Identification of *NR5A1* (*SF-1/AD4BP*) gene expression modulators by large-scale gain and loss of function studies

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Abstract

Nuclear receptor subfamily 5, group A, member 1 (*NR5A1*) previously known as *SF-1/AD4BP* is a transcription factor involved in the development of adrenal/gonadal tissues and steroidogenic lineage cell differentiation in adult somatic stem cells. To understand the cellular signaling network that regulates *NR5A1* gene expression, loss of function screening with an siRNA kinome library, and gain of function screening with an addressable full-length cDNA library representing one quarter of the human genome was carried out. The *NR5A1* gene expression was activated in mesenchymal stem cells by siRNA directed against protein kinase C (PKC)- δ , erb-B3, RhoGAP (ARHGAP26), and hexokinase 2, none of which were previously known to be

involved in the *NR5A1* gene expression. Among these, we identified crosstalk between erb-B3 and PKC- δ signaling cascades. In addition, the gain of function studies indicated that sex-determining region Y (*SRY*)-box 15 (*SOX15*), TEA domain family member 4, *KIAA1257* (a gene of unknown function), ADAM metallopeptidase with thrombospondin type 1 motif 6, Josephin domain containing 1, centromere protein, TATA box-binding protein-associated factor 5-like RNA polymerase, and inducible T-cell co-stimulator activate *NR5A1* gene expression. These results provide new insights into the molecular mechanisms of *NR5A1* gene expression.

Journal of Endocrinology (2008) **198**, 489–497

Introduction

Ovarian folliculogenesis has an essential role in oocyte maturation and steroid production (Palter *et al.* 2001). The wall of a mature preovulatory follicle consists of granulosa, theca interna, and theca externa cells that produce three classes of steroid hormones, progestins, androgens, and estrogens (Vanderhyden 2002). Theca interna cells produce androgens, whereas granulosa cells convert androgens to estrogens. Progesterone is the major product of granulosa cells (Vanderhyden 2002). The human follicle pool decreases significantly with age and the number of follicles is reduced to almost zero at menopause (Broekmans *et al.* 2007). The decline in estrogen at menopause may cause symptoms such as dizziness and depression, and post-menopausal women are at risk of developing osteoporosis (Broekmans *et al.* 2007). Hormone replacement is effective for menopause disorders; however, large-scale clinical studies demonstrated that estrogen treatment can be a risk factor for myocardial infarction, stroke, breast cancer, and uterine cancer (Rossouw *et al.* 2002). At present, the

FDA (Food and Drug Administration) recommends using the lowest dose of hormone replacement therapy (HRT) for the shortest duration (Cirigliano 2007). One of the major problems of estrogen replacement therapy is the optimization of the dose of estrogen in each individual patient (Cirigliano 2007). Production of steroids in the ovary is tightly regulated by the hypothalamus–anterior pituitary axis. Androgen production from theca interna cells is stimulated by luteinizing hormone (LH; Magoffin 2005). Follicle-stimulating hormone (FSH) stimulates the conversion of androgen to estrogen in granulosa cells by inducing aromatase, which catalyzes estrogen biosynthesis from C19 steroids (Simpson & Davis 2001). On the other hand, estrogen negatively regulates the production of LH and FSH in the anterior pituitary (de Ziegler *et al.* 2007). Thus, a treatment that can regenerate this hormone loop might have advantages compared with standard HRT, as it should result in tight control of hormone levels in blood by the body's natural feedback loops.

One possible therapeutic strategy to achieve this is to develop transplantable estrogen-producing cells *in vitro*.

To achieve this, nuclear receptor subfamily 5, group A, member 1 (*NF5A1* that was previously known as *SF-1* or *AD4BP*) is a key molecule. *NF5A1* is the steroidogenic tissue-specific transcription factor that controls the expression of the steroidogenic cytochrome *P450* genes in endocrine tissues, such as the adrenal cortex, testis, and ovary (Morohashi *et al.* 1992). Previously, Crawford *et al.* (1997) using stable ectopic expression of *NF5A1* in mouse embryonic stem (ES) cells, showed that *NF5A1* regulates differentiation and promotes steroidogenesis in ES cells, demonstrating that *NF5A1* is a dominant regulator of the steroidogenic cell phenotype. Also, it has been reported that primary long-term-cultured mesenchymal stem cells (MSCs) infected with an adenovirus-encoding bovine SF-1 (bSF-1) synthesized *de novo* multiple steroid hormones (Gondo *et al.* 2004, Tanaka *et al.* 2007). This has been confirmed by Yazawa *et al.* (2006) who established steroidogenic cells by *NF5A1* forced expression.

