Nuclear receptor subfamily 1 group H member 2 (*LXRB*) is the predominant liver X receptor subtype regulating transcription of 2 major lipogenic genes in goat primary mammary epithelial cells

H. B. Shi,^{*1} C. H. Zhang,^{†1} Z. A. Xu,^{*} X. F. Xu,[†] Z. B. Lv,^{*} J. Luo,^{‡2} and J. J. Looor^{‡2}

^{*}Key Laboratory of Silkworm Bioreactor and Biomedicine of Zhejiang Province, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou, China 310018

[†]College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, China 712100

[‡]Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana 61801

ABSTRACT

In ruminants, recent research has identified a crucial role for liver X receptors (LXR) in regulating lipid metabolism in mammary cells. However, the differences between LXR subtypes in regulating ruminant lipid metabolism are unknown. We used overexpression and knockdown of *LXRA* and *LXRB* in goat primary mammary epithelial cells to distinguish subtype-specific regulation of sterol regulatory element binding protein 1c (*SREBP1c*) and fatty acid synthase (*FASN*) mRNA expression and their promoter activity. Incubation with siRNA targeting LXR decreased expression of *LXRA* and *LXRB*. Knockdown of *LXRA* and *LXRB* in cells incubated with dimethyl sulfoxide (DMSO, control) had no effect on the expression of *SREBP1c* or *FASN*. Knockdown of *LXRB* in cells incubated with LXR ligand T0901317 (T09) led to decreased expression of *FASN*, but not *SREBP1c*. Overexpression of *LXRB* plus T09 dramatically upregulated *SREBP1c* and *FASN* to levels higher than overexpression of *LXRA* with T09. Luciferase reporter assays in cells with site-directed mutagenesis of LXR response elements (LXRE; LXRE1 from –286 to –251 bp or LXRE2 from –235 to –219 bp) revealed that the *SREBP1c* promoter with the wild type or either LXRE mutation in cells supplemented with T09 decreased markedly only when *LXRB* was knocked down. Knockdown of *LXRA* and *LXRB* had no effect on the *SREBP1c* promoter when cells had a double LXRE mutation. Overexpression of *LXRA* only in cells incubated with T09 increased the activity of the *SREBP1c* promoter with the wild type and the LXRE2 mutation. In contrast, compared with each control group, overexpression of *LXRB* dramatically increased *SREBP1c* promoter activity, regardless

of LXRE mutation. Furthermore, in cells stimulated with T09, knockdown of either *LXRA* or *LXRB* did not alter wild-type *FASN* promoter activity. Knockdown of *LXRA* increased wild-type and LXRE-site-mutated (LXRE from –677 to –662 bp) *FASN* promoter activity. Overexpression of *LXRB* increased wild-type and LXRE-site-mutated *FASN* promoter activity regardless of treatment with DMSO or T09, but overexpression of *LXRA* altered LXRE-site-mutated *FASN* promoter activity only in cells treated with DMSO. Increased activation of *SREBP1c* or *FASN* promoters containing LXRE mutations after overexpression of *LXRB* suggested that *LXRB* activates endogenous *SREBP1c*, which can then bind to the promoter of *SREBP1c* via an auto-loop circuit regulatory mechanism. Collectively, these results highlight an important role for *LXRB* in the transcriptional regulation of *SREBP1c* and *FASN* in goat mammary epithelial cells. Activation of this nuclear receptor controls lipogenesis via different mechanisms.

Key words: ruminant, mammary cells, gene expression, promoter activation

INTRODUCTION

Liver X receptors (**LXR**) are key nuclear receptors (also referred to as nuclear receptor subfamily 1 group H) that regulate the synthesis of lipid and cholesterol homeostasis (Ulven et al., 2005). In human preadipocytes, LXR activation with a synthetic agonist T0901317 (**T09**) increased the mRNA abundance of fatty acid synthase (*FASN*) and stearoyl-CoA desaturase 1 (*SCD1*; Darimont et al., 2006). To date, 2 protein subtypes of LXR (*LXRA* and *LXRB*) have been identified. Although some evidence supports a different functional role for these subtypes in humans and rodents (Quinet et al., 2006; Ishibashi et al., 2013), that role remains unclear.

Upon activation by oxysterols or synthetic agonists in humans and rodents, LXR bind to LXR response

Received December 22, 2016.

Accepted May 2, 2017.

¹These authors contributed equally to this paper.

²Corresponding authors: luojun@nwsuaf.edu.cn and jloor@illinois.edu

elements (**LXRE**) in the promoters of target genes as a heterodimeric complex with retinoid X receptor, leading to transcription of lipogenic enzymes (Laurenckiene and Ryden, 2012). Sterol regulatory element binding protein 1 (**SREBP1**) is a key transcription factor associated with milk fat depression when diets supplemented with fish oil or plant oil are fed to dairy cows (Bionaz et al., 2015). Subtypes **SREBP1a** and **SREBP1c** are controlled by different promoters; **SREBP1a** activates transcription of all **SREBP**-responsive genes, but **SREBP1c** specifically activates genes involved in fatty acid synthesis in bovines (Lengi and Corl, 2010). Studies to date have identified **LXRE** in the **SREBP1c** promoter in goats (Xu et al., 2016), cows (Lengi and Corl, 2010), and mice (Repa et al., 2000), indicating a direct regulation of **SREBP1c** by **LXR**. In vitro dose-response studies with macrophages from **LXRA**^{-/-} and **LXRB**^{-/-} mice confirmed an equivalent role for **LXRA** and **LXRB** in the regulation of **SREBP1** expression. The fact that selective **LXRB** activation increased high-density lipoprotein but circumvented hepatic **LXRA**-dominated lipogenesis indicated that **LXR** isoforms play different roles in fatty acid metabolism (Quinet et al., 2006).

