta exhibit differential expression patterns and may perform different functional roles, *i.e*., LXR-alpha is localized to the liver, adipose tissue, adrenal glands, intestine, kidney, macrophages, and lungs, whereas LXR-beta is widely distributed throughout the body, with high levels in the developing brain (Kainu *et al*., 1996; Zhang and Mangelsdorf, 2002). LXRs has been expected to have the therapeutic potential against atherosclerosis, since the synthetic ligands that act on both LXR-alpha and LXR-beta upregulate the ATP-binding cassette transporter A1 (ABCA1) transcription thereby stimulating cholesterol

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efflux from macrophages (Costet *et al*., 2000; Schwartz *et al*., 2000). On the other hand, the synthetic LXR ligands may also provide the adverse effect by increasing liver lipogenesis via sterol response element binding protein 1c (SREBP-1c), which is primarily activated by LXR-alpha (Peet *et al*., 1998). Among the attempts to fish out the beneficial LXR agonists that hardly induce SREBP-1c-mediated hyperglyceridemia, LXR-beta-selective ligands are thought to be the possible candidates minimizing the unexpected adverse effects (Koura *et al*., 2015, 2016).

Importantly, we have recently demonstrated that both LXR-alpha and LXR-beta mutually affect the expression levels of their counterpart (Fujino *et al*., 2020). Briefly, ectopically overexpressed LXR-beta upregulates LXR-alpha expression in both normal type of renal cells and renal adenocarcinoma-derived cells, whereas ectopically overexpressed LXR-alpha upregulates LXR-beta expression in renal adenocarcinoma-derived cells but not in normal type of renal cells, suggesting that different types of crosstalk between LXR-alpha and LXR-beta exist in normal or carcinoma-derived renal cells.

Recently, Tamura *et al*. (2018) reported that ouabagenin (OBG), an aglycone of cardiotonic steroid ouabain, acts as a functional LXR ligand. OBG exhibited agonistic activities towards both LXR-alpha and LXR-beta in luciferase reporter assay using 293T cells that overexpressed LXR-alpha or LXR-beta (Tamura *et al*., 2018). Interestingly, while the LXR agonist T0901317 stimulated the expression of genes involved in liver lipogenesis in human hepatocellular carcinoma cell lines, OBG did not stimulated the expression of these genes in these cells, suggesting that OBG may not act as the ligand for LXR-alpha in the liver (Tamura *et al*., 2018). In addition, suppression of epithelial sodium channel (ENaC) mRNA expression by OBG as well as the LXR agonist T0901317 in kidney cell lines was cancelled by the treatment with LXR-beta siRNA but not with LXR-alpha siRNA, indicating that OBG preferentially activates LXR-beta in the kidney (Tamura *et al*., 2018).

In the present study, we studied whether the mutual regulation between LXR-alpha and LXR-beta also exists in the hepatocyte-derived cell line using OBG as the LXR ligand. Given that OBG only weakly stimulated SREBP-1c expression in LXR-beta-overexpressing cells, in which LXR-alpha expression was upregulated, while LXR agonist GW3965 strongly induced SREBP-1c expression in these cells, we examined the effect of LXR-agonists, OBG and GW3965; on the expression of KLF15. KLF15 is one of the members of Krüppel-like factors, a family of phylogenetically conserved, broadly expressed transcription factors involved in diverse physiological and pathological processes (Hsieh *et al*., 2019). KLF15 is known to form a complex with LXR/RXR on the promoter of SREBP-1c gene, resulting in the recruitment of the corepressor RIP140 instead of the coactivator SRC1, thereby reducing the expression of SREBP-1c (Takeuchi *et al*., 2016).

MATERIALS AND METHODS

Materials

Antibody (Ab) specific for β-actin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Abs specific for LXR-alpha (ab28478) and LXR-beta (ab56237) were purchased from abcam (Cambridge, UK). Ouabagenin was obtained from Sigma-Aldrich (Tokyo, Japan). ECL™ anti-mouse IgG, horseradish peroxidase-linked whole antibody (from sheep) and ECL™ anti-rabbit IgG, horseradish peroxidase-linked whole antibody (from donkey) were purchased from GE Healthcare (Buckinghamshire, UK). GW3965 was purchased from Sigma-Aldrich. The human normal hepatocyte-derived cell line Fa2N-4 was obtained from ATCC.

