Regulation of Kruppel-like factor 4, 9, and 13 genes and the steroidogenic genes LDLR, StAR, and CYP11A in ovarian granulosa cells

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Natesampillai S, Kerkvliet J, Leung PC, Veldhuis JD. Regulation of Kruppel-like factor 4, 9, and 13 genes and the steroidogenic genes LDLR, StAR, and CYP11A in ovarian granulosa cells. Am J Physiol Endocrinol Metab 294: E385–E391, 2008. First published December 4, 2007; doi:10.1152/ajpendo.00480.2007.—Kruppel-like factors (KLFs) are important Sp1-like eukaryotic transcriptional proteins. The LDLR, StAR, and CYP11A genes exhibit GC-rich Sp1-like sites, which have the potential to bind KLFs in multiprotein complexes. We now report that KLF4, KLF9, and KLF13 transcripts are expressed in and regulate ovarian cells. KLF4 and 13, but not KLF9, mRNA expression was induced and then repressed over time (P < 0.001). Combined LH and IGF-I stimulation increased KLF4 mRNA at 2 h (P < 0.01), whereas LH decreased KLF13 mRNA at 6 h (P < 0.05), and IGF-I reduced KLF13 at 24 h (P < 0.01) compared with untreated control. KLF9 was not regulated by either hormone. Transient transfection of KLF4, KLF9, and KLF13 suppressed LDLR/luc, StAR/luc, and CYP11A/luc by 80–90% (P < 0.001). Histone-deacetylase (HDAC) inhibitors stimulated LDLR/luc five- to sixfold and StAR/luc and CYP11A/luc activity twofold (P < 0.001) and partially reversed suppression by all three KLFs (P < 0.001). Deletion of the zinc finger domain of KLF13 abrogated repression of LDLR/luc. Lentiviral overexpression of the KLF13 gene suppressed LDLR mRNA (P < 0.001) and CYP11A mRNA (P = 0.003) but increased StAR mRNA (P = 0.007). Collectively, these data suggest that KLFs may recruit inhibitory complexes containing HDAC corepressors, thereby repressing LDLR and CYP11A transcription. Conversely, KLF13 may recruit unknown coactivators or stabilize StAR mRNA, thereby explaining enhancement of in situ StAR gene expression. These data introduce new potent gonadal transregulators of genes encoding proteins that mediate sterol uptake and steroid biosynthesis.

sterol; gonad; luteal; low-density lipoprotein receptor; steroidogenic acute regulatory protein; cytochrome P-450 cholesterol side-chain cleavage

OVARIAN STEROIDOGENESIS is critical for the physiological regulation of both reproductive and nonreproductive tissues, such as the uterus, breast, vascular endothelium, adipose tissue, skeleton, and brain (19). De novo synthesis of steroids is directly dependent on the mitochondrial availability of free cholesterol. The primary source of intracellular sterol substrate for steroidogenesis is in the cow, pig, monkey, and human is blood-borne low-density lipoprotein (LDL) cholesterol, which is taken up by cognate membrane receptors (2, 16, 40, 50, 51). Steroidogenic acute regulatory protein (StAR) transports cytoplasmic cholesterol from the outer to the inner mitochondrial membrane, where the cytochrome P-450 cholesterol side-chain cleavage (CYP11A) complex resides. StAR and CYP11A are rate limiting in absolute terms and enzymatically, respectively.

These genes contain several response elements, including steroidalogenic factor 1 (SF-1), Sp1, C/EBP enhancer-binding protein-β (C/EBPβ), cAMP response-element-binding protein (CRE), sterol regulatory element-binding protein-1 (SREBP-1), and GATA-4 sites (24, 27). Regulation of LDL receptor (LDLR) transcription in mammalian cells is also complex, requiring interactions among cholesterol-sensitive SREBP-1 and sequence-specific but generic coregulatory factors like Sp1, CBP, C/EBPβ, YY1, and NF-Y (15). In addition, a recent study disclosed that the Sp1-like protein Krüppel-like factor 13 (KLF13) can repress the proximal LDLR promoter in a sterol-independent manner (28).