Another therapeutic strategy is to develop pharmacological interventions to activate endogenous steroidogenesis in the ovary. Primordial follicles appear to contain only granulosa cells. It is well known that theca cells differentiate from a population of unspecialized MSCs in the ovarian stroma (Magoffin 2005). Consistent with these findings, theca stem cells, which appear similar to fibroblasts and MSCs, were isolated recently (Honda *et al.* 2007). The presence of theca stem cells/ovarian MSCs in post-menopausal women has not been shown. However, it is likely that ovarian MSCs share common features with MSCs in other tissues. Thus, the therapeutic strategy for pharmacological interventions is to develop drugs that can stimulate the differentiation of endogenous theca/ovarian MSCs into estrogen-producing cells.

Adipocytes, which differentiate from MSCs, are known to produce estrogen in an LH/FSH-independent manner. Although the expression of estrogen biosynthesis enzymes is regulated by *NF5A1* in the ovary, a different transcription factor (*NF5A2* previously known as *LRH-1*) regulates the expression of aromatase in adipocytes (Clyne *et al.* 2002). *NF5A2* also regulates aromatase expression in the ovary (Saxena *et al.* 2007); however, *NF5A1* is not expressed in adipocytes. Thus, we have focused our studies on the identification of *NF5A1* gene expression modulators in MSCs.

To date, only limited factors have been identified that regulate *NF5A1* gene expression (Scherrer *et al.* 2002). However, as an siRNA kinome library has been shown to be useful to identify new regulators of the target of the interest (Chan *et al.* 2007, Tyner *et al.* 2008), to identify these modulator genes, we carried out large-scale screening to clarify the cellular signaling network regulating *NF5A1* gene expression in a comprehensive manner, by loss of function screening with an siRNA kinome library in MSCs. Moreover, gain of function screening with an addressable full-length cDNA library representing approximately one quarter of the human genome were also carried out using an *NF5A1* promoter reporter system in human embryonic kidney (HEK)293 cells.

Materials and Methods

Cell culture

Two lines of HEK293, HEK293 FT cells and HEK293 low cells, were obtained from Invitrogen and Microbix (Ontario, Canada) respectively. HEK293 FT cells were established from a fast-growing variant of the 293 cell line that stably expresses the SV40 large T antigen and these cells were mainly used in our study. HEK293 low cells were used when lower background of reporter gene expression is required in the confirmation study, due to its lower transfection efficiency compared with HEK293 FT cells. HEK293 were maintained in Iscove's modified Dulbecco's medium (IMDM; supplemented with 10% fetal bovine serum (FBS); Sigma-Aldrich). Human bone marrow-derived MSCs (lot number 4F1560; Lonza, Basel, Switzerland) were cultured in MSC medium (MSCGM; Lonza). When the cultures reached subconfluence, the cells were harvested with 0.05% trypsin and 1 mM EDTA and were replated at 5×10^3 cells per cm^2 .

siRNAs, cDNAs, and reagents

Both siRNA kinome library and validated siRNAs for hit molecules were purchased from Qiagen. The human cDNA library Mammalian gene collection (MGC), consisting of 6344 clones, was purchased from ATCC. IMAGE clones for protein kinase C (PKC)- δ , sex-determining region Y (SRY)-box 15 (SOX15), TEA domain family member 4 (TEAD-4), KIAA1257, ADAM metalloproteinase, Josephin domain containing 1 (JOSD1), centromere protein (CENP), TATA box-binding protein-associated factor 5-like RNA polymerase (TAF5)-like RNA polymerase, and inducible T-cell co-stimulator (ICOS) were all purchased from Open BioSystems. Rottlerin (R5648) was purchased from Sigma.

Plasmids

bNF5A1 (bSF-1) cDNA was cloned into both pMXs (kindly donated by Dr Kitamura, University of Tokyo, Japan) and pcDNA3.1 vector. The promoter region of human *NF5A1* sequence (5 kbp) was digested (BamHI and NotI) from human BAC clone 10J18 (Invitrogen) and was ligated with 77mer double stranded oligo DNAs (5'-GGC GGC CGC GGG GAC CCC AGG CTG CCG GTC TCC GCC GGC CCT CCC TGA CCC GCT GTC CCT CCG CAG GCG GAC GCC GC-3'). Then the DNA fragment was inserted into a pGL4.20 luciferase expression vector (Promega), named hSF-1 promoter-Luc. A 6.5 kbp DNA fragment for SF-1 promoter-luciferase was digested from hSF-1 promoter-Luc and ligated to pMXs-puro-SIN (self inactivating) vectors in reverse orientation, named pMXs-SIN-hSF-1 promoter-Luc. For creating the human aromatase promoter II reporter construct, 517 bp promoter II region of the aromatase gene, upstream part of exon 2, was cloned from human genomic DNA by PCR with primers 5'-CTC GAG GAT CCT TTG

