Nuclear Receptor 5A (NR5A) Family Regulates *5-Aminolevulinic Acid Synthase 1* (*ALAS1*) Gene Expression in Steroidogenic Cells

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5-Aminolevulinic acid synthase 1 (ALAS1) is a rate-limiting enzyme for heme biosynthesis in mammals. Heme is essential for the catalytic activities of P450 enzymes including steroid metabolic enzymes. Nuclear receptor 5A (NR5A) family proteins, steroidogenic factor-1 (SF-1), and liver receptor homolog-1 (LRH-1) play pivotal roles in regulation of steroidogenic enzymes. Recently, we showed that expression of SF-1/LRH-1 induces differentiation of mesenchymal stem cells into steroidogenic cells. In this study, genome-wide analysis revealed that *ALAS1* was a novel SF-1-target gene in differentiated mesenchymal stem cells. Chromatin immunoprecipitation and reporter assays revealed that SF-1/LRH-1 up-regulated *ALAS1* gene transcription in steroidogenic cells via binding to a 3.5-kb upstream region of *ALAS1*. The *ALAS1* gene was up-regulated by overexpression of SF-1/LRH-1 in steroidogenic cells and down-regulated by knockdown of SF-1 in these cells. Peroxisome proliferator-activated receptor-γ coactivator-1α, a coactivator of nuclear receptors, also strongly coactivated expression of NR5A-target genes. Reporter analysis revealed that peroxisome proliferator-activated receptor-γ coactivator-1α strongly augmented *ALAS1* gene transcription caused by SF-1 binding to the 3.5-kb upstream region. Finally knockdown of ALAS1 resulted in reduced progesterone production by steroidogenic cells. These results indicate that ALAS1 is a novel NR5A-target gene and participates in steroid hormone production. **(*Endocrinology* 153: 5522–5534, 2012)**

Orphan nuclear receptor steroidogenic factor 1 [SF-1, also known as Ad4BP, encoded by the nuclear receptor 5A1 (*NR5A1*) gene] plays a pivotal role in regulation of reproductive and endocrine functions at multiple levels during adrenal and gonadal development and differentiation, including regulation of steroidogenesis-related gene expression (1–3). Previously, we reported that bone marrow-derived mesenchymal stem cells (MSCs) are differentiated into steroidogenic cells by expression of SF-1 and treatment with 8-bromo-cAMP (8Br-cAMP) (4). Expression of SF-1 in MSCs induces the expression of steroidogenesis-related genes including steroidogenic acute regulatory protein (StAR) and various steroidogenic enzymes (4). In addition to these genes, we attempted to identify novel SF-1-target genes during differentiation into steroidogenic cells by genome-wide analyses, DNA microarray,

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Abbreviations: ALAS1, 5-Aminolevulinic acid synthase 1; ALAS2, 5-aminolevulinic acid synthase 2; APOA1, apolipoprotein A1; 8Br-cAMP, 8-bromo-cAMP; ChIP, chromatin immunoprecipitation; FOXO1, forkhead box protein O1; GFP, green fluorescent protein; HSD, hydroxysteroid dehydrogenase; LRH-1, liver receptor homolog-1; MOI, multiplicity of infection; MSCs, mesenchymal stem cells; NR5A, nuclear receptor subfamily 5 group A; NRF-1, nuclear respiratory factor-1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; POR, P450 oxidoreductase; SF-1, steroidogenic factor-1; siSF-1, SF-1-specific siRNA; siRNA, small interfering RNA; shRNA, small hairpin RNA; StAR, steroidogenic acute regulatory protein.

and a promoter tiling array. Using these analyses, we identified 14 SF-1-target genes during MSC differentiation. In this study, we focused on one SF-1 target gene, namely *5-aminolevulinic acid synthase 1* (*ALAS1*), an initiating and rate-limiting enzyme for mammalian heme biosynthesis (5).

ALAS catalyzes a reaction between glycine and succinyl-coenzyme A to form 5-aminolevulinic acid. In humans, ALAS enzymes are encoded by two genes: *ALAS1*, a ubiquitously expressed gene mapped to chromosomal band 3p21, and *5-aminolevulinic acid synthase 2* (*ALAS2*), a erythroid-specific gene mapped to the X chromosome (6). *ALAS1* participates in heme synthesis for all cytochrome family proteins in various tissues, including P450 enzymes in steroidogenic pathways. Heme is essential for the catalytic activities of P450 enzymes, and therefore *ALAS1* may be involved in steroid metabolism. For example, heme affects corticosterone and aldosterone synthesis in the rat adrenal gland (7) and stimulates P450 aromatase activity in human trophoblast cells (8), suggesting that *ALAS1* plays an important role in steroid hormone biosynthesis.

Regulation of *ALAS1* gene expression has been studied mainly in the liver, partly because of abundant expression of P450 drug-metabolizing enzymes in the tissue. In the mouse liver, binding sites for an insulin-sensitive forkhead box protein O1 (FOXO1) and nuclear respiratory factor-1 (NRF-1) within the promoter region of *ALAS1* have been shown to be involved in the induction of *ALAS1* gene expression by fasting (9). Upon exposure to drugs that induce the gene expression of cytochrome P450 and other drug-metabolizing enzymes, *ALAS1* is transcriptionally up-regulated via two enhancer elements at 20 and 16 kb upstream of the transcription start site, resulting in an increase of the biosynthetic rate of heme by cytochrome P450 in the liver (10). However, transcriptional regulation of *ALAS1* in steroidogenic tissues has not been well characterized.

Liver receptor homolog 1 (LRH-1) is another member of the NR5A nuclear receptor family and binds to the same or very similar DNA elements as those for SF-1 binding (11–13). LRH-1 is a transcription factor that mainly participates in the homeostasis of bile acid production in the liver (14, 15). Recently, LRH-1 was found to be active in the ovary as a regulator of steroidogenesis (16). Conditional knockout of LRH-1 in the mouse ovary results in infertility due to failure of ovulation (17). We also have shown that both LRH-1 and SF-1 induce similar changes in the chromatin structure near the *StAR* gene in steroidogenic tissues, particularly in the ovary, to regulate steroid hormone production (18). However, it is unclear whether LRH-1 can activate *ALAS1* gene expression in the ovary to regulate steroidogenesis.

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional coactivator of nuclear receptors and some other transcription factors (19). It regulates adaptive thermogenesis in brown adipose tissues (20), fiber type switching in skeletal muscle (21), β-oxidation of fatty acids (22), and gluconeogenesis (23, 24) in the liver. PGC-1α is also a coactivator of NRF-1 and FOXO1, both of which directly bind to the *ALAS1* promoter, and is an important factor for controlling *ALAS1* gene expression in the liver (9). Recently, we found that PGC-1α is expressed in the ovary and acts as a coactivator of both SF-1 and LRH-1 to transactivate NR5A-target genes (25). However, it is unclear whether PGC-1α acts on the *ALAS1* gene in a coordinated fashion with SF-1 and LRH-1 in steroidogenic tissues.

In this study, we investigate the regulation of *ALAS1* gene expression in steroidogenic cells by examining the upstream region of *ALAS1* using differentiated MSCs and KGN cells (a human granulosa tumor cell line) to reveal the importance of SF-1-binding sites at 3.5 kb upstream of the transcription start site. We will show that LRH-1 and SF-1 play important roles in *ALAS1* gene expression, and PGC-1α is involved in the transactivation of *ALAS1* gene expression in a coordinated fashion with SF-1 and LRH-1. Finally, we will show the involvement of *ALAS1* in progesterone production by steroidogenic cells.