Triple Cys2/His2 (C2H2) zinc finger sequences characteristic of the Sp1/KLF superfamily constitute the most common DNA-binding domain in humans, making up ~2% of the genome (7, 42). At least 24 such nuclear proteins, which are involved in a variety of cellular activities, such as cell growth, development, differentiation and apoptosis (23, 41), have been identified in mammals. Sp1/KLF13s bind conservatively to GC- or GT-rich sites containing either a CCGCC or CT/ACC core sequence (7). DNA microarray technology and other exploratory analyses have identified several KLF genes (KLF2, KLF4, KLF5, KLF6, KLF9, KLF13, KLF15, and KLF17) in the mammalian ovary, but their functions in this organ are not known (8, 9, 14, 21, 52). In addition, KLF4 is highly expressed in the testis and acutely regulated by FSH (33), but its role in ovary, which requires interactions among cholesterol-sensitive SREBP-1 and sequence-specific but generic coregulatory factors like Sp1, CBP, C/EBPβ, YY1, and NF-Y (15).

On the basis of the physiological importance of KLFs, the current study had two goals: 1) to assess regulation of KLF gene expression in ovarian granulosa luteal cells by LH and/or IGF-I, and 2) to evaluate control of three key steroidogenic gene promoters: LDLR, StAR, and CYP11A, by the particular KLFs identified in granulosa-luteal cells.

MATERIALS AND METHODS

Reagents. Ovine FSH (NIDDK oFSH-18; potency 65× NIH-oFSH-S1), OLH-26, and human IGF-I were obtained from the National Hormone and Pituitary Program, NIH (Bethesda MD), porcine insulin, trichostatin A, and 17β-estradiol from Sigma Chemical (St. Louis, MO); Eagle’s minimum essential medium (MEM), penicillin-streptomycin, gentamicin, fetal bovine serum (FBS), trypsin-EDTA, and lipofectamine reagent from Life Technologies (Grand Island,

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sodium butyrate from Upstate Cell Signaling Solutions (Lake Placid, NY), and the Luciferase Reporter Assay System from Promega (Madison, WI). Oligonucleotides were synthesized by IDT (Integrated DNA Technologies, Coralville, IA) or by the Advanced Genomics Technology Center, Mayo Clinic, Rochester, MN.

Ovarian granulosa cell culture. Ovaries from prepubertal swine (60–70 kg) were collected at an abattoir and transported to the laboratory in iced saline. Granulosa cells were obtained from small and medium-sized (1–5 mm) antral Graafian follicles by fine-needle aspiration under sterile conditions and washed three times with low-speed centrifugation (1,000 g) in MEM. Approximately 5 × 10⁶ viable cells were plated in 12-well culture dishes (Corning, NY) containing bicarbonate-buffered MEM, 3% FBS, insulin (1 µg/ml), estradiol (0.5 µg/ml), and FSH (5 ng/ml) to permit partial luteinization (52) and attachment for 48 h at 37°C in 5% CO₂. The resultant cells were termed granulosa-luteal cells. The human granulosa cell line SVOG-4o was produced as described previously from cells otherwise discarded at in vitro fertilization and immortalized by SV40 Tag (20).

**Transient transfection.** Transient transfection analyses utilized porcine LDLR/luc (1076 to +11 bp), StAR/luc (1423 to +130 bp), and CYP11A (−2320 to +23 bp) proximal 5′-upstream regulatory sequences driving cytoplasmically targeted firefly luciferase (17, 30, 35). Granulosa-luteal cell monolayers were incubated in serum-free MEM without antibiotics for 20–30 min. Transfection medium (0.5 ml/well) comprised serum-free MEM without antibiotics containing 1 µg of total plasmid DNA and 6 µl of lipofectamine. On the basis of optimizing time course experiments, 5% serum-containing MEM was replaced after 6 h of transfection. After an additional 24 h of recovery to allow expression, cells were exposed to serum-free MEM containing antibiotics and the indicated effectors or vehicle for 24 h. Where indicated, cells were cotransfected with 0.3 µg/well pCDNA3.1/His-C-KLF13 or pCDNA3.1/His-C-KLF9 or pCDNA 3.1/His-C-KLF4 and/or empty control pCMV/His-C vector. Full-length KLF13 expression vector, a COOH-terminal zinc finger-deleted KLF13 construct (−1–172 aa) or a COOH-terminal deleted KLF13 construct retaining only NH₂-terminal aa 1–35 were generated from a pCMV/His-C vector by PCR. Expression vectors containing just the zinc finger motif or the zinc finger combined with any one of three different NH₂-terminal repression domains (1–24 or 55–74 or 75–114 aa) were reported previously (12). To quantify reporter expression, cultures were rinsed once at room temperature with Dulbecco’s phosphate-buffered saline (PBS), lysed in 100 µl of 1× lysis buffer (Luciferase Assay System), and stored at −70°C until later assay. Luciferase activity was measured using 100 µl of firefly luciferin substrate (Promega) and 20 µl of cellular lysate in a Turner TD-20/20 DLR luminometer (Turner Designs, Sunnyvale, CA). Total protein was used to normalize luciferase activity and the indicated multiplicity-of-infection units and incubated overnight in growth medium containing 6 µg/ml polybrene followed by complete medium for 48 h to allow gene and protein expression.