AAT TCA ATA GAC AAA C-3' and 5'-GAA GCA ACA GGA GCT ATG GTA CCA AGC-3'. The fragment was cloned into pGL4.20 vector, named hARO promoter-Luc. A DNA fragment for aromatase promoter-luciferase was digested from hARO promoter-Luc, and ligated to pMXs-puro-SIN vectors in reverse orientation, named pMXs-SIN-hARO promoter-Luc.

Adenovirus vectors

Adenovirus vectors carrying bSF-1 gene (Ad-bSF-1) or human SF-1 promoter luciferase (Ad-hSF-1 promoter-Luc) were prepared using Adeno-X Expression System (Clontech). EGFP-expressing adenovirus vector (Ad-EGFP) was purchased from Cosmo Bio. Co. Ltd (Tokyo, Japan).

Detection of hormone production from cultured MSCs

Prior to the start of stimulation, the MSCs were seeded at 5×10^3 cells/well in 96-well plates, then cells were infected with Ad-bSF-1 or control Ad-EGFP at multiplicity of infection (MOI)=2. One day after infection, the medium was replaced with Phenol red-free Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% charcoal-treated FBS, and cells were cultured with or without 0.5 mM cAMP. Seven days post-infection, supernatants were collected and 17 β -estradiol, testosterone, dehydroepiandrosterone (DHEA), and progesterone concentrations measured with luminescence immunoassay kits (Immuno Biological Laboratories) following the manufacturer's procedure using an EnVision Multilabel Reader (2103; Perkin-Elmer).

Screening of human cDNA library

From the human cDNA library (MGC collection), 25 ng of each plasmid clone were spotted onto one well of a 384-well plate. hSF-1 promoter-Luc was transfected into 1500 HEK293 cells in each well containing the cDNA library array by using FuGENE HD transfection reagent (Roche). After 3 days, the luciferase activity was measured by SteadyGlo (Promega) using an Arvo Multilabel Reader (1420; Perkin-Elmer).

For the confirmation study with HEK293 cells, cells were grown in 96-well plates at an initial seeding density of 1×10^4 cells/well and cultured for 24 h. Here, we used pMXs-SIN-hSF-1 promoter-Luc, which had a lowest base background luciferase activity among the promoter-reporter constructs we created, and showed clear response to stimuli such as cAMP, instead of using hSF-1 promoter-Luc. We used 25–80 ng pMXs-SIN-hSF-1 promoter-Luc and 10–16 ng target cDNAs, and they were co-transfected and cells were analyzed for luciferase expression using Steady-Glo by EnVision Multilabel Reader (2103; Perkin-Elmer) after 4 days.

For the confirmation study with the MSCs, 500 ng of the targeted cDNA and 200 ng reporter construct were introduced into 5×10^4 MSCs by electroporation using a

MicroPorator MP-100 (Digital Bio; pulse voltage 990, pulse width 40, pulse number 1). Cells were transferred to 0.5 ml MSCGM and cultured for 2 days in 24-well plates. Luciferase activity was measured with BrightGlo (Promega), and Stop & Glo (Promega) was used for internal control.

siRNA library screening

The MSCs were infected with Ad-hSF-1 promoter-Luc at MOI=5–10. After 1 h, a human kinome siRNA library (final concentration was 20 nM) was transfected into the infected cells in 96-well plates (3000 cells/well) by using LipofectAMINE RNAiMax (Invitrogen). Three days after transfection, luciferase activity in each well was measured by Steady-Glo (Promega). Cell growth was also analyzed using CellTiter-Glo (Promega). The similar procedure was used for confirmation studies for siRNA hits coming out of the serial screenings (used MOI=10 of adenovirus vector for reporter construct instead of using MOI=5 to acquire stronger response of luciferase activity).

For the confirmation study with HEK293 cells, especially for the study of PKC- δ function, cells were grown in 96-well plates at an initial seeding density of 1×10^4 cells/well and cultured for 24 h. pMXs-SIN-hSF-1 promoter-Luc and PKC- δ cDNAs were co-transfected and cells were analyzed for luciferase expression using Steady-Glo.