Cell culture

Fa2N-4 cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/mL penicillin G sodium salt, and 50 μg/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% CO_2 at 37°C.

Quantification of mRNAs

Quantification of mRNA was performed using real-time PCR. Briefly, 4 μg of total RNA was reverse-transcribed using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resultant cDNA was subjected to real-time PCR analysis using a TaqMan Gene Expression Assay kit (Applied Biosystems, Tokyo, Japan). mRNA levels were determined using TaqMan assay mixtures for LXR-alpha (Hs00172885), LXR-beta (Hs00173195), SREBP-1c (Hs01088679), SRC1 (Hs00186661), KLF15 (Hs00362736), and β-actin (4310881E). Amplification and quantification were performed using the StepOne Real-Time PCR System (Applied Biosystems). mRNA levels were normalized to those of β-actin as an internal control.

Construction of expression vectors containing open reading frames of LXR-alpha and LXR-beta

To construct expression vectors containing open reading frames (ORF) of LXR-alpha (NM_005693.4) and

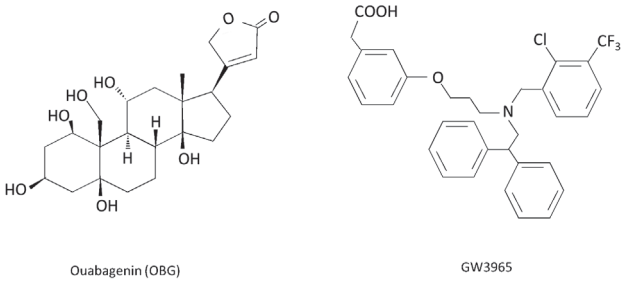

Fig. 1. Structure of OBG and GW3965.

LXR-beta (NM_007121.7), cDNAs encoding full-length human LXR-alpha and LXR-beta were synthesized by GENEWIZ (Saitama, Japan) and subcloned into pcDNA3.1(+). The resultant plasmids were called pc-LXR-alpha and pc-LXR-beta.

Ectopic expression of LXR-alpha and LXR-beta

Fa2N-4 cells were seeded at 5.0×10^5 cells and cultured for 24 hr. The cells were then transfected with pcDNA3.1(+), pc-LXR-alpha, or pc-LXR-beta using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).

Immunoblotting

Cells were washed with PBS, and cell extracts were prepared using SDS sample buffer without loading dye. After normalizing the protein content via the protein assay, dye was added to the samples. The samples were subjected to SDS-PAGE and immunoblotting analyses. For detection of LXR-alpha, LXR-beta, and β-actin, PVDF membranes were incubated with primary antibody (1:100) for 2 hr and incubated with secondary antibody (ECL™ anti-mouse IgG, horseradish peroxidase-linked whole antibody or ECL™ anti-rabbit IgG, horseradish peroxidase-linked whole antibody) for 1 hr. Immunocomplexes on the PVDF membranes were visualized on X-ray film using enhanced chemiluminescence western blotting detection reagents (GE Healthcare). Quantification of bands was conducted using densitometric analysis (IMAGE GAUGE 4.0).

Statistical analysis

Data are presented as the mean ± S.E.M. of three experiments performed in triplicate and were analyzed using Student's t-test.

RESULTS

OBG significantly upregulated LXR-alpha expression through the activation of LXR-beta but did not stimulate SREBP-1c expression