**Real-time quantitative PCR.** Total RNA was isolated using TRIzol reagent and reverse transcribed using the SuperScript III reverse transcriptase kit (Invitrogen) with 2.5 µM oligo(dT)₁₅, 0.2 µM 18S reverse template, and 1 µg of RNA. The cDNA was amplified in 25 µl of PCR buffer with IQ SYBR Green Master Mix (Bio-Rad, Hercules, CA) in the presence of specific primers for KLF4, 9, and 13 as well as for LDLR, StAR, and CYP11A coding sequences (Table 1). The PCR conditions comprised a hot-start by 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Samples were run in duplicate on the MyiQ single color real-time PCR detection system (Bio-Rad) to determine the threshold cycle (Ct) (5). Expression levels were normalized to 18S by the ΔΔCt method (30).

**Statistical methods.** Observations were based on three or more independent experiments conducted using separate batches of 150–200 ovaries. Data from separate experiments were subjected to one- or two-way ANOVA in a repeated-measures design (22). Ratio values (observed to control) were log-transformed to limit the dispersion of residual variance. Means were compared by the post hoc Tukey multiple comparison test at P < 0.05. For mRNA expression data, a one-sided F-ratio test was performed against the null hypothesis of no difference from time-matched control.

**RESULTS**

**KLF mRNA expression in granulosa-luteal cells.** To understand the expression and regulation of ovarian KLFs, gene transcripts were assessed by real-time PCR. Granulosa-luteal cells were exposed to control solvent, LH, and/or IGF-I for 2, 6, and 24 h (n = 4–14 experiments; Fig. 1). In unstimulated cultures, relative abundances of KLF4, 9, and 13 mRNA were

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**Table 1. Real-time PCR primer sequences**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
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<tr>
<td>KLF4</td>
<td>CACTGTCCTCAGCGAGACTACC</td>
<td>CATTGCGGAGGCCGATTTTC</td>
<td>397</td>
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<tr>
<td>KLF9</td>
<td>CGAGGGCGCGGCGACTACCCTG</td>
<td>GCCGTGCTGAAGGAGCTGAC</td>
<td>234</td>
</tr>
<tr>
<td>KLF13</td>
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<td>AACCTCTTCTTCGCGGCTGTT</td>
<td>150</td>
</tr>
<tr>
<td>LDLR</td>
<td>GAGCGAGGAGAACGCGGATGTT</td>
<td>GCCGACTATACGCGGATTT</td>
<td>337</td>
</tr>
<tr>
<td>StAR</td>
<td>GTGTCTCGCGTGAAAGCAC</td>
<td>GGTACGCGGAACCTCAA</td>
<td>393</td>
</tr>
<tr>
<td>CYP11A</td>
<td>CTGGGACCTCTGAGGAGCTGTT</td>
<td>AGCGCATACGCGGATTT</td>
<td>204</td>
</tr>
<tr>
<td>18S</td>
<td>GAGATGCTCTTGAGGAGCTGTT</td>
<td>GGAACTAAGGTTATGCA</td>
<td>315</td>
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</tbody>
</table>
showed that exogenous KLF13, KLF9, and KLF4 decreased KLF9, and KLF13. Statistical analyses (along with cDNAs for full-length coding sequences of KLF4, KLF9 and KLF13 (C) mRNA were quantified using SYBR Green (MATERIALS AND METHODS). Data reverse-transcribed and amplified using gene-specific primers (Table 1). PCR products were quantified using SYBR Green (MATERIALS AND METHODS). Transfected cells were allowed to recover for 24 h (P < 0.05) and by IGF-I at 24 h (P < 0.001; Fig. 2). Exposure to histone deacetylase (HDAC) inhibitors trichostatin A (10 ng/ml) or sodium butyrate (1.0 mM) for 24 h after transfection increased basal LDLR/luc reporter expression four- to fivefold (P < 0.001) and significantly attenuated repression of LDLR/luc activity by each of the three KLFs (P < 0.001).