Materials and Methods

Reagents

8Br-cAMP and a GenomePlex Complete Whole Genome Amplification Kit were purchased from Sigma (St. Louis, MO). A Human/Promoter Tiling Array Deluxe was purchased from Roche NimbleGen (Madison, WI; catalog no. C7291-00-02). A Human oligonucleotide glass array (Human Genome U133 Plus 2.0 Array) was purchased from Affymetrix (Santa Clara, CA). ν-serum IV was purchased from BD Biosciences (Franklin Lakes, NJ). A dual luciferase reporter assay system, pGL4.11-basic, pGL4.24 containing a minimal promoter, pRL-CMV, and pGL4.74 (hRluc/TK) vectors were purchased from Promega Corp. (Madison, WI). Trizol regent, SuperScript III reverse transcriptase, Opti-MEM, a BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP, Power SYBR Green PCR Master Mix, murine SF-1-specific siRNA (siSF-1) (Nr5a1MSS240944), Lipofectamine RNAiMAX, Lipofectamine, and Lipofectamine Plus reagents were purchased from Life Technologies (Carlsbad, CA). ExTaq Hot Start Version was purchased from Takara Bio (Otsu, Japan). A human siSF-1 (sc-37901) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human siALAS1 (MQ-009276-00-0002), mouse siALAS1 (M-046807–01-0005), and siGENOME Non-Targeting small interfering RNA (siRNA) no.1 (D-001210-01-20) were purchased from

Thermo Scientific (Waltham, MA). A QuickChange site-directed mutagenesis kit was purchased from Agilent Technologies (Santa Clara, CA). KOD-Plus, KOD-FX, and pTA2 vectors were purchased from Toyobo (Osaka, Japan). Enhanced chemiluminescence Western blot reagents and HistoVT One were purchased from Nacalai Tesque (Kyoto, Japan). A Vectastain Elite ABC kit was purchased from Vector Laboratories (Burlingame, CA). A Progesterone EIA Kit (EA74) was purchased from Oxford Biomedical Research (Oxford, MI). All other regents were obtained from commonly used suppliers.

Antibodies

Dynabeads protein G and M-280 sheep antimouse IgG were purchased from Life Technologies. Anti-FLAG M2 agarose (A2220), as well as anti-FLAG M2 (F1804) and anti-Flag M2-HRP (A8592) antibodies were purchased from Sigma. Anti-Myc tag (ab9132) and anti-*ALAS1* (ab22153) (for Western blotting) antibodies were purchased from Abcam (Cambridge, MA). An anti-*ALAS1* (LS-B2013) for immunohistochemistry was purchased from LifeSpan Bioscience (Seattle, WA). An anti-Ad4BP/SF-1 for immunohistochemistry was kindly given by Dr. K Morohashi and that for Western blotting was purchased from Millipore Corp. (07-618) (Billerica, MA). Anti-Ku (p80) (MS-285-P1) and anti-Ku (p70) (MS-329-P1) antibodies were purchased from Thermo Scientific (Rockford, IL). Antirabbit IgG, goat, F(ab′)2, peroxidase conjugated (013–17941), and antimouse IgG, goat, F(ab′)2, peroxidase conjugated (019-17921) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture, transient transfection, luciferase assay, and progesterone production

Human MSC (hMSC) lines, hMSC-hTERT-E6/E7 (26) and UE7T-13 (27), Phoenix cells, and the human granulosa tumor cell line KGN (28) were cultured as described elsewhere. HeLa cells, HepG2 cells, and Y1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and gentamycin. The human adrenocortical tumor cell line H295R (29) was maintained in Opti-MEM supplemented with 2% ν-serum IV and gentamycin. H295R cells stably expressing small hairpin RNA (shRNA) were maintained in Opti-MEM supplemented with 2% ν-serum IV, blasticidin, and gentamycin. HeLa and KGN cells were transfected using Lipofectamine and Lipofectamine Plus reagents according to the manufacturer's instructions. Luciferase assays were performed as described elsewhere (30). Each data point represented the mean of at least four independent experiments.

For knockdown of *ALAS1* gene, *ALAS1*-targeting siRNA, or nontargeting siRNA were transfected into KGN (5×10^4 cells) or Y1 cells (2×10^5 cells) in a 24-well plate with Lipofectamine RNAiMAX. The final siRNA concentration in the medium was 10 nM. After transfection, KGN cells were treated with or without 8Br-cAMP for 48 h. The medium was replaced every 24 h and was collected for progesterone measurement at the end of the culture. Progesterone concentrations in medium were measured by a Progesterone EIA Kit according to the manufacturer's instructions.

For knockdown of *SF-1* gene, SF-1-targeting siRNA or nontargeting siRNA were transfected into Y1 cells with Lipofectamine RNAiMAX. The final siRNA concentration in medium was 10 nM. The cells were treated again 72 h after transduction with the siRNAs and further cultured for 72 h (total 144 h). The cells were collected for measurement of ALAS1 expression by RT-quantitative PCR and Western blotting at the end of the culture.

SF-1-targeting shRNAs were generated according to the manufacturer's instructions. Plasmids for SF-1-targeting and control shRNAs were linearized with *Apa*LI and then transfected into H295R cells in a six-well plate. Transfected H295R cells were cultured for 48 h and then selected by blasticidin to generate stably transfected cell lines. Two independent cell lines were generated from each transfectant. Primers used for generating shRNA plasmids are shown in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Adenovirus and retrovirus preparation and infection

Preparation of adenoviruses expressing Adx-MycSF-1, Adx-MycLRH-1, and Adx-FlagPGC-1α has been described elsewhere (18, 25). Cells were infected 1 d after plating with adenoviruses expressing green fluorescent protein (GFP) (Adx-GFP), MycSF-1 (Adx-MycSF-1), MycLRH-1 (Adx-MycLRH-1), or FlagPGC-1α (Adx-FlagPGC-1α) and then incubated for 48 h. Retrovirus preparation and infection was performed as described elsewhere (25). MSCs were infected with a retrovirus expressing FlagSF-1 for 48 h and then selected by puromycin to generate stably transfected cell lines. A cell line was chosen from about 20 cell lines to perform chromatin immunoprecipitation (ChIP)-on-chip, ChIP, and gene expression analyses.

ChIP-on-chip analysis

ChIP-on-chip analysis was performed as described elsewhere (18). Briefly, hMSC-hTERT-E6/E7 cells that were stably expressing Flag-tagged SF-1 (1×10^8 cells) were used to prepare samples for ChIP. Immunoprecipitation with anti-FLAG M2 affinity gel was performed overnight at 4 C with rotation. After washing, reverse cross-linking, and recovering DNA, a whole-genome amplification method was used to amplify sufficient amounts of DNA for hybridization to the microarray. Labeling of samples, DNA hybridization, and scanning of the arrays were performed by NimbleGen. The microarray experiment was performed with a Human/Promoter Tiling Array. Log_2 ratios were computed for a sample pair and, after Tukey biweight normalization, visualized by SignalMap software (Roche NimbleGen).

Microarray

Expression analysis by DNA microarray has been described elsewhere (31, 32). Briefly, labeled cRNA was prepared from UE7T-13 cells with adenovirus-mediated expression of GFP (Adx-GFP) or MycSF-1 (Adx-MycSF-1). After fragmentation of cRNA, hybridization was performed with a human U133 Plus 2.0 Affymetrix GeneChip (Santa Clara, CA).

Plasmids

pCMV/Flag- and pCMV/Myc-tagged SF-1 expression vectors (pCMV/FlagSF-1 and pCMV/MycSF-1, respectively), the PGC-1α expression vector, and retroviral plasmid for expressing Flag-tagged SF-1 (pQCXIP/FlagSF-1) have been described elsewhere (25, 33). Human *ALAS1* upstream regions consisting of

various 5′-ends cloned into the pGL4.11 luciferase basic vector were generated as follows. The 5′-flanking regions (−2981/+106) were amplified by PCR using KOD-FX and genomic DNA from KGN cells as a template, and the PCR product was ligated into pTA2. To create a *Sal*I site, the identical region was reamplified using KOD-Plus and pTA2/hALAS1 (−2981/+106) as a template. The PCR product was digested with *Sal*I/*Hind*III and ligated into *Xho*I/*Hin*dIII-digested pGL4.11, which was designated as pGL4/hALAS1 (−2981/+106). The 5′-flanking regions (−3573/+39) were amplified by PCR using KOD-FX and genomic DNA from KGN cells as a template, and PCR products were ligated into pTA2. A *Xba*I/*Bgl*II (−3573/+39) fragment from pTA2/hALAS1 (−3573/+39) was ligated into *Nhe*I/*Bgl*II-digested pGL4/hALAS1 (−2981/+106), which was designated as pGL4/hALAS1 (−3573/+106). The 5′-flanking regions (−732/+39) were amplified by PCR with KOD-Plus and pGL4/hALAS1 (−2981/+106) as a template. The PCR product was digested with *Hin*dIII and ligated into *Eco*ICRI/*Hin*dIII-digested pGL4.11, which was designated as pGL4/hALAS1 (−732/+106). The region at −3573/−3419 was amplified by PCR with KOD-plus and pGL4/hALAS1 (−3573/+106) as a template and digested with *Acc*65I/*Xho*I. The *Acc*65I/*Xho*I fragment was ligated into *Acc*65I/*Xho*I-digested pGL4.24 (a reporter vector with minimal promoter activity), which was designated as pGL4.24/hALAS1 (−3573/−3419). Mutations of SF-1-binding sites in the *ALAS1* promoter (−3573/+106) or the region at −3573/−3419 were created using QuikChange site-directed mutagenesis. Nucleotide numbering was relative to the transcription start site of the human *ALAS1* gene. The nucleotide sequences of all PCR products were confirmed by DNA sequencing. Primers used for plasmid construction are shown in Supplemental Table 2.