Fa2N-4 cells transfected with pcDNA3.1(+) carrying LXR-alpha cDNA or with pcDNA3.1(+) carrying LXR-beta cDNA were subjected to the immunoblotting analyses to confirm that ectopically introduced each LXR isoforms were sufficiently expressed (Fig. 2A). Using these transfection conditions, we examined whether or not LXR-alpha and LXR-beta mutually affect the expression levels of their counterparts in Fa2N-4 cells as observed in renal type of cell lines (Fujino *et al*., 2020). As shown in Fig. 2B, ectopically overexpressed LXR-alpha stimulated LXR-beta expression. In the presence of LXR ligands, OBG or GW3965 (Fig. 1), expression levels of LXR-beta were lowered (Fig. 2B and 2F). Interestingly, although ectopically overexpressed LXR-beta did not significantly elevate LXR-alpha expression in the absence of LXR ligands, OBG rather than GW3965 strongly stimulated LXR-alpha expression in in Fa2N-4 cells that overexpress LXR-beta (Fig. 2C and 2F). We next examined the expression levels of SREBP-1c, a target gene product of LXR-alpha, in Fa2N-4 cells that overexpress LXR-alpha or LXR-beta. While LXR-alpha overexpression did not lead to the elevation of SREBP-1c mRNA level in the absence of LXR ligands, GW3965 rather than OBG strongly stimulated the SREBP-1c mRNA expression in Fa2N-4 cells that overexpress LXR-alpha (Fig. 2D). Surprisingly, OBG only weakly stimulated the SREBP-1c mRNA expression in LXR-beta-overexpressing Fa2N-4 cells, in which significant levels of LXR-alpha were expressed as shown in Fig. 2C and 2F, whereas remarkable induction of SREBP-1c was observed in LXR-beta-overexpressing Fa2N-4 cells treated with GW3965 (Fig. 2E). Given that adverse effect was observed on LXR-beta expression in LXR-alpha-overexpressed cells, depending on with or without LXR agonists (Fig. 2B and 2F), LXR-beta mRNA stability assay was performed. Figure 2G shows that LXR-beta mRNA stability was increased by ectopic overexpression of LXR-alpha. Thus, OBG and GW3965 probably down-regulated LXR-beta expression by activating LXR-alpha.

OBG stimulates the expression of KLF-15 that recruits the corepressor RIP140 instead of the coactivator SRC1 on the SREBP-1c promoter

SREBP-1c is known to be responsible for liver lipogenesis and the progress hyperglyceridemia. Recent study revealed that fasting-induced transcription factor KLF15