In a bovine mammary epithelial cell line, activation of **LXR** with T09 enhanced de novo fatty acid synthesis (McFadden and Corl, 2010) and altered cellular fatty acid profiles (Wang et al., 2012). Recent data in goat mammary epithelial cells (**GMEC**) indicated a role for **LXR** in regulating fatty acid synthesis via direct binding to the promoters of **FASN** (Li et al., 2015) and **SREBP1c** (Xu et al., 2016). The **LXR** also promoted the synthesis of monounsaturated fatty acids in **GMEC** via control of **SCD1** in an **SREBP1**-dependent manner (Yao et al., 2016). However, neither of these studies distinguished differences between **LXRA** and **LXRB** subtypes.

Work to date has demonstrated a role for **LXR** in directly regulating **SREBP1c** and **FASN**, but there is no molecular information about which **LXR** subtype might be central to this mechanism. We designed the present study to characterize the different transcriptional regulation mechanisms for **LXRA** and **LXRB** in **GMEC** by assessing **SREBP1c** and **FASN** responses.

MATERIALS AND METHODS

Adenovirus Generation

The cDNA for **LXRA** (GenBank No.: NM_001285751) and **LXRB** (GenBank No.: XM_012190735.2) were cloned from **GMEC** and then subcloned into a pAdTrack-CMV plasmid vector to generate pAdTrack-CMV-**LXRA** and pAdTrack-CMV-**LXRB**. The vectors

were inserted into an adenoviral vector (pAdEasy-1) to generate adenoviral plasmids in BJ5183 cells. The adenoviral plasmids linearized by *PacI* (New England Biolabs, Ipswich, MA) were transfected into 293A cells to generate the adenoviruses Ad-**LXRA** and Ad-**LXRB**. Generation and proliferation of the adenoviruses was performed as described previously (Luo et al., 2007; Shi et al., 2013a).

RNA Interference

To knock down expression of **LXRA** and **LXRB**, small interfering RNA (**siRNA**) were designed and synthesized by Jima Corp. (Shanghai, China). A scrambled **siRNA** was designed and used as a negative control. The **siRNA** sequences are reported in Supplementary Table S1 (<https://doi.org/10.3168/jds.2016-12510>).

Vector Construction

The vectors containing the **SREBP1c** promoter region were gifts from Huifen Xu (Northwest A&F University, Shaanxi, China). The **SREBP1c** promoter contained 2 **LXRE** sites (**LXRE1** from -286 to -251 bp and **LXRE2** from -235 to -219 bp). We performed overlap extension PCR to generate 2 single **LXRE** site-directed mutants (**SREBP1c-LXRE1-mut** and **SREBP1c-LXRE2-mut**) and a double **LXRE** site-directed mutant (**SREBP1c-2LXRE-mut**). The wild-type **SREBP1c** promoter (**SREBP1c-wild**) and the mutants were inserted into a pGL3-basic vector. Details of the luciferase vector construction are described elsewhere (Xu et al., 2016).

The wild-type **FASN** promoter region (-838 to +129 bp; **FASN-wild**) was a gift from Jun Li (Northwest A&F University). The **LXRE** site at -677 to -662 bp on the **FASN** promoter was mutated using site-direct mutant technology to generate a mutant plasmid (**FASN-LXRE-mut**; Li et al., 2015).

Cell Culture and Treatments

The **GMEC** were isolated from peak lactation Xinong Saanen goats as described previously (Wang et al., 2010; Shi et al., 2014a). Details of the cell culture are described elsewhere (Lin et al., 2013; Shi et al., 2013b). Briefly, to promote lactogenesis, **GMEC** were cultured in a lactogenic medium for 24 h before initial experiments. The lactogenic medium was composed of Dulbecco's modified Eagle's medium/F12 (HyClone, Beijing, China), insulin (5 mg/L; Sigma-Aldrich, St. Louis, MO), hydrocortisone (5 mg/L; Sigma-Aldrich), penicillin/streptomycin (10,000 U/L; Harbin Pharmaceutical Group, Harbin, China), prolactin (2 µg/mL;

Sigma-Aldrich) and BSA (1 g/L; Sigma-Aldrich). The 293A cell line for adenovirus generation was cultured in basal Dulbecco's modified Eagle's medium (Gibco, Langley, OK) containing 10% fetal bovine serum.

For the adenovirus infection, the GMEC cultured in 6-well plates were incubated with adenovirus media Ad-GFP (adenovirus with green fluorescent protein; control), Ad-LXRA, or Ad-LXRB, respectively. For RNA interference, the GMEC cultured in 6-well plates were transfected with the siRNA oligonucleotides for negative control (siNC), LXRA (siLXRA), and LXRB (siLXRB), respectively, using Lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen, Carlsbad, CA; http://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_RNAiMAX_Reagent_protocol.pdf). Treated GMEC were cultured with 5 μ M T09 [final concentration, diluted in dimethyl sulfoxide (DMSO); Sigma-Aldrich] or control (DMSO; Sigma-Aldrich) after 24 h of initial culture, and then harvested at 48 h (24 h later) for RNA extraction.