ChIP

ChIP samples were prepared as described elsewhere (18). Each sample was analyzed by a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Primers used for ChIP are shown in Supplemental Table 3.

Real-time RT-PCR and RT-PCR

Real-time RT-PCR (4) and RT-PCR (34) were performed as described elsewhere. Primers used for real-time RT-PCR and RT-PCR are shown in Supplemental Tables 4 and 5, respectively.

Western blotting

Western blotting was performed as described elsewhere (4). Briefly, cells in a 35- or 60-mm dish were lysed with 200–300 μl radioimmunoprecipitation assay buffer. Aliquots of the lysates containing same amounts (20–40 μg) of proteins were resolved on 10% or 12.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Western blot analysis was performed with antibodies described above. All immune complexes were ultimately visualized and quantitated using enhanced chemiluminescence Western blot reagents and a LAS4000 mini (GE Healthcare Japan, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed as described previously (25, 33). Briefly, male rat adrenals (6 wk of age) were fixed in 4% paraformaldehyde solution, dehydrated in a graded ethanol series, and embedded in paraffin wax. Section of 7 μm thickness were subjected to an antigen-retrieval technique with HistoVT One and treated with normal rabbit IgG, anti-*ALAS1*, or anti-Ad4BP/SF-1 antibodies. They were developed using a Vectastain Elite ABC kit.

Statistical analysis

Values are the mean ± SE of the mean. Data were analyzed by Student's *t* test.

Results

Recently, we showed that SF-1/LRH-1 expression induces differentiation of MSCs into steroidogenic cells (4, 33). In this study, we attempted to identify novel SF-1-target

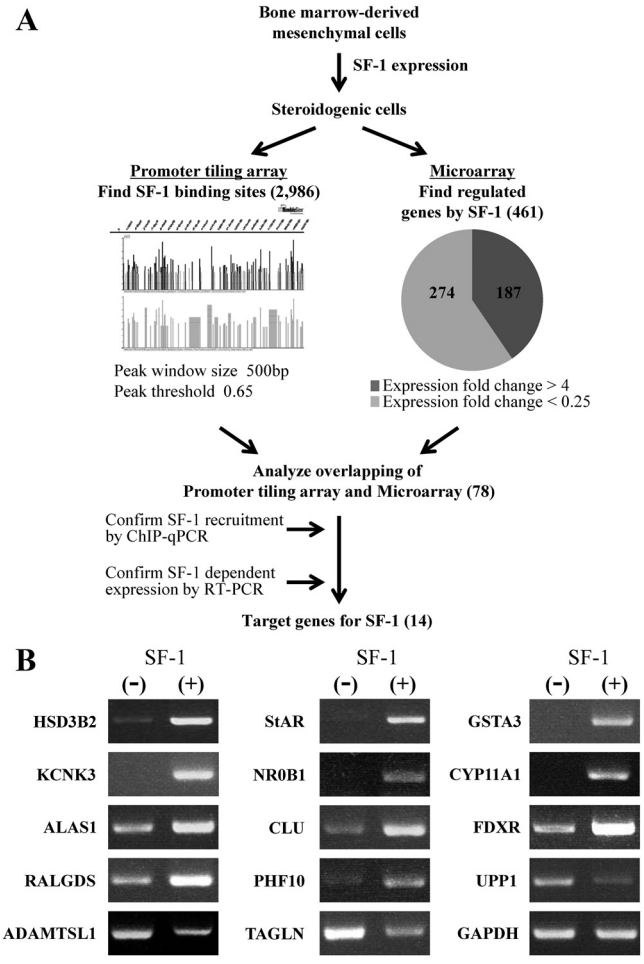

FIG. 1. Identification of SF-1-target genes by DNA microarray and ChIP-on-chip analysis. A, Scheme for identification of SF-1-target genes. Candidate SF-1-target genes were identified by a promoter tiling array (2986 genes) and DNA microarray (461 genes) using hMSCs with or without expression of SF-1. Overlapping candidate genes (78 genes) were further verified by conventional ChIP and RT-PCR analyses, and 14 genes were finally identified as SF-1-target genes. B, Expression of SF-1-target genes in hMSCs by adenovirus-mediated expression of SF-1. Human MSCs were infected with adenoviruses expressing GFP (−) or SF-1 (+). Expression levels of the 14 genes and control *GAPDH* gene were examined by RT-PCR at 2 d after infection.

TABLE 1. List of SF-1-target genes as determined by DNA microarray and ChIP-on-chip analysis

Gene symbol	Peak score	Peak location (bp)	Fold change	Accession no.	Description
HSD3B2	0.75	390	319.5	NM_000198	Hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 2
STAR	0.76	58, −141	289.7	NM_001007243	Steroidogenic acute regulator
GSTA3	0.94	10, 31, 44	77.0	NM_000847	Glutathione *S*-transferase A3
KCNK3	1.02, 0.63	1142, −472	43.4	NM_002246	Potassium channel, subfamily K, member 3
NR0B1	0.69, 0.66	−944, −3094	16.2	NM_000475	Nuclear receptor subfamily 0, group B, member 1
CYP11A1	1.06, 0.77	−854, −1922	13.9	NM_000781	Cytochrome P450, family 11, subfamily A, polypeptide 1
ALAS1	0.69, 0.63	−3243, −3271, −3328	11.7	NM_199166	Aminolevulinate, δ-, synthase 1
CLU	0.59, 0.92	910, −2283	7.6	NM_001831	Clusterin
FDXR	1.44	1101, 1102, 1104	6.1	NM_024417	Ferredoxin reductase
RALGDS	0.74, 0.61	−3562, −3627, −3645	5.1	NM_006266	Ral guanine nucleotide dissociation stimulator
PHF10	0.7	−1617, −1710	4.1	NM_018288	PHD finger protein 10
UPP1	0.67	−3253, −3367, −3578, −3846, −3985	0.24	NM_003364	Uridine phosphorylase 1
ADAMTSL1	0.8	−607	0.24	NM_052866	ADAMTS-like 1
TAGLN	1.09	−3911, −3968, −3981	0.08	NM_003186	Transgelin

genes in differentiated cells by genome-wide analyses, a promoter tiling array (ChIP-on-chip), and DNA microarray. Seventy eight genes were double positive among 2986 and 461 positive genes identified by the ChIP-on-chip assay and the expression array, respectively (Fig. 1A). Those genes were individually verified by a conventional ChIP assay and RT-PCR (Fig. 1B and Table 1), and 14 genes were finally identified as SF-1-target genes. In addition to well-known steroidogenesis-related genes such as hydroxysteroid dehydrogenase 3B2 (*HSD3B2*), *StAR*, *NROB1*, and *CYP11A1*, we found that *ALAS1*, *CLU*, *GSTA3*, *KCNK3*, *FDXR*, *PHF10*, and *RALGDS* genes were upregulated, whereas *ADAMTSL1*, *TAGLN*, and *UPP1* genes were down-regulated (Fig. 1B).

Among the 14 genes, *ALAS1*, a rate-limiting enzyme for heme biosynthesis, was chosen to examine the regulation of gene expression and to reveal the participation of the gene in steroidogenesis.

First, SF-1-binding *in vivo* was examined by a conventional ChIP assay of the upstream region of the *ALAS1* gene in SF-1-transduced hMSCs. As shown in Fig. 2A, SF-1 bound to −3.5 kb upstream of the transcriptional start site of *ALAS1*. The figure also shows positive (STAR −158) and negative (STAR +6000) controls of the ChIP assay. Similar results were obtained using SF-1-transduced KGN cells derived from a human granulosa tumor (Supplemental Fig. 1).