In steroidogenic tissues, cholesterol is transported from the outer to the inner mitochondrial membrane by StAR protein and converted into pregnenolone by the CYP11A enzyme. Cotransfection of KLF13, KLF9, and KLF4 with StAR or CYP11A promoter fragments repressed luciferase activity by 80–90% compared with basal (in each case P < 0.001; Fig. 2). In addition, StAR/luc and CYP11A/luc were upregulated by TSA or SB treatment approximately twofold over basal (P < 0.01). Thus, each of the three KLFs can repress promoter-reporter constructs of all three of LDLR, StAR, and CYP11A. HDAC inhibitors relieved such repression, suggesting that histone deacetylases contribute to promoter silencing.

To evaluate whether transcriptional repression is consistent, SVOG-4o cells derived from a human granulosa cell line (20) were also studied. Transfection of KLF4, 9, or 13 reduced the expression of LDLR/luc (by >95%) and StAR/luc (by 50%, P < 0.001; Fig. 3). Exposure to SB partially reversed inhibition of LDLR/luc but not StAR/luc. CYP11A/luc did not express well in the immortalized cells.

To assess the structural requirements for gene repression by KLF13, we created a series of deletional pcDNA3.1/His C constructs containing or excluding the NH2-terminal regulatory or COOH-terminal triple zinc finger DNA-binding domains. As a positive control, transfection of CMV-driven full-length LDLR/luc activity by 92, 78, and 85%, respectively (P < 0.001; Fig. 2). Exposure to histone deacetylase (HDAC) inhibitors trichostatin A (10 ng/ml) or sodium butyrate (1.0 mM) for 24 h after transfection increased basal LDLR/luc reporter expression four- to fivefold (P < 0.001) and significantly attenuated repression of LDLR/luc activity by each of the three KLFs (P < 0.001).

KLFs suppress gene expression in ovarian cells. To assess regulation of ovarian steroidogenic genes, porcine granulosa-luteal cells were transiently transfected with the promoter-reporter constructs LDLR/luc (−1076 to +11bp), StAR/luc (−1423 to +130bp), and CYP11A/luc (−2320 to +23 bp) along with cDNAs for full-length coding sequences of KLF4, KLF9, and KLF13. Statistical analyses (n = 8 experiments) showed that exogenous KLF13, KLF9, and KLF4 decreased LDLR/luc expression four- to sixfold compared with untreated control (P < 0.001). KLF4 and KLF9, but not KLF13, mRNA expression differed significantly (but not alone) for 2 h increased KLF4 mRNA expression four- to sixfold compared with untreated control (P < 0.01). KLF9 mRNA expression was unaffected by different experimental stimuli. KLF13 mRNA expression was reduced by LH at 6 h (P < 0.05) and by IGF-I at 24 h (P < 0.01) compared with respective untreated controls obtained at the same time points. Thus, all three KLF genes are expressed in pig granulosa-luteal cells and regulated (KLF13 and KLF4) or not regulated (KLF9) by hormone(s) over time.