Study of function of a PKC- δ inhibitor, Rottlerin

The MSCs were infected with Ad-hSF-1 promoter-Luc at MOI=10. After 3 h, cells were re-seeded at 3×10^3 /well in 96-well plates and treated with 0.1% DMSO containing Rottlerin or 0.1% DMSO as a control. Luciferase activity and cell growth were analyzed 4 days after treatment.

DNA-binding assay for SOX15

Biotin-modified DNA oligonucleotides for wild-type SOX-binding motif (WT-SBM; 5'-GAA ACA CCA ACA AAG AAG GCG AG-3') and mutant SBM (MT-SBM; 5'-GAA ACA CCT CGA GTC AAG GCG AG-3') were created. Non-modified DNA oligonucleotides for WT-SBM or MT-SBM were also created as specific and non-specific competitors respectively. To perform the DNA-binding assay, the NoShift Transcription Factor Assay Kit (Novagen, San Diego, CA, USA) was used according to the manufacturer's instructions. In brief, biotinylated DNA oligonucleotides of WT-SBM or MT-SBM were mixed with a nuclear extract of SOX15-overexpressing or control pcDNA3.1 introduced into HEK293 cells. In a competition assay, a tenfold excess of competitor (non-modified DNA oligonucleotide of WT-SBM or MT-SBM) was also added. The SOX15-DNA complex was captured on a streptavidin-coated microassay plate. The plate-bound SOX15 protein was then detected with anti-SOX15 antibody (R&D, Cat#AF4070) by using the manufacturer's standard procedure.

Results

Selection of cell lines for the high-throughput functional screening

We have chosen human bone marrow-derived MSCs and HEK293 cells for the large-scale functional screening.

Human bone marrow-derived MSCs were chosen as an alternative to ovarian MSCs, since it has been reported that MSCs isolated from different tissues share common features (Minguell *et al.* 2001). Indeed, following ectopic expression of *bNF5A1* cDNA using an adenovirus vector (Ad-bSF-1), the MSCs produced 17 β -estradiol (Fig. 1a) and progesterone (Fig. 1b). cAMP was added because it is known to induce steroidogenesis in adrenal and gonadal cells (Monté *et al.* 1998), and indeed production of 17 β -estradiol was additively stimulated by cAMP. In addition to ovarian steroids, ectopic expression of *NF5A1* in MSCs resulted in synthesis of DHEA, which is normally produced in adrenal cortex zona reticularis (Fig. 1c) and testosterone (Fig. 1d). DHEA and testosterone production was also stimulated by cAMP. On the other hand, however, endogenous *NF5A1* gene expression, which is known to be positively regulated by SF-1 itself (Nomura *et al.* 1996), was not observed after ectopic expression of SF-1 (data not shown).

MSCs were a suitable cell source for our study; however, the disadvantage of these cells is that its difficulty in cDNA introduction, even siRNA introduction method in these cells was well established in-house, so that they are not practicable for high-content cDNA library screening. To overcome this problem with MSCs, HEK293 cells were chosen for the high-content cDNA library screening, since this cell line is easily transfected with cDNAs. Moreover, it originated from nephrogenic mesoderm that shares a common origin, intermediate mesoderm, with the genital ridge so that we judged HEK293 to be suitable for the initial gain of function screen with cDNA libraries.

Large-scale gain and loss of function screening

To gain a comprehensive understanding of *NF5A1* gene expression modulators, a human siRNA kinome library and a human cDNA library (MGC cDNA library containing 6344 clones) were screened. The 5 kb of the *NF5A1* promoter (Nomura *et al.* 1996), including exon 1, intron 1, and the first part of exon 2 (up to 4 bp upstream of the ATG starting site), was inserted 76 bp upstream of the ATG starting site of a luciferase reporter gene in a pGL4.20 vector (Zhou *et al.* 2001), then the promoter-reporter constructs were introduced into MSCs and HEK293 cells using adenovirus vector and plasmid transfection respectively. The 517 bp gonad-specific human aromatase promoter II region (from 517 bp upstream of the beginning of exon 2 to one base before exon 2, which is located 90 bp upstream of the ATG starting site in exon 2; Zhou *et al.* 2001) was inserted 38 bp upstream of the ATG site of a luciferase reporter gene in pGL4.20 vector and used to validate the function of genes that came up as screening hits (Fig. 2b). For the primary screening, MSCs and

Figure 1 Steroid hormones were produced by the MSCs upon ectopic expression of bovine *NF5A1*. MSCs were seeded at 5×10^3 cells/well in 96-well plates and infected with adenovirus carrying bovine *NF5A1* or EGFP (control) at (a), (b), and (c) multiplicity of infection (MOI) = 2 or (d) MOI = 2.5. The next day, infected MSCs were treated with or without 0.5 mM cAMP and, 6 days later, (a) 17 β -estradiol, (b) progesterone, (c) DHEA, and (d) testosterone production was measured by luminescence immunoassay. Data represent mean $\pm$ S.D. of (a), (b) two, (c) one, and (d) three independent experiments.