To examine the effects of the upstream region on the transcriptional activity of *ALAS1*, a luciferase reporter assay was performed using constructs harboring various lengths of the upstream region of the *ALAS1* gene (Fig. 2B). Inconsistent with the fact that the *ALAS1* gene is expressed in most tissues, basal activities were high in HeLa cells, which do not express SF-1, among all deletion constructs (*white bar*). Expression of SF-1 caused a marked increase in activity only with the construct containing −3573/+106 ($P < 0.01$), suggesting that two putative SF-1-binding sites near −3.5 kb upstream were responsible for the SF-1-dependent activity of *ALAS1* gene expression.

Next, we examined the two putative SF-1-binding sites for *ALAS1* gene transcription by mutation analysis. One construct contained short DNA fragments (−3573/−3419) with a minimal promoter fragment, and another contained long fragments (−3573/+106) without a promoter fragment. Both constructs showed SF-1-dependent activation of the *ALAS1* gene. Mutation analysis (Fig. 2, C and D) revealed that each SF-1-binding site was important for SF-1-dependent activation of *ALAS1* gene transcription.

SF-1-dependent activation of the *ALAS1* gene was further examined using SF-1-transduced and -knockdown cells. Adenovirus mediated transiently expression of SF-1 in hMSCs (Fig. 3, A and B) and retrovirus mediated stably expression in other hMSCs (Fig. 3, C and 3D) revealed that the SF-1-dependent induction of *ALAS1* gene expression occurred in a similar manner to that of the *StAR* gene, a well-known SF-1-target gene. Dose-dependent activation was also observed by transduction of SF-1 into KGN cells that are of a human ovarian granulosa origin (Fig. 3, E and F). Adenovirus-mediated transduction of SF-1 into H295R human adrenocortical cells also resulted in activation of *ALAS1* gene expression (Fig. 3G). On the other hand, suppression of

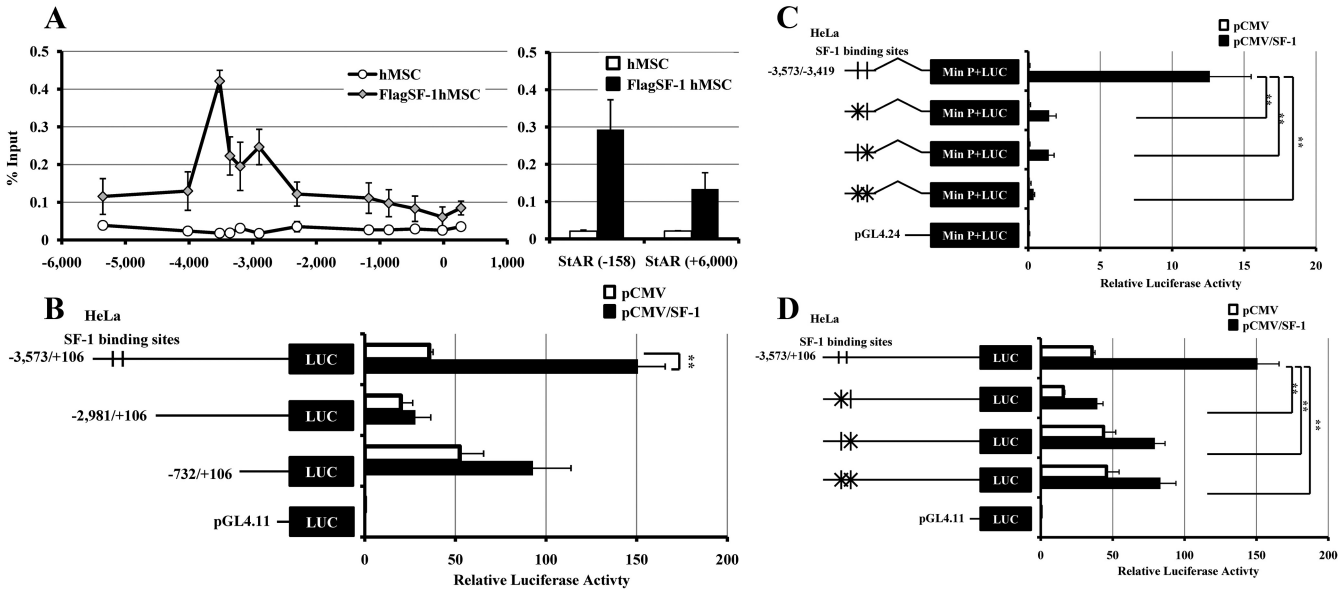

FIG. 2. Identification of SF-1-binding sites within the upstream region of the *ALAS1* gene and their roles in *ALAS1* gene transcription. A, ChIP analysis of SF-1 binding to the region upstream of the human *ALAS1* gene. Flag-tagged SF-1 was expressed in hMSC-hTERT-E6/E7 cells, a hMSC line, by retroviral infection, and an anti-Flag antibody was used to detect SF-1 binding to the region. Parent cells, hMSC-hTERT-E6/E7, were used as negative control for immunoprecipitation with anti-Flag antibody. A *StAR* promoter region (StAR −158) and downstream region (StAR +6000) were used as positive and negative controls, respectively. B, Luciferase reporter assay of the upstream region of the *ALAS1* gene. Reporter constructs containing DNA fragments of various lengths from the upstream region of the *ALAS1* gene were cotransfected with a control vector (pCMV) or Flag-tagged SF-1 expression vector (pCMV/SF-1) into HeLa cells. Reporter deletion constructs are schematically drawn at the *left side of the figure*. Luciferase activities were measured at 48 h after transfection. **, $P < 0.01$ *vs.* control pCMV. C, Effects of mutations in putative SF-1-binding sites −3.5 kb upstream of the *ALAS1* gene on enhancement of luciferase activity of a reporter vector harboring a minimal promoter. Single or double mutations were introduced in two putative SF-1-binding sites in the upstream DNA fragment (−3573/−3419) that was fused to a minimal promoter, and reporter activities were measured by a luciferase assay using HeLa cells with or without expression of SF-1. Mutations in each or both SF-1 sites markedly decrease promoter activity in SF-1-transduced HeLa cells. **, $P < 0.01$ *vs.* wild-type construct. D, Effects of mutations in putative SF-1-binding sites in the upstream region of the *ALAS1* gene on luciferase activity. Single or double mutations were introduced in the SF-1 sites of a −3573/+106 DNA fragment, and reporter activities were measured by a luciferase assay using HeLa cells with or without expression of SF-1. Mutations in each or both SF-1 sites result in decreased luciferase activity in SF-1-transduced HeLa cells. **, $P < 0.01$ *vs.* wild-type construct. LUC, Luciferase; Min, minimal.

endogenous SF-1 gene expression by SF-1-specific short-hairpin RNA or siSF-1 resulted in decreases of both *ALAS1* and *StAR* gene expression in H295R cells (Fig. 3, H and I) or in Y1 cells (Fig. 3, J and K), respectively. Similar results were observed when using KGN cells (Supplemental Fig. 3). Protein levels of *ALAS1* were also declined by SF-1 knockdown both in H295R cells (Fig. 3L) and Y1 cells (Fig. 3M). Immunohistochemical studies showed that *ALAS1* was predominantly expressed in cytoplasm of adrenocortical cells where SF-1 was also expressed but in nucleus of the cells (Fig. 3, N and O). These observations indicate that, although *ALAS1* is expressed in various tissues or organs, it is also a target gene of SF-1 in steroidogenic cells.