Vector Transfection

For the luciferase assay, the GMEC cultured in 48-well plates at 80 to 90% confluent were cotransfected with 290 ng of a vector (SREBP1c-wild, SREBP1c-LXRE1-mut, SREBP1c-LXRE2-mut, SREBP1c-2LXRE-mut, FASN-wild, or FASN-LXRE-mut) plus 10 ng of control vector (renilla luciferase) per well using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol (http://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_2000_Reagent_protocol.pdf). After 12 h of initial culture, the transfected GMEC were incubated with an adenovirus medium (Ad-GFP, Ad-LXRA, or Ad-LXRB), or with siRNA using Lipofectamine RNAiMAX (Invitrogen). The transfected GMEC were treated with 5 μ M T09 (final concentration, diluted in DMSO; Sigma-Aldrich) or control (DMSO; Sigma-Aldrich) after 36 h of initial culture, and then harvested at 56 h for luciferase activity assays.

RNA Extraction and Quantitative PCR

Total RNA was extracted using the RNA Prep pure cell kit (Tiangen Biotech Co. Ltd., Beijing, China), and quantitative PCR (qPCR) was performed according to the manufacturer's instructions using SYBR Green (SYBR Premix Ex Taq II, Perfect Real Time; Takara Bio Inc., Kyoto, Japan; Shi et al., 2014b). Genes studied included *LXRA*, *LXRB*, *FASN*, and *SREBP1* (i.e., *SREBP1a* + *SREBP1c*). Based on current knowledge of *SREBP1a* and *SREBP1c* goat sequences, the coding DNA sequence of *SREBP1c* completely overlaps that of *SREBP1a*, making it impossible to detect *SREBP1c*

solely with qPCR. The *SREBP1* gene was assessed to reflect the mRNA expression of *SREBP1c*. All qPCR reactions were performed using a Bio-Rad CFX96 sequence detector (Bio-Rad Laboratories Inc., Hercules, CA), and data were normalized to the geometric mean of ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RPS9*). The sequences of the primers for the genes are reported in Supplementary Table S2 (<https://doi.org/10.3168/jds.2016-12510>).

Luciferase Activity Assay

After collection, the GMEC were washed 3 times with PBS and then incubated with a passive lysis buffer (Promega, Madison, WI) for 30 min at room temperature. The dual Luciferase Reporter assay kit (Promega) was used to measure luciferase activity on a Fluoroskan Ascent apparatus (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol. Relative luciferase activity was calculated as the ratio of firefly luciferase to renilla luciferase activity.

Data Analysis

Treatments were replicated at least 3 times, and results are expressed as mean $\pm$ standard error of the mean. Data from qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to the respective control (siNC + DMSO or Ad-GFP + DMSO). Data for the relative luciferase activity of SREBP1c-wild, SREBP1c-LXRE1-mut, SREBP1c-LXRE2-mut, SREBP1c-2LXRE-mut, FASN-wild, or FASN-LXRE-mut and the mRNA expression of *SREBP1* and *FASN* were expressed within DMSO or T09 groups. This approach allowed us to directly assess the effect of different treatments for a given factor. Statistical differences within DMSO or T09 groups were determined using one-way ANOVA in SPSS 19.0 (SPSS, Inc./IBM Corp., Chicago, IL). Significance was declared at $P < 0.05$.

RESULTS

Knockdown of LXRA and LXRB Altered the mRNA Expression of SREBP1 and FASN

To examine whether inhibiting *LXRA* and *LXRB* decreased *SREBP1* and *FASN* mRNA expression, we used specific siRNA to knock down *LXRA* or *LXRB*. The qPCR results (Figure 1A) showed that incubation with siLXRA and siLXRB decreased the mRNA expression of *LXRA* and *LXRB* by more than 80% ($P = 0.001$) and 92% ($P = 0.001$), respectively, without

significantly affecting ($P = 0.99$) the other subtype. Incubation with T09 had no effect ($P = 0.99$) on expression of *LXRA* or *LXR*B compared with the control (siNC + DMSO).

In the cells treated with DMSO, the GMEC with siLXRA or siLXRB had no effect on the mRNA abundance of *SREBP1* or *FASN* compared with the control (siNC; Figure 1B). In cells supplemented with T09, knockdown of *LXRA* increased expression of *SREBP1* ($P = 0.01$) compared with the control (siNC) but did not increase expression of *FASN* ($P = 0.35$). In cells supplemented with T09, knockdown of *LXR*B decreased expression of *FASN* ($P = 0.003$) compared with the control (siNC; Figure 1C). Compared with knockdown of *LXRA*, knockdown of *LXR*B led to a lower expression of both *FASN* ($P = 0.001$) and *SREBP1* ($P = 0.02$) in cells supplemented with T09.

Overexpression of *LXR*B or *LXRA* Altered the mRNA Expression of *SREBP1* and *FASN*

To examine whether overexpression of *LXRA* or *LXR*B increased *SREBP1* and *FASN* mRNA expression, we overexpressed *LXRA* or *LXR*B by generating a specific adenovirus for each subtype (Ad-LXRA and Ad-LXRB). As shown in Figures 2A and B, the mRNA abundance of *LXRA* and *LXR*B increased dramatically in the GMEC incubated with Ad-LXRA (~150-fold; $P = 0.001$) and Ad-LXRB (~140-fold; $P = 0.001$), respectively, compared with the control (Ad-GFP + DMSO).