HEK293 cells were used for the siRNA and cDNA screening respectively. Four kinases previously unknown to be involved in regulating *NF5A1* gene expression were identified by siRNA screening. *NF5A1* gene expression was activated in MSCs by siRNA targeted to PKC- δ , erb-B3, RhoGAP (ARHGAP26), and hexokinase 2 (Fig. 2c). The gain of

Figure 2 Summary of large-scale functional screening. Structure of reporter construct for (a) human SF-1 promoter (hSF1 promoter-Luc) and (b) human aromatase promoter (hARO promoter-Luc). The transcription start site for each promoter is indicated by an arrow. Gray boxes indicate exons of *NF5A1* gene, hatched boxes indicate exons for aromatase gene, and black boxes indicate luciferase cDNA (from pGL4.20 vector). White boxes indicate spacer sequences derived from pGL4.20 and the lengths are 103 bp for the SF-1 reporter construct and 38 bp for the aromatase reporter construct respectively. SOX9-binding sites are shown by hatched ovals, CRE-binding sites by black ovals, SF-1-binding sites by open ovals, and TEAD (putative)-binding sites by gray ovals. The luciferase reporter gene is driven by each promoter. (c) Four kinases (PKC- δ (PRKCD_11), erb-B3 (ERBB3_6), RhoGAP (ARHGAP26_4), and hexokinase 2 (HK-2_5)) were identified as negative regulators of *NF5A1* gene expression by human kinome siRNA screening. The MSCs were infected with adenovirus carrying hSF-1 promoter-Luc (Ad-hSF-1 promoter-Luc) at MOI=10. After 3 h, infected MSCs were re-seeded at 3×10^3 /well in 96-well plates and transfected with human kinome siRNA library. (d) SOX15, TEAD-4, KIAA1257, ADAMTS6, JOSD1, CENP, TAF5-like RNA polymerase, and ICOS were identified as positive regulators of *NF5A1* gene expression by human cDNA library screening. HEK293 cells were seeded at 6×10^3 /well in 96-well plates and transiently transfected with 10 ng hSF-1 promoter-Luc together with 25 ng human cDNA. The luciferase activity was measured 3–4 days after transfections. Data were normalized and represent mean $\pm$ S.D. of two to three independent experiments.

function screening by introducing cDNAs revealed that *SOX15*, *TEAD-4*, *KIAA1257* (a gene of unknown function), *ADAM* metallopeptidase with thrombospondin type 1 motif 6 (*ADAMTS6*), *JOSD1*, *CENP*, *TAF5*, and *ICOS* activate *NF5A1* gene expression in HEK293 cells (Fig. 2d).

Identification of the PKC- δ /erb-B3 module

Among the four kinase siRNAs identified in this study, the PKC- δ and erb-B3 siRNA showed significant stimulation of *NF5A1* gene expression in MSCs. As the MSCs used in this study express five PKC subtypes, PKC- α , PKC- δ , PKC- ϵ , PKC- η , and PKC- ι (data not shown), we first studied the subtype specificity (Dempsey *et al.* 2000, Woods & Johnson 2007). Although multiple siRNAs for PKC- δ activated the SF-1 promoter reporter system in the MSCs, none of the siRNAs for PKC- α , PKC- ϵ , PKC- η , or PKC- ι activated the SF-1 promoter reporter system (Fig. 3a). In accordance with the siRNA studies, ectopic expression of PKC- δ cDNA by transfection dose dependently inhibited the SF-1 promoter reporter system in HEK293 cells (Fig. 3b). In

addition, Rottlerin, an inhibitor of PKC- δ , dose dependently activated the SF-1 reporter promoter in MSCs (Fig. 3c).

Next, we examined the relationship between PKC- δ and erb-B3. It has been shown that erb-B3-binding protein (Ebp1; Liu *et al.* 2007), a member of the PA2G4 family, binds to nuclear Akt by phosphorylation mediated by PKC- δ . Consistent with this finding, siRNA against Ebp1 increased SF-1 promoter reporter activity (Fig. 3d). These results indicate that erb-B3, PKC- δ , and Ebp1 make up an inhibitory signaling module for *NF5A1* gene expression in MSCs.

