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Transcriptional regulation of human *ferredoxin 1* in ovarian granulosa cells

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ABSTRACT

Ferredoxin 1 (FDX1; adrenodoxin) is an iron–sulfur protein that is involved in various metabolic processes, including steroid hormone synthesis in mammalian tissues. We investigated the transcriptional regulation of *FDX1* in ovarian granulosa cells. Previously, we reported that the NR5A family, including steroidogenic factor-1 (SF-1) and liver receptor homolog-1 could induce differentiation of human mesenchymal stem cells (hMSCs) into steroidogenic cells. A ChIP assay showed that SF-1 could bind to the *FDX1* promoter in differentiated hMSCs. Luciferase reporter assays showed that transcription of *FDX1* was synergistically activated by the NR5A family and 8Br-cAMP treatment through two SF-1 binding sites and a CRE-like sequence in a human ovarian granulosa cell line, KGN. Knockdown of FDX1 attenuated progesterone production in KGN cells. These results indicate transcription of *FDX1* is regulated by the NR5A family and cAMP signaling, and participates in steroid hormone production in ovarian granulosa cells.

1. Introduction

In the ovary, gonadotropins, FSH and LH promote ovarian functions, including follicular development and steroid hormone synthesis (Richards, 1994). FSH and LH control many ovarian steroidogenic enzymes, with their cognate receptors, through activation of an adenylate cyclase, which catalyzes the conversion of ATP to cAMP. Subsequently, the generated cAMP activates signal transductions, such as the protein kinase A (PKA) and phosphatidylinositol-3 kinase pathways. Their signal pathways then affect downstream molecules in ovarian granulosa cells (Richards, 2001). Previously, we cloned many genes rapidly induced by pregnant mare's serum gonadotropin in immature rat ovaries using the subtraction cloning method (Mizutani et al., 1997). In addition, we examined rapidly induced genes with stimulation by a cAMP analogue, 8Br-cAMP, in rat ovarian granulosa cells using DNA microarray analysis. We found that the expression of ferredoxin 1 (FDX1, also known as adrenodoxin), as well as immediate early genes, ovulation-related genes and steroidogenic genes, is rapidly increased by cAMP stimulation in primary cultured rat granulosa cells. FDX1 is an iron–sulfur protein, which transfers electrons from NADPH to mitochondrial cytochrome P450 enzymes, through a flavoprotein, ferredoxin reductase. FDX1 is involved in various metabolic processes, including iron–sulfur cluster biogenesis, bile acid synthesis, vitamin D synthesis and steroidogenesis (Sheftel et al., 2010; Shi et al., 2012; Miller, 2005). FDX1 is ubiquitously expressed in various tissues, but abundantly in steroidogenic tissues, such as adrenal glands and gonads (Picado-Leonard et al., 1988). In steroidogenic cells, cholesterol is transported from the cytosol into mitochondria to synthesize steroid hormones by steroidogenic acute regulatory protein (STAR). The conversion of cholesterol to pregnenolone, which is catalyzed by the cholesterol side chain cleavage cytochrome P450 enzyme (P450scc, encoded by *CYP11A1*), is the first enzymatic step of steroid hormone synthesis in mitochondria. In this step, FDX1 functions as the electron donor for the catalytic activity of P450scc (Miller, 1988). Subsequently, the synthesized pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD, encoded by *HSD3B*). Transcriptional mechanisms of *STAR*, *CYP11A1* and *HSD3B* are well understood and are clearly regulated by steroidogenic factor-1 (SF-1, also known as Ad4BP) in steroidogenic cells (Parker and

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; CRE, cAMP response element; CREB, cAMP regulatory element binding protein; P450scc, cholesterol side chain cleavage cytochrome P450 enzyme; ChIP, Chromatin Immunoprecipitation; EMSA, electrophoretic mobility shift assay; FDX1, ferredoxin 1; hMSCs, human mesenchymal stem cells; LRH-1, liver receptor homolog-1; NR5A, nuclear receptor 5A; PKA, protein kinase A; PKI, protein kinase-inhibiting peptide; StAR, steroidogenic acute regulatory protein; SF-1, steroidogenic factor-1.

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Schimmer, 1997). SF-1 belongs to the nuclear receptor 5A (NR5A) family and has critical roles in hypothalamic functions, the normal development of adrenal gland and gonads, and steroidogenesis (Kim et al., 2011; Luo et al., 1994; Morohashi et al., 1992). SF-1 regulates transcription of many gonadotropin-induced ovarian genes, such as *CYP19A1*, *CYP11B1* and *INHA* (Michael et al., 1997; Carlone and Richards, 1997; Wang et al., 2000; Ito et al., 2000). Another member of the NR5A family, LRH-1 also regulates ovarian genes that function in steroidogenesis and ovulation (Duggavathi et al., 2008; Saxena et al., 2004, 2007). However, it is not known whether the NR5A family regulates transcription of *FDX1*, which is a gonadotropin-induced gene and is essential for ovarian steroidogenesis.