In cells treated with DMSO, *LXRA* overexpression had no significant effect on the mRNA abundance of *SREBP1* or *FASN* compared with the control (Ad-GFP; Figures 2C and D). Overexpression of *LXR*B had no effect on *SREBP1* ($P = 0.35$) compared with the control (Ad-GFP), but did increase *FASN* expression ($P = 0.01$) in cells with DMSO.

In cells treated with T09, overexpression of *LXRA* had no effect on *SREBP1* ($P = 0.83$), but it increased *FASN* expression ($P = 0.004$) compared with the control (Ad-GFP). In cells supplemented with T09, overexpression of *LXR*B dramatically upregulated the mRNA abundance of both *SREBP1* ($P = 0.01$) and *FASN* ($P = 0.001$) compared with control (Ad-GFP). As well, the mRNA expression level of *SREBP1* ($P = 0.03$) and *FASN* ($P = 0.01$) in cells incubated with T09 and Ad-LXRB was higher than in the group supplemented with Ad-LXRA and T09.

Knockdown of *LXR*B Decreased the Full Activity of the *SREBP1c* Promoter

In the cells treated with DMSO, knockdown of *LXRA* increased *SREBP1c*-wild activity ($P = 0.01$), and knock-

down of *LXR*B downregulated *SREBP1c*-wild activity ($P = 0.01$) compared with the control (siNC; Figure 3A). In the group transfected with *SREBP1c*-LXRE1-mut (Figure 3B; $P = 0.01$) or *SREBP1c*-LXRE2-mut (Figure 3C; $P = 0.02$), the DMSO cells incubated with siLXRA had higher levels of promoter activity than the control (siNC + DMSO). Incubation with siLXRB decreased the activity of *SREBP1c*-LXRE2-mut ($P = 0.042$; Figure 3 C) and had no effect on *SREBP1c*-LXRE1-mut ($P = 0.05$; Figure 3 B) compared with the control (siNC + DMSO).

In cells supplemented with T09, cells incubated with siLXRB had lower *SREBP1c*-wild ($P = 0.001$), *SREBP1c*-LXRE1-mut ($P = 0.011$), and *SREBP1c*-LXRE2-mut ($P = 0.002$) activity compared with the control (siNC; Figures 3A, B, and C). We observed no significant change in cells incubated with siLXRA and T09 compared with the control (siNC + T09; Figure 3A, B, and C). When both LXRE were mutated (*SREBP1c*-2LXREs-mut), incubation with siLXRA or siLXRB had no effect on the activity of the promoter, regardless of treatment with T09 or DMSO (Figure 3D).

Overexpression of *LXR*B Dramatically Increased *SREBP1c* Promoter Activity

In GMEC supplemented with DMSO, overexpression of *LXRA* had no effect on the activity of *SREBP1c* promoter activity compared with the control (Ad-GFP), regardless of LXRE mutation (Figures 4A, B, C, and D). However, overexpression of *LXR*B always increased the activity of *SREBP1c*-wild ($P = 0.001$), mutations of either LXRE ($P = 0.001$), or mutations of both LXRE ($P = 0.001$), compared with the control (Ad-GFP; Figures 4 A, B, C, and D).

In the GMEC supplemented with T09, overexpression of *LXRA* also increased the activity of *SREBP1c*-wild ($P = 0.02$) and *SREBP1c*-LXRE2-mut ($P = 0.014$) compared with the control (Ad-GFP + T09; Figures 4 A and C), but it did not have this effect in cells with double LXRE mutations ($P = 0.917$) or an LXRE1 mutation ($P = 0.30$; Figures 4 B and D). In contrast, overexpression of *LXR*B in GMEC supplemented with T09 increased ($P < 0.05$) *SREBP1c* promoter activity dramatically regardless of LXRE mutation, compared with each control group (Ad-GFP + T09) and with the groups incubated with Ad-LXRA and T09 (Figure 4 A, B, C, and D).

***LXR*B Is Responsible for the Activity of the *FASN* Promoter**

In GMEC containing DMSO, compared with the control (siNC), we observed a significant increase of

Figure 1. Changes in gene expression in goat mammary epithelial cells in response to knockdown of liver X receptor α or β [*LXRA* or *LXRB*; siLXRA + dimethyl sulfoxide (DMSO) or siLXRB + DMSO], incubation with agonist T0901317 (T09; siNC + T09), control (siNC + DMSO), or knockdown of *LXRA* or *LXRB* plus T09 (siLXRA + T09 or siLXRB + T09). Liver X receptor α or β were knocked down using small interfering RNA (siRNA). (A) mRNA expression of *LXRA* and *LXRB*; (B) mRNA expression of sterol regulatory element binding protein 1 (*SREBP1*); (C) mRNA expression of fatty acid synthase (*FASN*). Values are means $\pm$ SEM from 3 individual cultures and expressed relative to the control group. The different letters denote significant ($P < 0.05$) differences, and the absence of letters denotes the absence of statistical differences. In panel A, uppercase and lowercase letters denote different statistical comparisons.