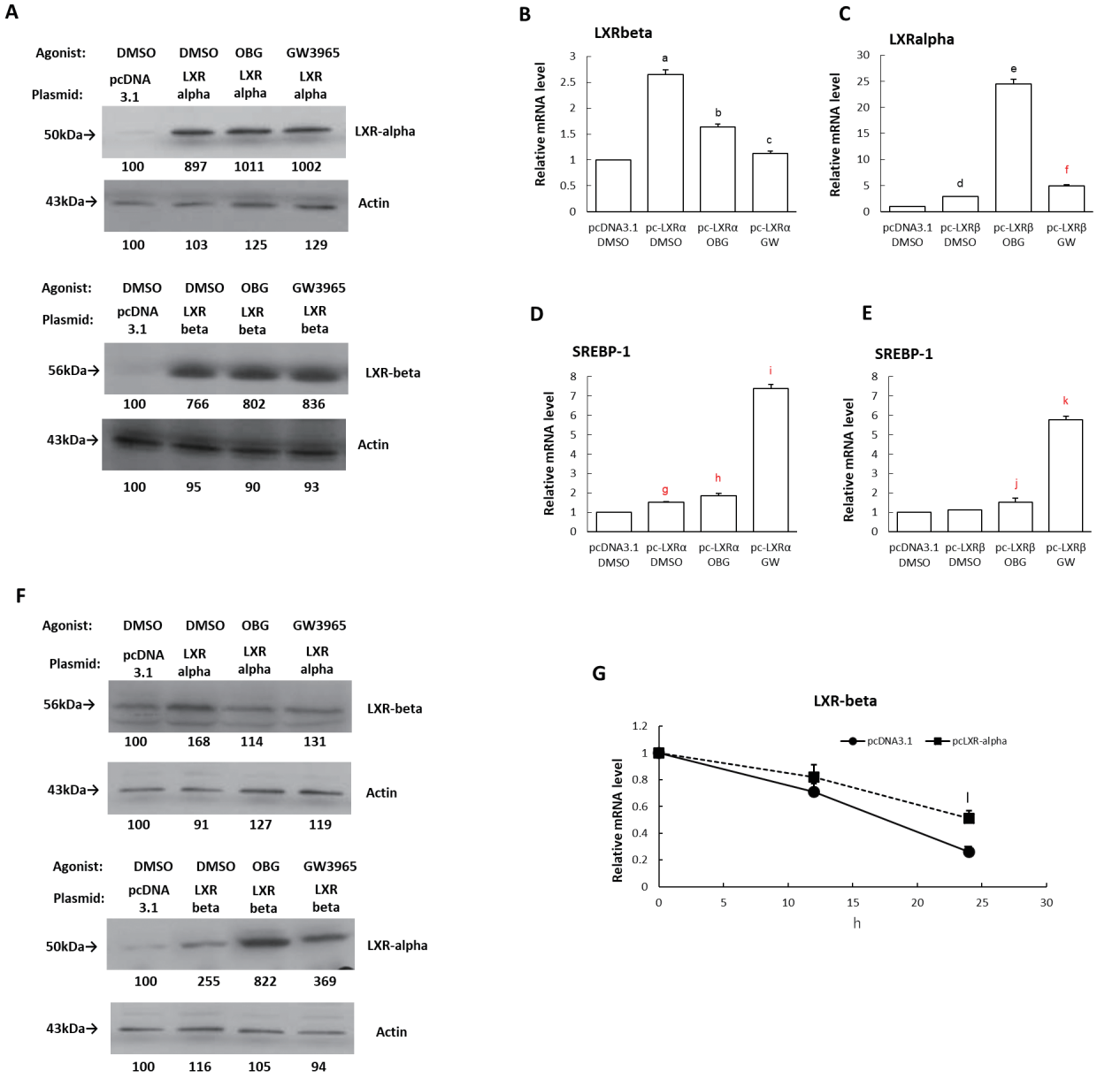

Fig. 2. Change in SREBP-1c expression in human hepatocyte-derived Fa2N-4 cells transfected with expression vectors for LXR-alpha and LXR-beta in the presence or absence of OBG and LXR agonist GW3965. A-F: Fa2N-4 cells seeded at 5.0×10^5 cells/60-mm dish were transfected with pcDNA3.1 (+), pc-LXR-alpha, or pc-LXR-beta in the presence or absence of 2 μM OBG and 5 μM GW3965. (A and F) After 24 hr, cell extracts were subjected to immunoblotting to detect LXR-alpha, LXR-beta, and β-actin protein as described in the MATERIALS AND METHODS. (B and D) After transfection with pcDNA3.1 (+) or pc-LXR-alpha, total RNA was extracted, and quantification of LXR-beta (B) and SREBP-1c (D) mRNA was performed as described in the MATERIALS AND METHODS. (C and E) After transfection with pcDNA3.1 (+) or pc-LXR-beta, total RNA was extracted, and quantification of LXR-alpha (C) and SREBP-1c (E) mRNA was performed. G: Fa2N-4 cells seeded at 5.0×10^5 cells/60-mm dish were transfected with pcDNA3.1 (+) or pc-LXR-alpha. After 24 hr, cells were treated with 1 μg/uL actinomycin D for 12 and 24 hr, then total RNA was subjected to quantification of LXR-beta. Data were analyzed using Student's t-test and are presented as the mean ± S.E.M. of three experiments performed in triplicate. a, d, g: Significant to "pcDNA3.1/DMSO"; a: $P < 0.01$, d: $P < 0.001$, g: $P < 0.05$. b, c, h, i: Significant to "pc-LXRα/DMSO"; b: $P < 0.01$, c: $P < 0.01$, h: $P < 0.05$, i: $P < 0.001$. e, f, j, k: Significant to "pc-LXRβ/DMSO"; e: $P < 0.001$, f: $P < 0.05$, j: $P < 0.05$, k: $P < 0.001$. l: Significant to "pcDNA3.1" (24 hr); $P < 0.05$.