There are two members of the NR5A nuclear receptor family, SF-1 and LRH-1 (35). They regulate gene expression via binding to similar response elements and are simultaneously active in steroidogenic tissues, particularly in the ovary, to regulate steroid hormone production (25). LRH-1, but not SF-1, is also a known transcription factor that regulates bile acid production in the liver (14, 15). Therefore, we examined the effects of LRH-1 on *ALAS1* expression in both KGN cells, of an ovarian origin, and HepG2 cells, of a liver origin. As shown in Fig. 4, A and B, LRH-1 strongly bound to −3.5 kb upstream of the *ALAS1* gene in KGN cells, whereas no specific binding of LRH-1 to the corresponding region was observed in HepG2 cells. In HepG2 cells, LRH-1 bound to the promoter region of apolipoprotein A1 (*APOA1*) that is a known target gene of LRH-1 in hepatic cells (Fig. 4B) (36). The difference in LRH-1 binding between KGN and HepG2 cells may reflect a difference in chromatin structure near −3.5 kb upstream of the *ALAS1* gene between these cell types. Therefore we next examined histone modifications in both KGN cells and HepG2 cells. The levels of H3K4me2 (binding sites of certain DNA-binding proteins) (37) and H3Ac (positive marks of transcription) were increased by transduction of LRH-1 into KGN cells, but not HepG2 cells, to this region (Supplemental Fig. 4). Inconsistent with the above observation, transduction of LRH-1 into KGN cells induced *ALAS1* gene expression in a dose-dependent manner (Fig. 4C), whereas no such induction was observed by transduction of LRH-1 into HepG2 cells (Fig. 4D). Well-

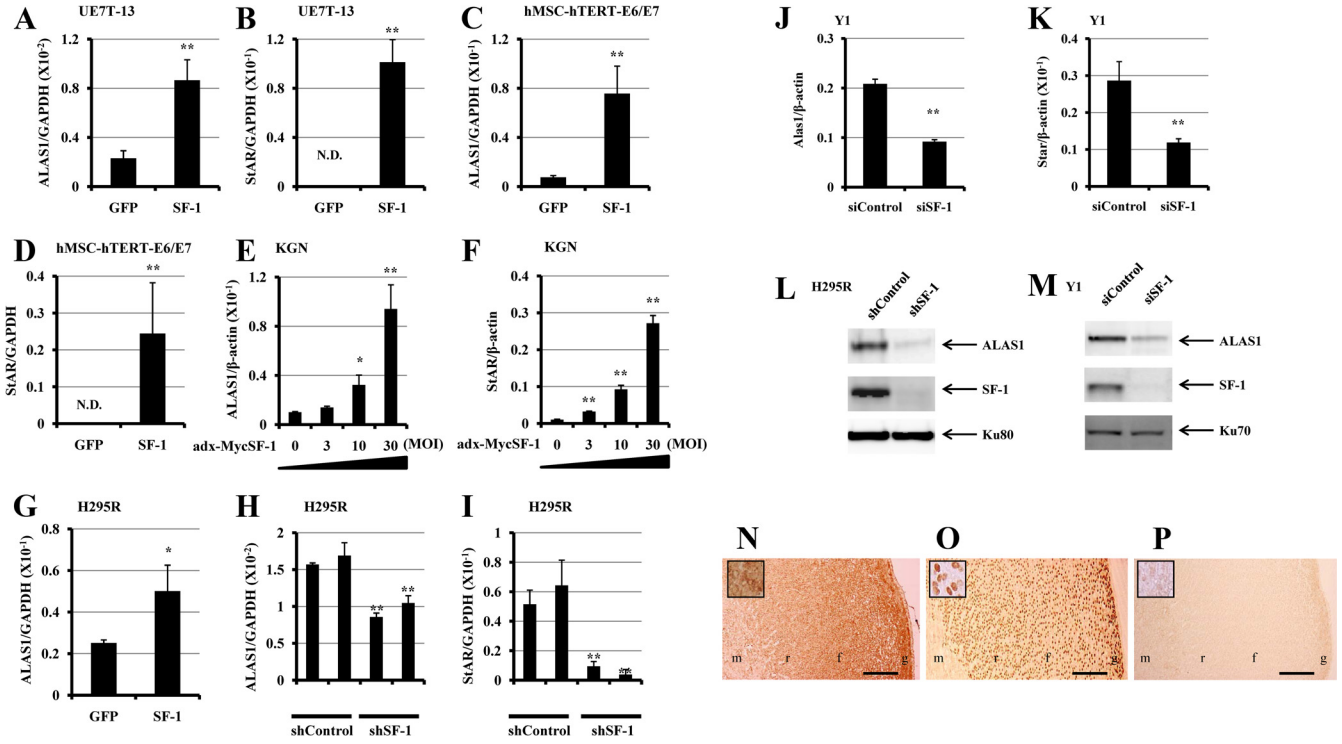

FIG. 3. Involvement of SF-1 in transcription of the *ALAS1* gene. hMSCs (UE7T-13) were infected with adenoviruses to transiently express GFP (control) or SF-1, and the gene expression of *ALAS1* (A) and *StAR* (B) was examined at 48 h after infection. The effects of retrovirus-mediated stable expression of GFP (control) and SF-1 on *ALAS1* (C) and *StAR* (D) gene expression were also examined in hMSC-hTERT-E6/E7 cells. Dose-dependent effects of adenovirus-mediated expression of SF-1 on *ALAS1* (E) and *StAR* (F) were examined in KGN cells, a tumor cell line derived from human ovarian granulosa cells. The effects of adenovirus-mediated expression of SF-1 were also examined using H295R cells, a human adrenocortical cell line (G). The effects of knockdown of endogenous SF-1 in H295R cells on *ALAS1* (H) and *StAR* (I) gene expression were examined by stably expressing an shRNA against SF-1 (shSF-1). The effects of knockdown of endogenous SF-1 in Y1 cells on *ALAS1* (J) and *StAR* (K) gene expression were examined by transiently expressing an siRNA against SF-1 (siSF-1). The gene expression of *ALAS1* and *StAR* was measured by real-time RT-PCR, and mRNA levels were normalized against *β-actin* or *GAPDH*. The effects of knockdown of endogenous SF-1 in H295R cells (L) or Y1 cells (M) on protein levels of *ALAS1* were examined. Localization of *ALAS1* (N) and SF-1 (O) proteins in the rat adrenal gland was examined. Negative control using a nonimmune IgG (P) was also shown. The *insets* showed higher magnification of zona glomerulosa. m, Adrenal medulla; r, zona reticularis; f, zona fasciculate; g, zona glomerulosa. *Scale bar*, 100 μm. N.D., Not detected. *, $P < 0.05$ and **, $P < 0.01$ *vs.* control. Note: protein expression of SF-1 in these cells is shown by Western blotting in Supplemental Fig. 2.

known LRH-1-target genes (*StAR* in steroidogenic cells and *APOA1* in hepatic cells) showed induced expression by LRH-1 in corresponding cell types (Fig. 4, C and D). These observations indicate that cell type-specific induction of the *ALAS1* gene in KGN cells was dependent on LRH-1 binding to the −3.5 kb upstream region of the *ALAS1* gene. We previously found that PGC-1α works together with SF-1 and LRH-1 as a coactivator, which markedly enhances the expression of target genes of the NR5A family (25). In this study, we examined whether PGC-1α enhanced the expression of *ALAS1* in steroidogenic cells endogenously expressing the NR5A family.

Transduction of PGC-1α into KGN cells (Fig. 5A) and HepG2 cells (Fig. 5B) markedly increased the expression of *ALAS1* in a dose-dependent manner. PGC-1α transduction also increased expression of the *StAR* gene in KGN cells (Fig. 5A) and the *APOA1* gene in HepG2 cells (Fig. 5B). These results indicate that PGC-1α strongly enhanced the expression of *ALAS1* in both ovarian and hepatic cells in a tissue-specific manner. It is likely that, in contrast to the effects of PGC-1α in KGN cells, the enhancement of *ALAS1* gene expression in hepatic cells by PGC-1α may be LRH-1 independent, because LRH-1 did not bind to the upstream region of *ALAS1* in these cells (Fig. 4B).

Next, we examined the importance of SF-1-binding sites near the −3.5 kb upstream region for synergistic activation of the *ALAS1* gene by SF-1 and PGC-1α. As shown in Fig. 6A, coexpression of SF-1 and PGC-1α in HeLa cells caused marked synergistic activation of the reporter construct harboring the DNA fragment at −3573/−3419, which included the two SF-1 sites. Mutations in either SF-1 site caused a marked decrease of *ALAS1* activation, and mutations in both sites completely abolished *ALAS1* activity. Similar synergistic activation was observed when a construct harboring the −3573/+106 region was used (Fig. 6B), whereas little synergistic actions were observed when constructs har-