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KLF13 suppressed LDLR/luc activity by 80–90%. As a negative control, empty His C vector did not alter basal promoter expression (Fig. 4). Vectors lacking the COOH-terminal zinc finger motif (1–172 aa) or COOH-terminal deleted KLF13 construct retaining only NH2-terminal sequence (1–35 aa) did not repress LDLR/luc. Conversely, expression vectors containing just the zinc finger motif or the latter along with any of three different NH2-terminal repressor domains (1–24 or 55–74 or 75–114 aa) suppressed luciferase activity comparably to wild-type KLF13 (P < 0.001, n = 5 experiments). These observations suggest that the DNA-interacting zinc finger domain is essential for the repressive effects of KLF13 on the LDLR promoter.

Viral transfer of the KLF13 minigene. To ensure high expression efficiency and allow evaluation of native (endogenous) steroidogenic gene expression, a lentivirus expression vector, pSIN-CSGW-UNEm (see MATERIALS AND METHODS), was used to overexpress the full-length coding sequence of porcine KLF13. Granulosa-luteal cells were transduced with various concentrations or multiplicities of infection (5–50 MOI) of KLF13 lentivirus or empty lentivirus and cultured for 3 days to allow gene expression. Overexpression of KLF13 message was confirmed by real-time RT-PCR and of KLF13 protein by Western blot. Transcripts of the putatively targeted genes LDLR (Fig. 5A), StAR (Fig. 5B), and CYP11A (Fig. 5C), were quantified by real-time PCR in a total of 13–19 experiments. Regression analyses showed that increased expression of KLF13 message predicted decreased expression of LDLR mRNA (P < 0.001). A similarly strong negative correlation was found between KLF13 mRNA and CYP11A mRNA (P < 0.003). In contrast, the amounts of KLF13 and StAR mRNA were positively correlated in granulosa-luteal cells (P < 0.007).
DISCUSSION

The present work establishes the following new observations: 1) KLF4 and KLF13 mRNA expression is regulated by LH and/or IGF-I in granulosa-luteal cells; 2) all three KLFs (4, 9, and 13) suppress not only the LDLR promoter but also those of StAR and CYP11A; and 3) lentiviral overexpression of KLF13 downregulates endogenous LDLR and CYP11A mRNA but upregulates StAR mRNA in granulosa-luteal cells.

Activation and repression of selected genes by distinct transcriptional factors play important roles in gonadal function, including in cellular replication, apoptosis, differentiation, and steroid biosynthesis (32). The zinc finger family includes the gonadotropin-inducible transcription factors GIO1 and GIOT2, which are transiently regulated by FSH (26). Other zinc finger proteins, such as GATA4 and GATA6, are also expressed in the ovary and are positively regulated by FSH in granulosa cells (18). Recently, a novel nuclear zinc finger protein called GATA-like protein-1 (GLP1), a critical nuclear repressor, was discovered in somatic cells of developing gonads, including in Leydig and granulosa cells (19). Yan et al. (48) reported on a zinc finger protein, ZFP393, expressed exclusively in the testis and ovary (52), which was later shown to be KLF17. Furthermore, analyses of a rat gene database and DNA microarrays disclosed expressions of KLF4, KLF5, KLF9, KLF10, KLF13, and KLF15 in gonadal tissues (9, 14, 21, 43). These studies did not elucidate any functional roles of KLFs. However, clinical investigations point to dysregulation of KLF2 and KLF4 in the polycystic ovary syndrome (8) and of KLF2 (49) and KLF6 (5) in ovarian tumors. The present study delineates both expression and regulation of KLF4, KLF9, and KLF13 gene transcripts in granulosa-luteal cells.

The accompanying data document regulated expression of KLF4 and KLF13 gene transcripts in ovarian granulosa-luteal cells. KLF9 gene expression did not vary over time and was not responsive to LH or IGF-I stimulation. However, exposure to LH increased the abundance of KLF4 transcripts approximately fivefold. By real-time PCR, KLF4 mRNA concentrations peaked at 2 h and returned to control level after 6 and 24 h. FSH analogously induced KLF4 gene expression in the murine testis within 4 h with recovery to baseline before 24 h (33). In addition, in a fibroblastic cell line (NIH 3T3), KLF4 transcripts were elevated in growth-arrested compared with proliferating cells (36). Northern blot and RT-PCR analyses showed ubiquitous expression of the KLF13 gene in mouse and human tissues and in several cell lines (25, 34, 38). Hormonal regulation of gonadal KLF13 mRNA was inferred from DNA microarray data in which immature rats were treated with human chorionic gonadotropin. The latter increased KLF13 mRNA twofold at 6 h with a return to basal after 12 h (9). In contrast, we observed that a maximally stimulatory concentration of IGF-I (but not LH) induces KLF13 gene transcripts two- to threefold within 2 h in granulosa-luteal cells. However, further studies will be required to understand the full matrix of hormone concentration- and time-dependent regulation of the KLF13 gene in gonadal cells.