Figure 2. Changes in gene expression in goat mammary epithelial cells in response to overexpression of liver X receptor α or β [*LXRA* or *LXRβ*; Ad-LXRA + dimethyl sulfoxide (DMSO) or Ad-LXRβ + DMSO], incubation with agonist T0901317 (T09; Ad-GFP+T09), control (Ad-GFP + DMSO), or overexpression of *LXRA* or *LXRβ* plus T09 (Ad-LXRA + T09 or Ad-LXRβ + T09). Liver X receptor α or β were overexpressed using adenoviruses (Ad); Ad-GFP is an adenovirus with green fluorescent protein (GFP). (A) mRNA expression of *LXRA*; (B) mRNA expression of *LXRβ*; (C) mRNA expression of sterol regulatory element binding protein 1 (*SREBP1*); (D) mRNA expression of fatty acid synthase (*FASN*). Values are means $\pm$ SEM from 3 individual cultures and expressed relative to the control group (Ad-GFP + DMSO). The different letters denote significant ($P < 0.05$) differences, and the absence of letters denotes the absence of statistical differences. In panel D, uppercase and lowercase letters denote different statistical comparisons.

FASN-wild ($P = 0.035$) and *FASN*-LXRE-mut ($P = 0.001$) activity after knockdown of *LXRA*, but we found no effect ($P = 0.35$ and 0.05) after knockdown of *LXRβ* (Figures 5 A and B). In cells stimulated with T09, knockdown of *LXRβ* had no effect on *FASN*-wild ($P = 0.26$) and *FASN*-LXRE-mut activity ($P = 0.19$) (Figures 5A and B); knockdown of *LXRA* still increased *FASN*-LXRE-mut activity ($P = 0.02$) but had no effect on *FASN*-wild activity ($P = 0.75$) compared with the control (Figures 5 A and B).

Overexpression of *LXRβ* increased the activity of *FASN*-wild ($P < 0.001$) and *FASN*-LXRE-mut ($P < 0.001$) in cells incubated with either DMSO or T09 compared with the control (Ad-GFP; Figures 5C and D). Overexpression of *LXRA* had no effect on the activity of *FASN*-wild ($P = 0.06$). In cells incubated with DMSO, we observed a significant increase of *FASN*-LXRE-mut activity ($P = 0.01$) after overexpression of *LXRA* (Figure 5D) compared with the control (Ad-GFP + DMSO), but the effect of *LXRA* overexpression on *FASN*-LXRE-mut was lower than that of *LXRβ* overexpression ($P < 0.001$). In cells incubated with T09, *LXRA* overexpression had no effect on *FASN*-wild ($P = 0.93$) or *FASN*-LXRE-mut activity (Figure 5D).

DISCUSSION

It is well established that LXR are nuclear receptors associated with lipid synthesis in rodents (Bionaz et al., 2015). Recent studies in goats (Yao et al., 2016) and rodents (Varin et al., 2015) also provide evidence for LXR as central players in regulating cellular fatty acid profiles. Data from goats and cows have indicated that LXR regulate *SREBP1c* and *FASN* via binding to the promoter or controlling the activation of *SREBP1c* (Ma and Corl, 2012; Li et al., 2015). Our data show that the *LXRβ* subtype plays a predominant role in controlling the expression of *SREBP1c* and *FASN* in GMEC.

Activation of LXR in a bovine cell line with T09 induced *SREBP1* mRNA expression and enhanced the transcription of genes involved in fatty acid synthesis (Ma and Corl, 2012). In this study, we used T09 (stimulated level) and DMSO (basal level) to assess the activation of LXR in the context of the *SREBP1c* promoter (Xu et al., 2016). The weak effect on the expression of *SREBP1* and *FASN* after incubation with siLXRA and siLXRβ in cells containing DMSO (Figure 2 B and C) was consistent with previous findings in cattle

(Oppi-Williams et al., 2013) and goats (Li et al., 2015). It supports evidence that LXR may not be essential for the basal transcription of genes required for fatty acid synthesis, at least in cultured bovine mammary cells (Oppi-Williams et al., 2013). Because LXR are ligand-dependent nuclear receptors, the weak response of *SREBP1* and *FASN* after incubation with siLXRA and siLXRB in cells containing DMSO might be due to the absence of a ligand. The fact that T09 stimulation downregulated *FASN* expression when *LXRB* was knocked down but upregulated *SREBP1c* and *FASN* when *LXRB* was overexpressed indicated that *LXRB* had a strong capacity for inducing downstream genes related to lipogenesis in this ligand-stimulated culture system.

The *SREBP1c* gene is a target of LXR, and its promoter contains an LXRE (Repa et al., 2000; Lengi and Corl, 2010). A recent study in goats showed that the promoter of *SREBP1c* has 2 LXRE, and LXR regulates *SREBP1c* expression in vitro (Xu et al., 2016). Knockdown of *LXRB* in cells supplemented with T09 decreased activation of the wild-type and single-LXRE-mutated *SREBP1c* promoters, and only overexpression

of *LXRB* increased *SREBP1c* promoter activation in cells with DMSO; this finding suggested that the *LXRB* subtype had a strong capacity for regulating *SREBP1c* transcription in GMEC. Along with data from *SREBP1c*-wild and either *LXRE* mutation, the finding that specific knockdown of *LXRA* or *LXRB* had no effect on the *SREBP1c*-2LXRE-mut suggested that *LXRB* directly regulated *SREBP1c* via LXRE.