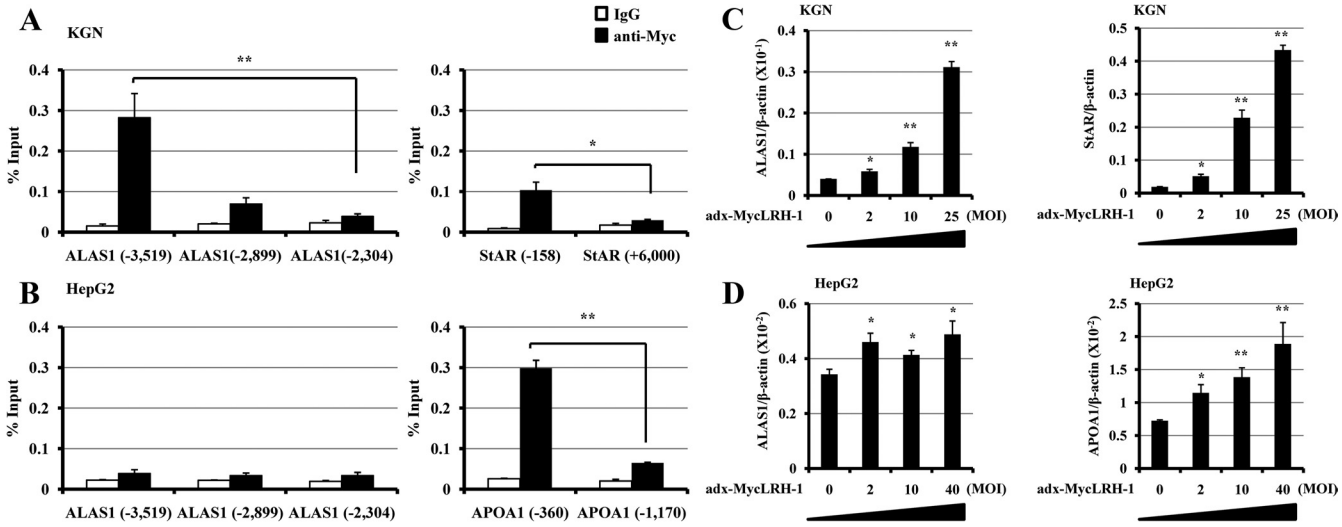

FIG. 4. Difference in responses by KGN and HepG2 cells to binding and induction of the *ALAS1* gene by LRH-1. A, LRH-1 binding to the upstream region of the *ALAS1* gene in KGN cells was examined by ChIP analysis. Myc-tagged LRH-1 was expressed in KGN cells by adenovirus infection [10 multiplicity of infection (MOI)], and LRH-1 binding was analyzed by a ChIP assay using an anti-myc antibody or a nonimmune IgG as negative control. Strong binding is observed near −3.5 kb upstream. LRH-1 binding to the neighborhood of the *StAR* gene (LRH-1-target in steroidogenic cells) is shown as positive (−158) and negative (+6000) controls. B, LRH-1 binding to the upstream region of the *ALAS1* gene in HepG2 cells was also examined. Myc-tagged LRH-1 was expressed in HepG2 cells by adenovirus infection (40 MOI). No specific binding is observed in HepG2 cells, even near the −3.5 kb upstream region. Strong LRH-1-binding is observed at the promoter region of the *APOA1* gene (LRH-1-target in hepatic cells). *Numbers* refer to the transcription start sites of each gene. C, Dose-dependent induction of *ALAS1* gene expression by adenovirus-mediated transduction of LRH-1 into KGN cells was examined. Induction of *StAR* gene expression is shown as a positive control. D, Effects of adenovirus-mediated transduction of LRH-1 on *ALAS1* gene expression in hepatic HepG2 cells. Induction of *APOA1* gene expression by LRH-1 in HepG2 cells is shown as a positive control. *, $P < 0.05$; and **, $P < 0.01$ *vs.* control. Note: protein expression of LRH-1 in these cells is shown by Western blotting in Supplemental Fig. 2.

boring shorter fragments were used. Essentially the same results shown in Fig. 6A were obtained when mutations were introduced within SF-1-binding sites in the −3573/+106 region, although synergistic actions were observed to a lesser extent (Fig. 6C).

Synergistic activations were also observed when KGN cells were used (Fig. 6, D–F). Furthermore, ChIP assays revealed that PGC-1α also bound to the SF-1-binding sites at −3.5 kb upstream of the *ALAS1* gene in KGN cells (Fig. 6G). These observations indicate that SF-1 and PGC-1α synergistically activated *ALAS1* gene expression via the two SF-1-binding sites near −3.5 kb upstream of the human *ALAS1* gene.

Finally, we examined the participation of SF-1-dependent activation of *ALAS1* gene expression in steroid hormone production by steroidogenic cells. As shown in Fig. 7, knockdown of *ALAS1* expression by siRNA resulted in decreased progesterone production by KGN cells ($P < 0.01$, Fig. 7A) and mouse adrenocortical Y1 cells ($P < 0.01$, Fig. 7B). In both cases, gene expression of *StAR*, *CYP11A1*, and 3β-HSD (*HSD3B2* in human and *Hsd3b1* in mouse), all of which are essential for synthesis of progesterone from cholesterol, was unchanged (Supplemental Fig. 5). Protein levels of P450scc (encoded by Cyp11a1) was also unchanged in Y1 cells (Supplemental Fig. 5). This observation indicates that expression levels of *ALAS1* reflected steroid hormone production in steroidogenic cells.

Discussion

Because we have shown previously that introduction of SF-1 into MSCs induces differentiation into steroidogenic cells (4), the identification of genes that are regulated by SF-1 during differentiation would be valuable to identify factors for steroidogenesis. Thus far, more than 30 genes have been identified as target genes of SF-1, including most steroidogenic enzymes (38). Furthermore, angiogenesis-related genes, such as *angiopoietin 2* (39) and *sterol O-acyltransferase 1* (40), have been identified as SF-1-target genes in H295R cells of a human adrenocortical origin by promoter tiling array (ChIP-on-chip analysis) and DNA microarray (gene expression analysis), respectively. In this study, we performed a combined analysis with promoter tiling array and DNA microarray using differentiated steroidogenic cells from hMSCs, and identified 10 genes as novel SF-1-target genes (Fig. 1B). Interestingly, SF-1 worked not only as an activator, but also a suppressor of three genes among the identified 10 genes. Although SF-1-suppressive genes have not been reported, nuclear re-