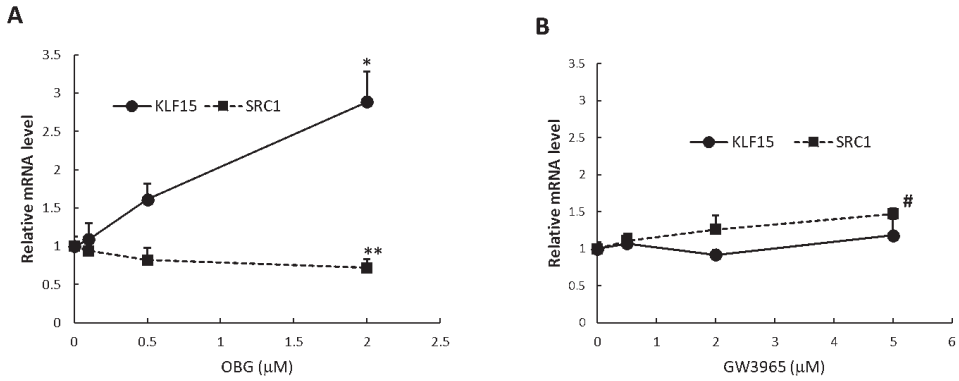

Fig. 3. Change in KLF15 and SRC1 expression in Fa2N-4 cells treated with OBG and GW3965. Fa2N-4 cells seeded at 5.0×10^5 cells/60-mm dish were treated with the indicated concentrations of OBG (A) and GW3965 (B). After 24 hr, total RNA was extracted, and quantification of KLF15 and SRC1 mRNA was performed as described in the MATERIALS AND METHODS. Data were analyzed using Student's t-test and are presented as the mean ± S.E.M. of three experiments performed in triplicate. *, **, #: Significant to DMSO-treated (control) cells: * $P < 0.001$, ** $P < 0.05$, # $P < 0.05$ (SRC1).

acts as negative regulator for SREBP-1c expression, by binding to the LXR-alpha/RXR-alpha complex, thereby recruiting corepressor RIP140 instead of coactivator SRC1 on the promoter of SREBP-1c gene (Takeuchi *et al*., 2016). Thus, we examined whether or not OBG affected the expression level of KLF15 in Fa2N-4 cells. As shown in Fig. 3A, OBG significantly stimulated KLF15 expression in a dose dependent manner, while it slightly reduced SRC1 expression. In contrast, GW3965 did not affect the expression levels of KLF15 at any concentration tested, while it slightly enhanced SRC1 expression (Fig. 3B).

DISCUSSION

In the present study, we revealed that LXR-alpha and LXR-beta mutually regulates the expression levels of their counterparts in the normal hepatocyte-derived cell line Fa2N-4. However, regulation of LXR-beta by LXR-alpha and that of LXR-alpha by LXR-beta are different in terms of ligand requirement. Results of Fig. 2B, 2F and 2G suggests that OBG, an aglycone of cardiac glycoside ouabain, and synthetic agonist GW3965 similarly activate LXR-alpha and down-regulates LXR-beta mRNA expression. On the other hand, OBG dramatically stimulated LXR-alpha expression in Fa2N-4 cells overexpressing LXR-beta, compared with the GW3965 (Fig. 2C and 2F). Thus, OBG activates both of LXR-alpha and LXR-beta, however, it may preferentially activate LXR-beta as compared with GW3965, resulting in dramatical increase in LXR-alpha. Importantly, under the condition where LXR-alpha was highly expressed, GW3695 rather than OBG significantly stimulated the expression of SREBP-1c that is primarily activated by LXR-alpha (Fig. 2D). On the other hand, OBG but not GW3695 failed to stimulate SREBP-1c expression in Fa2N-4 cells, even though the ectopically overexpressed LXR-beta caused the elevation of LXR-alpha expression in these cells (Fig. 2E). Moreover, we have demonstrated that OBG but not GW3695 significantly stimulated the expression of KLF15, which is known to form a complex with LXR/RXR on the promoter of SREBP-1c gene thereby releasing coactivator SRC1, instead recruiting corepressor RIP140. These results suggest that up-regulation of KLF15 by OBG counteracts the SREBP-1c upregulation induced by OBG-activated LXR-alpha. Glucocorticoid receptor (GR), a nuclear receptor activated by glucocorticoids, is known to regulate KLF15 expression (Asada *et al*., 2011). However, it is unlikely that GR directly participates in the upregulation of KLF15 in OBG-treated Fa2N-4 cells, since OBG fails to activate GR (Tamura *et al*., 2018).

As reported by Tamura *et al*., OBG was shown to suppress the expression of ENaC in the kidney. We can postulate that OBG may stimulate the expression of KLF15 in the kidney, promoting the formation of KLF/LXR/RXR complex, thereby replacing the coactivator with corepressor on the promoter of ENaC gene. It should be noted that KLF15 is a zinc-finger transcription factor that is highly expressed in the glomeruli and interstitial cells of the kidneys, with predominant roles in renal biology, making it a potential therapeutic target for kidney disease (Wang *et al*., 2019).

In this study, we focused on the regulatory mechanism of two LXR isoforms, LXR-alpha and LXR-beta, and further characterized OBG as a possible candidate to minimize the adverse effects of LXR-alpha-mediated lipogenesis by highlighting the involvement of a member of Krüppel-like factors, KLF15. Further insight should be directed for the characterization of isoform-selective ligands for LXRs and the elucidation of the role of crosstalk between LXRs and KLFs in various endocrine disorders.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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