The seemingly crucial role of *LXRB* in regulating *SREBP1c* challenges previous findings with rodent macrophages, which demonstrated that *LXRA* and *LXRB* play an equivalent role in the regulation of *SREBP1c* gene expression (Quinet et al., 2006). The importance of *LXRB* is supported by the response in promoter activation of *SREBP1c*. The discrepancy with rodents may be due to inherent species differences (e.g., use of glucose versus acetate for lipogenesis) and the existence of tissue- or cell-specific mechanisms. The fact that *LXRB* expression in lactating goat mammary tissue is higher than *LXRA* also supports the present data (transcriptome data: GEO Series GSE87089).

In the present study, the finding that knockdown of *LXRA* had no effect on *SREBP1c* promoter activation

Figure 3. Knockdown of liver X receptor α or β (*LXRA* or *LXRB*) in goat mammary epithelial cells alters the activation of sterol regulatory element binding protein 1c (*SREBP1c*) promoter containing a wild-type construct (SREBP1c-wild), mutation of one of the liver X receptor response elements (SREBP-LXRE1-mut or SREBP-LXRE2-mut), or a double mutation (SREBP-2LXREs-mut). A vector containing renilla luciferase was used as a control and cotransfected with each *SREBP1c* promoter in goat mammary epithelial cells. (A) Relative luciferase activation of SREBP1c-wild; (B) relative luciferase activation of SREBP1c-LXRE1-mut; (C) relative luciferase activation of SREBP1c-LXRE2-mut; (D) relative luciferase activation of SREBP1c-2LXREs-mut. Values are means $\pm$ SEM from 3 individual cultures. The different letters denote significant ($P < 0.05$) differences, and the absence of letters denotes the absence of statistical differences. Uppercase and lowercase letters denote different statistical comparisons.

Figure 4. Overexpression of liver X receptor α or β (*LXRA* or *LXRB*) in goat mammary epithelial cells alters the activation of sterol regulatory element binding protein 1c (*SREBP1c*) promoter containing a wild-type construct (*SREBP1c-wild*), mutation of one of the liver X receptor response elements (*SREBP1c-LXRE1-mut* or *SREBP1c-LXRE2-mut*), or a double mutation (*SREBP1c-2LXREs-mut*). A vector containing renilla luciferase was used as a control and cotransfected with each *SREBP1c* promoter in goat mammary epithelial cells. (A) Relative luciferase activation of *SREBP1c-wild*; (B) relative luciferase activation of *SREBP1c-LXRE1-mut*; (C) relative luciferase activation of *SREBP1c-LXRE2-mut*; (D) relative luciferase activation of *SREBP1c-2LXREs-mut*. Values are means $\pm$ SEM from 3 individual cultures. The different letters denote significant ($P < 0.05$) differences, and the absence of letters denotes the absence of statistical differences. Uppercase and lowercase letters denote different statistical comparisons.

in cells supplemented with T09 led us to hypothesize that *SREBP1c* is not a target of *LXRA*. The fact that specific knockdown of *LXRA* in cells with DMSO induced an increase of *SREBP1c* promoter activation was unexpected, but was consistent with previous rodent data (Zelcer and Tontonoz, 2006). The increased *SREBP1c* promoter activation we observed after knockdown of *LXRA* in cells with DMSO suggests that *LXRA* may have a counterregulatory role with respect to *LXRB*, and may indirectly affect its activation.

In addition to *LXRE*, the presence of *SREBP1c* response elements in the goat *SREBP1c* promoter implies that mature *SREBP1c* could bind to the *SREBP1c* response elements and regulate itself (Xu et al., 2016). In the present study, the finding that overexpression of *LXRB* still increased the activation of *SREBP1c-2LXREs-mut* supported the existence of an “auto-loop” regulatory mechanism: *LXRB* could cause an increase of endogenous *SREBP1c*, which could then enhance the activation of the *SREBP1c* promoter. The weak response of *SREBP1c-2LXREs-mut* to the overexpression of *LXRA* also confirmed the minor effect of *LXRA* on endogenous *SREBP1c* expression. In aggregate, the

data from the present study are highly suggestive that *LXRB* is the main *LXR* subtype controlling *SREBP1c* in GMEC.

Based on the effect of *LXRB* on *SREBP1c*, we deduced that the control of transcription of some lipogenic genes related to fatty acid synthesis (e.g., *FASN*) in ruminant cells is partly by controlling the expression of *SREBP1c* (Li et al., 2015), as the response in *FASN* indicates. The qPCR data in GMEC supplemented with T09 showed that knockdown of *LXRA* had no effect on *FASN* expression, but knockdown of *LXRB* significantly decreased *FASN* expression, indicating a key regulatory role for *LXRB* in *FASN* mRNA expression. In agreement with the higher expression of *FASN* after overexpression of *LXRB*, activation of the *FASN* promoters was enhanced by overexpression of *LXRB*, regardless of treatment with DMSO or T09. The finding that overexpression of *LXRB* induced an increase of *FASN-LXRE-mut* activation is in agreement with previous work in GMEC, demonstrating that *LXR* could indirectly regulate the activation of *FASN* via *SREBP1c* (Li et al., 2015). Furthermore, the finding that only overexpression of *LXRB* responded to the

increase in activation of FASN-LXRE-mut in T09-treated cells is consistent with the induction of endogenous SREBP1c activation by LXRB, which supports evidence for the hypothesis that LXR is responsive to SREBP1c induction, and that LXR regulates lipogenic genes in an SREBP1-dependent manner in GMEC (Yao et al., 2016).