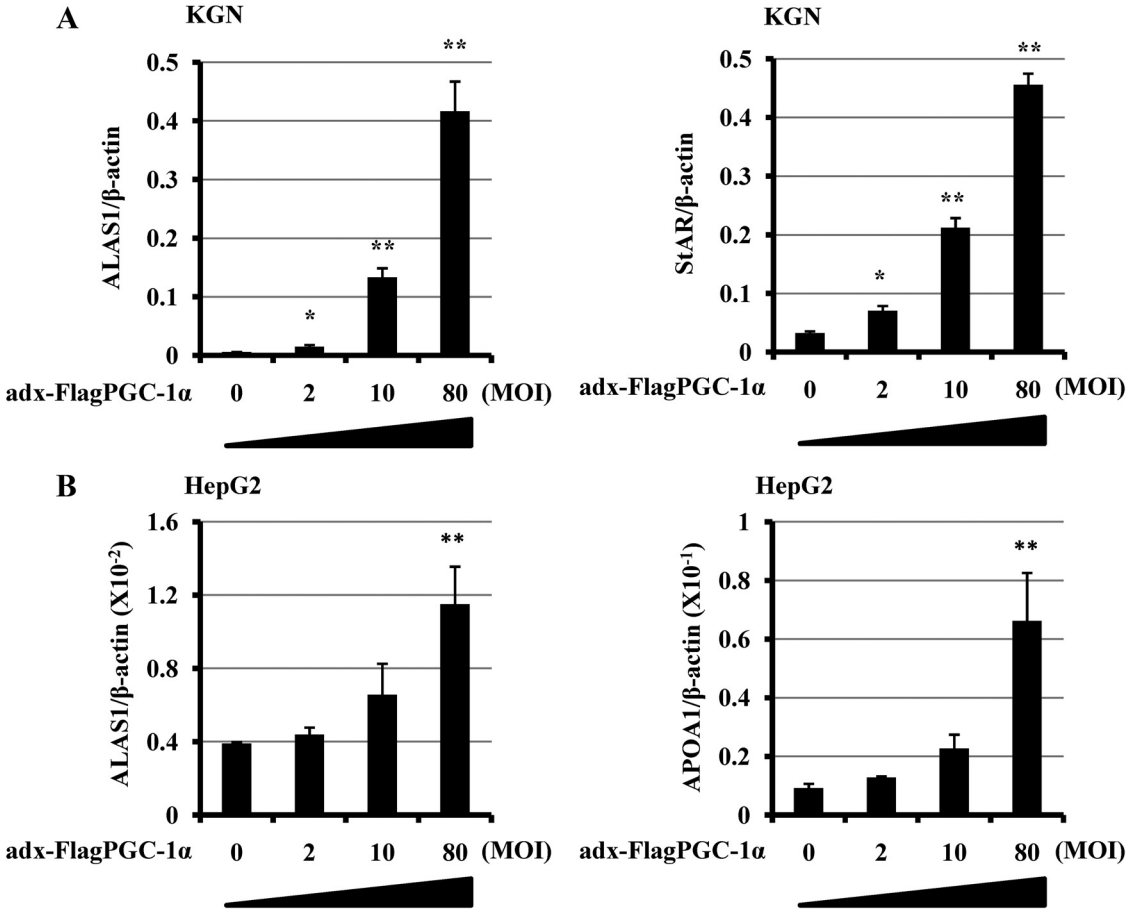

FIG. 5. Effects of adenovirus-mediated transduction of PGC-1α on *ALAS1* gene expression in KGN and HepG2 cells**.** A, *ALAS1* gene expression is markedly induced in a dose-dependent manner by adenovirus-mediated transduction of PGC-1α into KGN cells. Induction of *StAR* gene expression by PGC-1α is shown as a positive control. B, *ALAS1* gene expression is also induced in a dose-dependent manner by adenovirus-mediated transduction of PGC-1α into HepG2 cells. Induction of *APOA1* gene expression by PGC-1α is shown as a positive control. *, $P < 0.05$; and **, $P < 0.01$ *vs.* control. Note: protein expression of PGC-1α in these cells is shown by Western blotting in Supplemental Fig. 2.

ceptors can generally work as suppressors (41). Therefore, mechanisms for SF-1-dependent suppression of gene expression may exist.

We investigated *ALAS1* among the 10 novel genes for the following reasons. In humans, ALAS enzymes are encoded by two genes: *ALAS1*, a housekeeping gene, and *ALAS2*, a erythroid-specific gene (6). Some housekeeping genes are expressed at various levels among tissues and may be controlled in a tissue-specific manner due to their specific functions. For example, 3-oxoacid coenzyme A transferase 1, a key enzyme for ketone body degradation, provides an alternative energy source to many tissues during fasting, but it is repressed in the adult liver (42). Similarly, expression of ALAS1, a rate-limiting enzyme for heme biosynthesis, may be controlled in a tissue-specific manner, particularly in steroidogenic organs and the liver, because heme is essential for the activities of all cytochrome P450 enzymes that play essential roles in steroidogenesis and drug metabolism.

In the liver, transcriptional regulation of *ALAS1* is well characterized. Transcriptional complexes of brain and muscle arnt-like protein-1 and neuronal periodic acid Schiff PAS domain protein 2, which are clock-controlling genes, as well as bile acid-activated FXR regulate *ALAS1* in a liver specific-manner (43, 44). PGC-1α is also involved in *ALAS1* gene activation in the liver by forming a complex with FOXO1 and NRF-1 at the promoter region of *ALAS1* (9). On the other hand, in this study, two SF-1-binding sites −3.5 kb upstream were shown to be quite important for *ALAS1* gene transcription in steroidogenic cells. Introduction of SF-1 results in activation of *ALAS1* reporter genes containing the SF-1 sites (Fig. 2). In fact, both SF-1 and LRH-1 bind to the upstream SF-1 sites in steroidogenic cells [in differentiated MSCs (Fig. 2A) and in KGN cells (Fig.4A)], whereas no specific binding of LRH-1 is observed in hepatic HepG2 cells (Fig. 4B). Gene expression of *ALAS1* is also dependent on both SF-1 (Fig. 3) and LRH-1 (Fig. 4C) in steroidogenic cells including

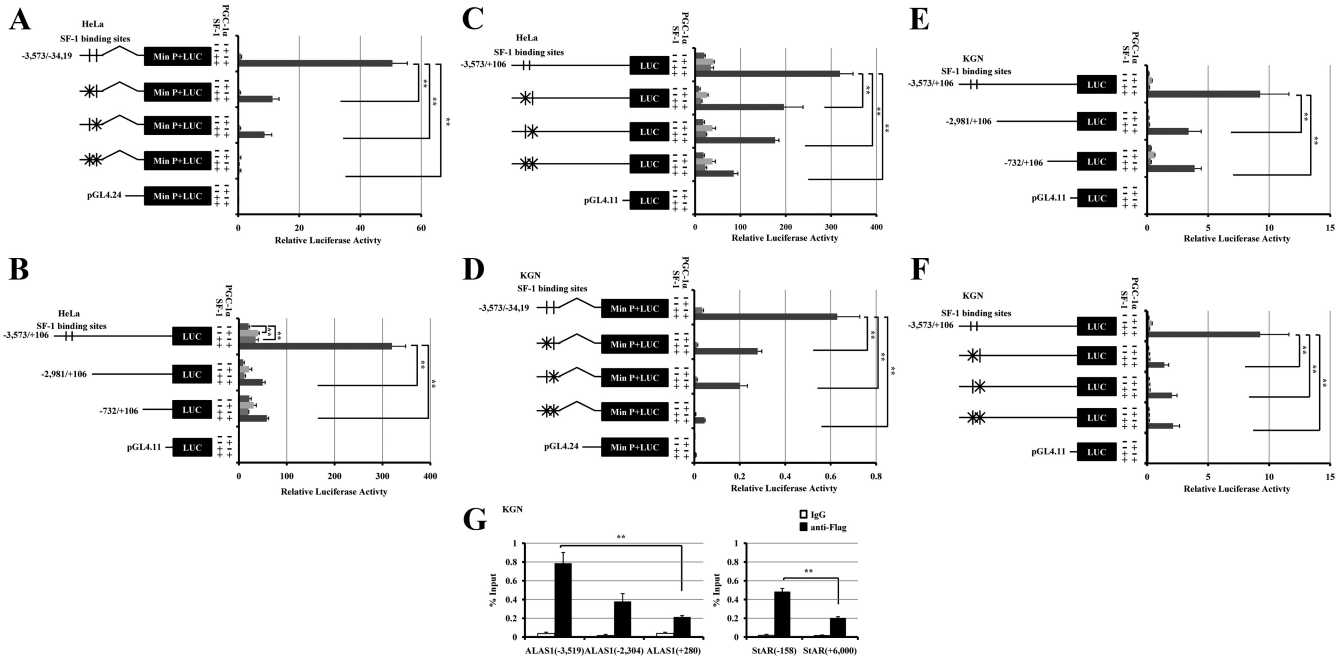

FIG. 6. Synergistic activation of an *ALAS1* reporter gene by SF-1 and PGC-1α. A, A reporter vector containing a DNA fragment (−3573/−3419) upstream of the *ALAS1* gene fused to a minimal promoter was cotransfected with SF-1 and/or PGC-1α expression vectors into HeLa cells. The effects of mutations in SF-1-binding sites of the reporter were also examined. Marked synergistic activation by SF-1 and PGC-1α is observed, and mutations in each or both SF-1 sites markedly decrease reporter activities caused by the synergistic effects of SF-1 and PGC-1α. B, Reporter constructs containing DNA fragments of various lengths from the upstream region of the *ALAS1* gene were cotransfected with SF-1 and/or PGC-1α expression vectors into HeLa cells. Synergistic activation by SF-1 and PGC-1α is observed only in the longest construct containing the SF-1 sites. C, Mutations were introduced into the SF-1-binding sites of the longest construct (−3573/+106), and reporter activities were measured in HeLa cells. Mutations in each or both SF-1 sites markedly decrease synergistic activation of the reporters. D, Synergistic activation by SF-1 and PGC-1α was also examined in KGN cells of an ovarian granulosa origin. A reporter vector in panel A was used and cotransfected into KGN cells with SF-1 and/or PGC-1α expression vectors. The effects of mutations were also examined. Marked synergistic activation by SF-1 and PGC-1α is observed, and mutations in each or both SF-1 sites markedly decrease the synergistic effects. E, Reporter constructs containing DNA fragments of various lengths from the upstream region of the *ALAS1* gene were cotransfected with SF-1 and/or PGC-1α expression vectors into KGN cells. F, Mutations were introduced in the SF-1-binding sites of the longest construct (−3573/+106), and reporter activities were measured in KGN cells. Mutations in each or both SF-1 sites decrease the synergistic activation of reporters. A–F, HeLa and KGN cells were transiently transfected with the indicated reporter or expression vectors. Luciferase activities were measured at 48 h after transfection. **, $P < 0.01$ *vs.* control. G, PGC-1α binding to the upstream region of the *ALAS1* gene in KGN cells was examined by ChIP analysis. Flag-tagged PGC-1α (20 MOI) along with SF-1 (10 MOI) were expressed in KGN cells by adenovirus infection, and PGC-1α binding was analyzed by a ChIP assay using an anti-Flag antibody or a nonimmune IgG for negative control. Strong binding is observed near −3.5 kb upstream. PGC-1α binding to the neighborhood of the *StAR* gene (SF-1-target in steroidogenic cells) is shown as positive (−158) and negative (+6000) controls. **, $P < 0.01$ *vs.* control. LUC, Luciferase; Min, minimal.

differentiated MSCs, whereas no such induction is observed by transduction of LRH-1 into HepG2 cells (Fig. 4D). These observations suggest that different tissue-specific regulation of the *ALAS1* gene exists in liver and steroidogenic tissues, and such regulation in the latter is dependent on NR5A. The idea is further supported by immunohistochemical observations that *ALAS1*, SF-1, and steroidogenic-enzymes were predominantly expressed in adrenocortical cells (Fig. 3, N and O). These observations strengthened the biological significance of *ALAS1* in adrenal steroidogenesis *in vivo*.