Effect of the transcription factors *SOX 15* and *TEAD-4* on *NF5A1* gene expression

Among the eight cDNAs that activated the SF-1 promoter in HEK293 cells, we focused our studies to two transcription factors, *SOX15* (Hiraoka *et al.* 1998) and *TEAD-4* (Thompson *et al.* 2003). These two factors, as well as *NF5A1*, activated the SF-1 promoter reporter system in MSCs and HEK293 cells (Fig. 4a and b). In addition, *SOX15*, *TEAD-4*, and *bNF5A1* activated the aromatase promoter (Fig. 4c). The bSF-1 expression vector

Figure 3 Effect of PKC- δ , Ebp1, and erb-B3 on SF-1 promoter inhibition. (a) From the PKC family, PKC- δ specifically regulated SF-1 promoter activity. (b) Ectopic expression of PKC- δ inhibited SF-1 promoter activity in a dose-dependent manner. (c) Rottlerin, a PKC- δ inhibitor, enhanced SF-1 promoter activity in a concentration-dependent manner. (d) Knockdown of erb-B3-binding protein Ebp1 enhanced SF-1 promoter activity. For (a), (c), and (d), the MSCs were infected with Ad-hSF-1 promoter-Luc at (a), (d) MOI=5, or (c) MOI=10. After 3 h, infected MSCs were re-seeded at 3×10^3 /well in 96-well plates and transfected with siRNA or treated with Rottlerin. The luciferase activity was analyzed 4 days after transfection or treatment. Data were normalized and represent mean $\pm$ s.d. of three independent experiments. For (b), HEK293 were seeded at 1×10^4 /well in 96-well plates and transiently transfected with 17 ng pMXs-SIN-hSF-1 promoter-Luc together with 0, 10.6, 21.25, and 42.5 ng PKC- δ expression vector or control pcDNA3.1 plasmid. The luciferase activity was analyzed 3 days after transfection. Data were normalized and represent mean $\pm$ s.d. of at least six independent experiments. PRKCA_7, PKC- α ; PRKCD_11, PKC- δ ; PRKCE_6, PKC- ϵ ; PRKCH_6, PKC- η ; PRKCI_11, PKC- ι ; ERBB3_6, erb-B3; EBP1_5, Ebp1/PA2G4.

was used as a positive control because the SF-1 and aromatase promoter reporter systems used in this study contain SF-1-binding sites (Fig. 2a and b). SOX15-binding sites on the SF-1 and aromatase promoters have not previously been studied. However, Shen & Ingraham (2002) described regulation of *NF5A1* expression by SOX proteins, particularly SOX3, SOX8, and SOX9, and they showed that all of these SOX proteins were able to bind to the proximal SOX-binding site within the SF-1 promoter. Thus, we expected that SOX15 is also binding to the same site. To identify whether SOX15 binds directly to the SOX-binding site in the SF-1 proximal promoter region, we performed DNA-binding studies using a DNA-binding ELISA (Fig. 4d). SOX15 specifically bound to the SOX responsive element on the SF-1 promoter, and this binding competed with that of an oligonucleotide with the SOX-binding sequence (Fig. 4d). This result indicates that SOX15 directly binds to the SOX-binding site on the SF-1 promoter. We could not identify a SOX15-binding site on the aromatase promoter, indicating that the effects of SOX15 in activating the aromatase reporter system may be via indirect pathways.

Next, we examined the subtype specificity for the SOX family using the SF-1 reporter system in HEK293 cells. The MGC cDNA library that was used for our gain of function study included five human SOX family transcription factors (Kawabe *et al.* 1999), SOX8, SOX9, SOX15, SOX18, and SOX30, and these SOX proteins were used for this analysis (Fig. 4e). Although all five factors activated the SF-1 reporter system, SOX15 and SOX30 showed significant activation.

cAMP response element-binding protein (CREB), is a transcription factor that activates the expression of steroidogenic genes, such as *STAR* and *P450aromatase* (Mendelson & Kamat 2007). Recently, it has been reported that CREB and SF-1 proteins recruit a complex of transcriptional coactivators to target gene promoters (Weck & Mayo 2006). It is also known that one of the SOX family proteins, SOX9, and CREB bind to regulatory regions on the SF-1 promoter to induce gene expression (Val *et al.* 2003). Therefore, we next studied whether SOX15 also had a synergistic effect on the SF-1 reporter system together with CREB (Fig. 4f). Ectopic expression of CREB clearly activated the SF-1 promoter reporter system and, like SOX9, SOX15 and CREB had a synergistic effect on the SF-1 promoter.