Despite the strong indication that LXRB is the isotype controlling lipogenic genes, it is noteworthy that *FASN* expression and FASN-LXRE-mut were still induced by overexpression of *LXRA*. These findings indicated that LXR could bind to the promoter of some lipogenic genes, such as *FASN* and *SCD1* (Yao et al., 2016). Based on current knowledge of the *SREBP1a* and *SREBP1c* goat sequences, the coding DNA sequence of *SREBP1c* completely overlaps that of *SREBP1a*, precluding us from detecting *SREBP1c* using qPCR alone. A limitation of this study was that we assessed mRNA expression of *SREBP1* (i.e., *SREBP1a* + *SREBP1c*) after overexpression or knockdown of LXR, which may have accounted for the inconsistency in mRNA expression and promoter activation of *SREBP1c* to knockdown of *LXRB* (Figure 1B and Figure 4A). Furthermore, this response suggests a complex regulation model for *SREBP1c*, whose promoter also contains several other

functional elements, such as nuclear factor-Y and Sp-1 sites (Lengi and Corl, 2010).

Although our findings provide a better understanding of the transcriptional regulatory mechanism by which LXR subtypes play a role in controlling lipogenic gene expression in goat mammary cells, some limitations still need to be considered. For instance, protein expression analyses will be required to verify the silencing or overexpressing “efficiency” of the genes. In addition, based on the findings of the present study, we deduced that LXR subtypes might function via different signaling pathways in regulating lipogenic genes. It also should be kept in mind that *LXRA* might compete with LXRB for binding to LXRE, indirectly affecting the transcriptional regulation of LXRB in GMEC.

CONCLUSIONS

In the present study, the combination of mRNA expression level and the promoter activation of *SREBP1c* and *FASN* underscored an important role of the LXRB subtype in regulating the expression of *SREBP1c* and *FASN* in GMEC. The subtype *LXRA* seems to have a weak effect in regulating these 2 genes, mainly due to the weak response of *SREBP1c*. A significant increase

Figure 5. Overexpression of liver X receptor α or β (*LXRA* or *LXRB*) or knockdown of *LXRA* or *LXRB* in goat mammary epithelial cells alters the activation of fatty acid synthase (*FASN*) promoter containing a wild-type construct (FASN-wild) or a mutation of the liver X receptor response element (FASN-LXRE-mut), incubated with the liver X receptor agonist T0901317 (T09) or dimethylsulfoxide. A vector containing renilla luciferase was used as a control and cotransfected with each *FASN* promoter in goat mammary epithelial cells. (A, C) Relative luciferase activation of FASN-wild; (B, D): relative luciferase activation of FASN-LXRE-mut. Values are means $\pm$ SEM from 3 individual cultures. The different letters denote significant ($P < 0.05$) differences, and the absence of letters denotes the absence of statistical differences. Uppercase and lowercase letters denote different statistical comparisons.

of *FASN* or *SREBP1c* promoter activity after knock-down of *LXRA* suggests that this subtype may have a counterregulatory role, as a way to control genes whose transcription is regulated in an LXRE-dependent manner. More controlled molecular experiments will have to be performed to address questions about the precise signaling pathway of the 2 LXR subtypes in ruminant cells. Collectively, results from the present study suggest a crucial role for the LXRβ-SREBP1c signaling pathway in the transcriptional regulation of lipogenic genes.

ACKNOWLEDGMENTS

This research was jointly supported by Zhejiang Sci-Tech University Grant (15042088-Y, Hangzhou, China), the Special Fund for Agro-scientific Research in the Public Interest of Zhejiang province (2016C32061, Hangzhou, China), and the Transgenic New Species Breeding Program of China (Beijing, China; 2014ZX08009-051B).