Cell type-specific regions of nuclear receptor binding have been demonstrated by ChIP-based genome-wide analyses of progesterone (45) and glucocorticoid (46) receptors. In this study, we also showed cell-type specific binding of the NR5A family to the upstream region of the *ALAS1* gene, which may cause up-regulation of the *ALAS1* gene in steroidogenic tissues to supply a sufficient amount of heme as a prosthetic group of all P450 cytochrome steroidogenic enzymes. Nakajima *et al.* (47) reported that *ALAS1* expression is quite high in adrenocortical and testicular Leydig cells, as well as in the liver by analysis of a GFP knock-in mouse at the *ALAS1* gene site, and speculated that the high expression was necessary to supply heme molecules for steroid synthesis. This hypothesis is supported by our observation in which knockdown of *ALAS1* expression results in decreased progesterone production by steroidogenic KGN and Y1 cells (Fig. 7).

In vivo studies have shown that *ALAS1* expression is induced by tropic hormones in steroid hormone-producing tissues, *i.e.* ovary (48), testis (49), and adrenal gland (50). It is well known that tropic hormones also induce the expression of steroid hormone-synthesizing enzymes including P450 heme proteins to strongly up-regulate ste-

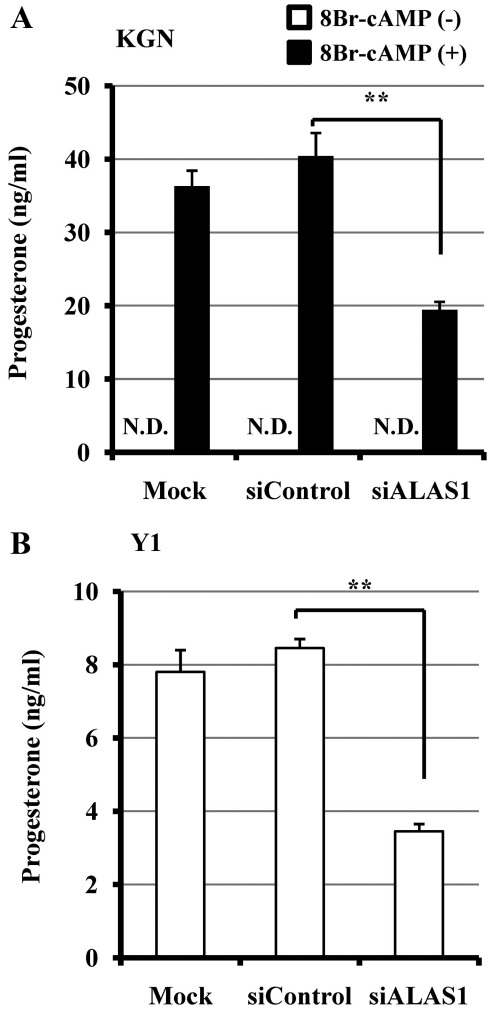

FIG. 7. Effects of *ALAS1* knockdown on progesterone production by steroidogenic cells. KGN cells (A) and Y1 cells (B) were transfected with siALAS1 or siControl oligo nucleotides. After transfection, KGN cells were treated with or without 8Br-cAMP for 48 h, whereas Y1 cells were not treated. Culture medium was replaced every 24 h. Medium was collected at 48 h after transfection for progesterone at the end of culture. Progesterone levels were measured by an ELISA kit. Progesterone production is reduced by siRNA treatments. N.D., Not detected. **, $P < 0.01$ *vs.* control.

roid hormone production. *ALAS1* could play important roles for steroidogenesis via heme supply to the enzymes. Tropic hormones activate cAMP-protein kinase A pathway, which leads to an ultimate increase in SF-1/LRH-1 activity (38). *ALAS1* expression could be enhanced by tropic hormone, at least in part, via SF-1/LRH-1 activity *in vivo*.

The importance of the electron-transfer system in steroidogenesis is also demonstrated by P450 oxidoreductase (POR), a microsomal enzyme that transfers electrons to various P450 steroidogenic enzymes, involvement in steroidogenesis as an SF-1-target gene (32), and mutations in the *POR* gene or deletion of the *POR* promoter region causes the congenital steroid hormone-deficient syndrome, Antley-Bixler (51, 52). We speculate that novel SF-1-target genes, including *ALAS1*, may be responsible, to a certain extent, for congenital steroid hormone deficiencies of an unknown cause.

PGC-1α is a key regulator of various metabolic pathways in skeletal muscle, fat, and liver (53). Recently, we reported that PGC-1α markedly increases progesterone production by ovarian granulosa cells via SF-1/LRH-1 (25). In this study, similar to other NR5A-target genes, we showed that PGC-1α strongly augments *ALAS1* gene expression induced by SF-1 (Figs. 5 and 6). Because mutations in SF-1-binding sites −3.5 kb upstream of *ALAS1* markedly reduce the coactivator activity of PGC-1α in steroidogenic cells, it is likely that PGC-1α forms a complex with SF-1 or LRH-1 bound to the −3.5 kb upstream SF-1 sites. However, the coactivator activity of PGC-1α was not completely abolished by mutation or deletion of the −3.5 kb upstream SF-1 sites (Fig. 6). These results were consistent with the observation that knockdown of SF-1 did not completely abolish the PGC-1α-induced *ALAS1* expression in KGN cells (Supplemental Fig. 3). Although PGC-1α is a strong transcriptional coactivator of NR5A family, PGC-1α could co-activate not only NR5A family but also certain nuclear factors that bind to the *ALAS1* upstream region elsewhere in steroidogenic cells to slightly up-regulate *ALAS1* transcription.

In the liver, on the other hand, it has been reported that PGC-1α augments *ALAS1* gene expression via coactivation of FOXO1 and NRF-1 transcription factors bound to the promoter region (9). We found that PGC-1α induces *ALAS1* gene expression not only in steroidogenic cells, but also in hepatic cells (Fig. 5). However, because LRH-1 cannot bind to the −3.5 kb upstream region of *ALAS1* and shows only slight effects on *ALAS1* gene expression in hepatic cells (Fig. 4, B and D), PGC-1α may not act via NR5A, but via other transcription factors such as FOXO1 and NRF-1 in hepatic cells. This suggestion is consistent with previous reports describing that PGC-1α can form complexes with various transcription factors to activate genes in various tissues.

Finally, we showed the importance of *ALAS1* gene expression for steroidogenesis. Knockdown of *ALAS1* expression in steroidogenic cells results in reduced progesterone production by cells, indicating that *ALAS1* is involved in steroidogenesis via the supply of heme as a prosthetic group of P450 steroid hormone-synthesizing enzymes.

In conclusion, we identified *ALAS1* as a novel SF-1-target gene, and SF-1/LRH-1 up-regulates *ALAS1* gene transcription via binding to a 3.5-kb upstream region of the *ALAS1* transcription start site in a cell type-specific manner. PGC-1α strongly augments *ALAS1* gene tran-

scription via coactivating SF-1 bound to the 3.5-kb upstream region. Knockdown of *ALAS1* results in reduced progesterone production by steroidogenic cells, suggesting that *ALAS1* is involved in steroidogenesis and may be a gene responsible for certain genetic disorders of steroidogenesis. Further study is clearly needed to elucidate such a possibility.

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