As mentioned above, *TEAD-4* forced expression also activated SF-1 and aromatase reporter gene expression. Therefore, we analyzed promoter regions in both the SF-1 and aromatase genes and found three putative TEAD-4-binding sites in the SF-1 promoter region we used for the reporter system and one in the aromatase promoter region of the reporter construct (Jacquemin *et al.* 1996, Anbanandam *et al.* 2006). Based on this finding and on the published information, we suggest that TEAD-4 could also be an important regulator of SF-1 and aromatase gene expression.

Discussion

HRT is widely used for patients suffering from deficiency in production of a number of steroid hormones, glucocorticoids (adrenal failure), or estrogens (menopausal disorders). However, a limitation of current HRT is the difficulty in dose

optimization. For this reason, some groups are now trying to develop a method to transplant *ex vivo*-generated steroidogenic cells to allow control of hormone concentrations in blood by the patients' own hypothalamus–anterior pituitary axis (Gondo *et al.* 2004, Yazawa *et al.* 2006, Tanaka *et al.* 2007).

Alternatively, as we suggest here, it might be possible to regulate *de novo* NF5A1 expression pharmacologically by targeting molecules involved in NF5A1 gene expression regulation.

Although genome-wide functional screening will pick up a large amount of non-druggable targets, *in silico* pathway analysis will be able to identify the druggable molecules directly or indirectly connected to these targets. Inhibitors for these druggable molecules also would not be sufficient to make pharmacological effect separately. However, the combination of these 'weak' inhibitors would be able to make pharmacological effect that is defined as 'long-tail' drugs (Kitano 2007).

In this study, a large-scale functional screening method allowed us to identify four kinase that were previously not known to be involved in regulating NF5A1 gene expression. In addition, we identified that PKC- δ and erb-B3 together with Ebp1 make up an inhibitory module for NF5A1 gene expression. It has been shown that EGF-like growth factors such as amphiregulin, epiregulin, and β -cellulin mediate the action of LH in the ovulatory follicle (Park *et al.* 2004). In addition, EGF-like growth factors are considered as negative regulators of FSH-related steroidogenesis (Park *et al.* 2004). Our study indicates that EGF-like growth factor-mediated inhibition of steroidogenesis may be mediated through the repression of NF5A1 gene expression.

In the gain of function screening, eight cDNAs that were previously not known to activate NF5A1 gene expression were identified. Four of them, SOX15, TEAD-4, CENP, and TAF5-like RNA polymerase, are nuclear proteins. In addition, ADAMTS6, JOSD1, ICOS, and KIAA1257 were identified. In this study, we focused our studies to two transcription factors, SOX15 and TEAD-4. Although it is well known that SOX9 regulates NF5A1 gene expression, we have identified that

Figure 4 The transcription factors SOX and TEAD-4 significantly increased the promoter activity for both SF-1 and aromatase. (a and b) Both SOX15 and TEAD-4 efficiently enhanced SF-1 promoter activity in (a) HEK293 cells and (b) the MSCs. (c) Both SOX15 and TEAD-4 also increased aromatase promoter activity by using pMXs-SIN-hAro promoter-Luc. (d) SOX15 specifically binds to the SOX-binding site on the SF-1 promoter region. The core SOX-binding site (AACAAAG) was used in a NoShift competitor assay to measure the DNA-binding activity of hSOX15. WT-SBM (WT) or MT-SBM (MT) was used in this assay in the presence or absence of a SBM-specific competitor (Com) or a non-specific competitor (NS.Com). ** $P < 0.05$ compared with WT. (e) Other SOX family members also increased SF-1 promoter activity. (f) Synergistic effects of SOX15 and CREB on SF-1 promoter activity. HEK293 cells or MSCs were transfected with the SF-1 or aromatase reporter gene together with the indicated expression plasmids. The expression plasmid of bNF5A1 was used as a positive control. After 2 days, cell extracts were prepared and measured for luciferase activity. Data were normalized and represent mean $\pm$ s.d. of at least three independent experiments.