An earlier study showed that KLF13 suppresses LDLR promoter expression in pig ovarian cells, which contain immunoreactive Sp1 and Sp3 and an unknown protein-DNA complex (13). Nuclear proteins bound all three Sp1-like 5'-TCCTCC-3' sequences contained in the porcine proximal LDLR promoter (28, 31). Here, we show by transient transfection that each of three KLFs represses a putative proximal promoter fragment of the LDLR, StAR, and CYP11A genes. Viral overexpression studies documented that KLF13 is a strong repressor of endogenous LDLR and CYP11A gene expression in granulosa-luteal cells but is unexpectedly a potent inducer of endogenous StAR gene expression. Mechanistic analyses demonstrated that HDAC inhibitors significantly overcome repression by each of KLF4, 9, and 13 of promoter-reporter constructs for LDLR, StAR, and CYP11A. Moreover, the COOH-terminal triple zinc finger motif of KLF13, rather than its NH2 terminus, provides a critical repressive signal for LDLR expression. The collective data introduce evidence for a novel nuclear transcriptional system that may participate in steroidogenic gene regulation in the ovary.

Several transcriptional factor-binding sequences exist 5'-upstream of the transcriptional start site of the StAR gene (3), including a critical GC-rich motif located at −180/−150 bp that binds Sp3 and mediates repression of murine StAR (4). The pig StAR promoter contains multiple Sp1-like binding sites within the region −170 to −145 bp (5'-CTGCCCTCCCC CCCCCATCCC CGGCC-3'). These elements may participate in StAR regulation (10), given that the segment −219 to −31 bp is hormonally responsive (17, 29). In MA-10 cells, addition of the HDAC inhibitor TSA increased wild-type mouse StAR promoter activity, whereas mutations within the CAGA/Sp3 repressor region (−174 5'-CAGAGTGCTGTTCCCTCCC-3' −157 bp) did the opposite.

Sp1-like sites might also mediate CYP11A gene induction. IGF-I increases CYP11A reporter activity through IGF-I-responsive elements (IGFRE, −130 to −100 bp), which represent GC-rich domains that can bind both Sp1 (44, 46, 47) and poly pyrimidine tract-binding protein (PTB) (45). In this context, the porcine CYP11A promoter exhibits two CCCCTCC-rich sequences located −211 to −190 bp and −130 to −110 bp upstream of the transcriptional start site. However, whether KLF4, 9, or 13 acts via these or other cis element is not yet known.

Many Sp1-like and KLF proteins have similar but not identical affinities for GC-rich motifs (11, 41). Both the particular GC-rich sequence and gene context appear to determine binding and recruitment of KLF and coactivators such as CBP/p300/pCAB (1, 39) or corepressors such as HDAC/mSin3A (4, 28, 53). Preliminary mechanistic analysis presented here indicates that, at least in the case of KLF13, the COOH-terminal triple zinc finger domain is needed to mediate transrepression of an LDLR promoter fragment in granulosa-luteal cells. Whether the same requirement is true for KLF4 and KLF9 is or relevant to other steroid-regulated or steroidogenic genes targeted by KLFs has not been defined.

In summary, potent Sp1-like transcriptional regulators KLF4, KLF9, and KLF13 are expressed in ovarian granulosa cells. Expression of the KLF4 and KLF13 transcripts is hormonally regulated, and all three KLFs can repress LDLR, StAR, and CYP11A hybrid promoter-reporter constructs. In addition, KLF13 decreases native gene transcripts for LDLR and CYP11A while increasing transcripts for StAR. Repression is antagonized by HDAC inhibitors, suggesting a role for histone deacetylation in gene silencing by these KLFs.
REFERENCES