REFERENCES

- Bionaz, M., J. Osorio, and J. J. Loor. 2015. Triennial lactation symposium: Nutrigenomics in dairy cows: Nutrients, transcription factors, and techniques. *J. Anim. Sci.* 93:5531–5553.
- Darimont, C., O. Avanti, I. Zbinden, P. Leone-Vautravers, R. Mansourian, V. Giusti, and K. Mace. 2006. Liver X receptor preferentially activates de novo lipogenesis in human preadipocytes. *Biochimie* 88:309–318.
- Ishibashi, M., R. Filomenko, C. Rébé, A. Chevriaux, A. Varin, V. Derangère, G. Bessède, P. Gambert, L. Lagrost, and D. Masson. 2013. Knock-down of the oxysterol receptor LXRα impairs cholesterol efflux in human primary macrophages: Lack of compensation by LXRβ activation. *Biochem. Pharmacol.* 86:122–129.
- Laurencikienė, J., and M. Ryden. 2012. Liver X receptors and fat cell metabolism. *Int. J. Obes. (Lond.)* 36:1494–1502.
- Leng, A. J., and B. A. Corl. 2010. Short communication: Identification of the bovine sterol regulatory element binding protein-1c promoter and its activation by liver X receptor. *J. Dairy Sci.* 93:5831–5836.
- Li, J., J. Luo, J. Zhu, Y. Sun, D. Yao, H. Shi, and W. Wang. 2015. Regulation of the fatty acid synthase promoter by liver X receptor alpha through direct and indirect mechanisms in goat mammary epithelial cells. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 184:44–51.
- Lin, X. Z., J. Luo, L. P. Zhang, W. Wang, H. B. Shi, and J. J. Zhu. 2013. MiR-27a suppresses triglyceride accumulation and affects gene mRNA expression associated with fat metabolism in dairy goat mammary gland epithelial cells. *Gene* 521:15–23.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25:402–408.
- Luo, J., Z. L. Deng, X. Luo, N. Tang, W. X. Song, J. Chen, K. A. Sharff, H. H. Luu, R. C. Haydon, K. W. Kinzler, B. Vogelstein, and T. C. He. 2007. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat. Protoc.* 2:1236–1247.
- Ma, L., and B. A. Corl. 2012. Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1. *J. Dairy Sci.* 95:3743–3755.
- McFadden, J. W., and B. A. Corl. 2010. Activation of liver X receptor (LXR) enhances de novo fatty acid synthesis in bovine mammary epithelial cells. *J. Dairy Sci.* 93:4651–4658.
- Oppi-Williams, C., J. K. Suagee, and B. A. Corl. 2013. Regulation of lipid synthesis by liver X receptor alpha and sterol regulatory element-binding protein 1 in mammary epithelial cells. *J. Dairy Sci.* 96:112–121.
- Quinet, E. M., D. A. Savio, A. R. Halpern, L. Chen, G. U. Schuster, J.-Å. Gustafsson, M. D. Basso, and P. Nambi. 2006. Liver X receptor (LXR)-β regulation in LXRα-deficient mice: Implications for therapeutic targeting. *Mol. Pharmacol.* 70:1340–1349.
- Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J.-M. A. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. *Genes Dev.* 14:2819–2830.
- Shi, H., H. Shi, J. Luo, W. Wang, A. B. Haile, H. Xu, and J. Li. 2014a. Establishment and characterization of a dairy goat mammary epithelial cell line with human telomerase (hT-MECs). *Anim. Sci. J.* 85:735–743.
- Shi, H. B., J. Luo, D. W. Yao, J. J. Zhu, H. F. Xu, H. P. Shi, and J. J. Loor. 2013a. Peroxisome proliferator-activated receptor-γ stimulates the synthesis of monounsaturated fatty acids in dairy goat mammary epithelial cells via the control of stearyl-coenzyme A desaturase. *J. Dairy Sci.* 96:7844–7853.
- Shi, H. B., J. Luo, J. Zhu, J. Li, Y. Sun, X. Lin, L. Zhang, D. Yao, and H. Shi. 2013b. PPARγ regulates genes involved in triacylglycerol synthesis and secretion in mammary gland epithelial cells of dairy goats. *PPAR Res.* 2013:310948.
- Shi, H., W. S. Zhao, J. Luo, D. W. Yao, Y. T. Sun, J. Li, H. P. Shi, and J. J. Loor. 2014b. Peroxisome proliferator-activated receptor γ1 and γ2 isoforms alter lipogenic gene networks in goat mammary epithelial cells to different extents. *J. Dairy Sci.* 97:5437–5447.
- Ulven, S. M., K. T. Dalen, J.-Å. Gustafsson, and H. I. Nebb. 2005. LXR is crucial in lipid metabolism. *Prostaglandins Leukot. Essent. Fatty Acids* 73:59–63.
- Varin, A., C. Thomas, M. Ishibashi, L. Ménégaut, T. Gautier, A. Trousson, V. Bergas, J. P. P. de Barros, M. Narce, J. M. A. Lobaccaro, L. Lagrost, and D. Masson. 2015. Liver X receptor activation promotes polyunsaturated fatty acid synthesis in macrophages: Relevance in the context of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 35:1357–1365.
- Wang, W., J. Luo, Y. Zhong, X. Lin, H. Shi, J. Zhu, J. Li, Y. Sun, and W. Zhao. 2012. Goat liver X receptor α, molecular cloning, functional characterization and regulating fatty acid synthesis in epithelial cells of goat mammary glands. *Gene* 505:114–120.
- Wang, Z., J. Luo, W. Wang, W. Zhao, and X. Lin. 2010. Characterization and culture of isolated primary dairy goat mammary gland epithelial cells. *Sheng Wu Gong Cheng Xue Bao* 26:1123–1127.
- Xu, H. F., J. Luo, H. P. Wang, H. Wang, T. Y. Zhang, H. B. Tian, D. W. Yao, and J. J. Loor. 2016. Sterol regulatory element binding protein-1 (SREBP-1)c promoter: Characterization and transcriptional regulation by mature SREBP-1 and liver X receptor alpha in goat mammary epithelial cells. *J. Dairy Sci.* 99:1595–1604.
- Yao, D. W., J. Luo, Q. Y. He, H. F. Xu, J. Li, H. B. Shi, H. Wang, Z. Chen, and J. J. Loor. 2016. Liver X receptor α promotes the synthesis of monounsaturated fatty acids in goat mammary epithelial cells via the control of stearyl-coenzyme A desaturase 1 in an SREBP-1-dependent manner. *J. Dairy Sci.* 99:6391–6402.
- Zelcer, N., and P. Tontonoz. 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest.* 116:607–614.