SOX15 can also stimulate *NF5A1* gene expression significantly. The *NF5A1* gene expression profile is still controversial. For example, *NF5A1* expression was reported to be upregulated in the developing male gonad (Sarraj *et al.* 2003); however, SOX15 protein was only found within the cytoplasm of 13.5 dpc Sertoli cells. In the same study, Sarraj *et al.* showed that SOX15 was not expressed in the adult ovary. In contrast, Assou *et al.* (2006) found that SOX15 was overexpressed in oocytes in their study of the mechanisms regulating human oocyte maturation. Although our result is consistent with the results and hypothesis of Shen & Ingraham (2002) that SOX15 is expressed in adult Leydig cells and is involved in *NF5A1* expression during Leydig cell differentiation, the role of SOX15 in the theca/granulosa cell differentiation is not yet to be concluded without the evidence that SOX15 is expressed in the somatic stem cells in ovary.

In the present study, we found that not only SOX15 but also SOX30 is a strong regulator of the SF-1 promoter. Osaki *et al.* (1999) found SOX30 to be exclusively expressed in adult testis and showed an exclusive germ cell-specific expression indicating that SOX30 might be involved in spermatogenesis. Although, in the same study, they reported that SOX30 was not detected in adult ovary by northern blot experiments, Assou *et al.* reported expression of SOX30 in oocytes. SOX30 might be an important molecule for germ stem cell differentiation. Like SOX15, SOX30 may be a regulator of steroidogenic cell differentiation in adult stem cells in the testis or ovaries. However, as mentioned above, in 2002, Shen & Ingraham studied the functions of SOX3, SOX8, and SOX9 in regulation of *NF5A1* expression, and showed that even though all three of them bind to the proximal SOX-binding site within the SF-1 promoter, only SOX9 is able to transactivate *NF5A1* gene expression so that each SOX protein required appropriate factors as its partners to activate *NF5A1* to achieve tissue-specific gene expression. Therefore, further study will be required to understand about the function of SOX15 and SOX30 in various types of steroidogenic tissues.

In addition, we have identified that *TEAD-4* significantly upregulated the SF-1 and aromatase promoters and putative TEAD-4-binding sites exist on the SF-1 promoter and the aromatase promoter. It has been reported that *TEAD-4* is expressed in the ovarian epithelial cancer cell line OVCAR-3 and a placenta cell line that expresses *NF5A1* and aromatase (JEG-3; Harris & Mellon 1998). Thus, *TEAD-4* might have a physiological role in regulating *NF5A1* expression. *TEAD-4* is a member of the transcription enhancer factor family that shares a common TEA-DNA-binding domain. Four transcription factors (*TEAD-1*, *TEAD-2*, *TEAD-3*, and *TEAD-4*) bind to the same DNA sequence. One of these transcription factors may have a physiological role in SF-1 promoter activation.

ADAMTS6 is a secreted protein that is a member of the extracellular metalloproteinase family containing disintegrin-like domains (ADAMs). The function of ADAMTS6 is still not well understood; however, it is known that extracellular matrix remodeling in ovaries is very important in folliculogenesis and ovulation (Berkholtz *et al.* 2006) and, moreover, one of

the ADAMTS family members, ADAMTS-1, is necessary for structural remodeling during ovarian follicle growth (Brown *et al.* 2006). Therefore, it is possible that secreted ADAMTS6 may also affect steroidogenesis in follicular cells.

ICOS is a member of the CD28 family of costimulatory molecules expressed primarily on activated human T cells (van Berkel & Oosterwegel 2006). ICOS activation of SF-1 reporter gene expression might occur through the action of ICOS ligand on HEK293 cells, and downstream signaling of ICOS ligand may activate *NF5A1* expression. This appears plausible as follicles are surrounded by lymphocytes and it is known that the immune system contributes to the regulation of gonadal function (Bukulmez & Arici 2000). Since our gene identification was based on functional screening, homologs of some of the identified genes may actually function in the steroid-producing cells. Further studies would be expected for the expression and function of all identified target genes on *NF5A1* gene regulations across various endocrine tissues. Expression of identified genes might be restricted to stem/progenitor/blast cells in tissues, which make up a very small part of the total cell population, or might be more widely expressed. Moreover, time course of their expression may also play a major role for *NF5A1* gene regulation. These points will also need to be clarified in the future study.

These results provide new insights into the molecular mechanisms of *NF5A1* gene expression as well as molecular targets for pharmacological intervention to treat diseases caused by a reduction of estrogen production in the ovary, such as menopausal disorders.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice its impartiality.

Funding

The work was supported by Bayer-Schering Pharma.

Acknowledgements

We thank M Adachi, H Masaki, K Kominami, H Migita, and S Takahashi for advice and technical support.

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Received in final form 11 June 2008

Accepted 25 June 2008

Made available online as an Accepted Preprint
25 June 2008