The aim of this study was to investigate the contribution of the NR5A family and cAMP to the transcriptional regulation of *FDX1* in ovarian granulosa cells. Recently, we reported that the introduction of a member of the NR5A family into human bone marrow-derived mesenchymal stem cells (hMSCs) resulted in the differentiation of the cells into steroidogenic cells (Yazawa et al., 2006, 2009; Miyamoto et al., 2011). By a ChIP assay using differentiated hMSCs, we found novel SF-1 binding sites in the promoter region of human *FDX1*. In this study, we show that transcription of *FDX1* is regulated by the NR5A family and cAMP signaling, and participates in progesterone production in ovarian granulosa cells.

2. Materials and methods

2.1. Reagents

Anti-phospho-CREB (S133) (#4276) and H-89 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH (sc32233) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 8Br-cAMP, forskolin and Anti-FLAG M2 (F1804) antibodies were purchased from Sigma (St. Louis, MO, USA). Anti-FDX1 (ab108257) and anti-Myc tag (ab9132) antibodies were purchased from Abcam (Cambridge, MA, USA). The dual luciferase reporter assay system, pGL4.11-basic and pRLpCMV vectors were purchased from Promega (Madison, WI, USA). [γ-32P] ATP was purchased from PerkinElmer (Waltham, MA, USA). Trizol regent, SuperScript III reverse transcriptase, Lipofectoamine LTX, Lipofectoamine Plus regents, Lipofectamine RNAiMAX and Dynabeads M-280 Sheep anti-mouse IgG were purchased from Life Technologies (Carlsbad, CA, USA). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). The Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). siGENOME Non-Targeting siRNA #1 (D-001210-01-20) was purchased from Thermo Scientific (Waltham, MA, USA). Progesterone EIA Kit was purchased from Oxford biochemical research (Oxford, MI, USA). Chemi-Lumi One Super was purchased from Nacalai Tesque (Kyoto, Japan). The Bio-Rad DC protein assay kit was purchased from Bio-Rad laboratories (Hercules, CA, USA). All other regents were obtained from commonly used suppliers.

Table 1
Genes induced by stimulation with 8-Br-cAMP in primary cultured rat granulosa cells.

Probe set ID	Accession number	Gene symbol	Ratio
1369067_at	NM_031628	Nr4a3	191.2
1387908_at	AF239157	Rasd1	178.3
1387410_at	U72345	Nr4a2	63.3
1387174_a_at	AB006007	Star	46.0
1369871_at	NM_017123	Areg	35.8
1369737_at	NM_017334	Crem	33.5
1368025_at	NM_080906	Ddit4	33.4
1370964_at	BF283456	Ass	31.7
1371237_a_at	AF411318	Mt1a	29.4
1374855_at	BI279017	Per1	28.1
1368596_at	AB020480	Snf1lk	24.9
1371194_at	AF159103	Tnfaip6	19.6
1368144_at	AY043246	Rgs2	18.2
1367940_at	NM_053352	Cmkor1	18.2
1389230_at	AA818910	Arrdc3	18.1
1387788_at	NM_021836	Junb	16.6
1369953_a_at	BI285141	Cd24	14.1
1368085_at	NM_133595	Gchfr	11.3
1369587_at	NM_021689	Ereg	10.9
1367982_at	NM_024484	Alas1	10.3
1369044_a_at	AF202733	Pde4b	10.2
1389680_at	BI291626	Ell2_predicted	7.6
1386956_at	NM_031541	Scarb1	7.6
1368223_at	NM_024400	Adamts1	7.3
1367915_at	NM_053437	Dgat1	6.9
1368641_at	NM_053402	Wnt4	6.6
1377163_at	BM385741	Inhbb	6.5
1370375_at	J05499	Gls2	6.0
1389025_at	BI278688	Taf11_predicted	5.5
1387260_at	NM_053713	Klf4	5.5
1370193_at	AI172261	Ptp4a1	5.3
1387087_at	NM_024125	Cebpb	5.3
1368336_at	NM_017126	Fdx1	5.2
1370569_at	BF565001	Pde4d	5.1
1368990_at	NM_012940	Cyp1b1	5.1
1368134_a_at	NM_133380	Il4r	5.1

2.2. Rat granulosa cell culture

Rat granulosa cell culture was performed as previously described (Minegishi et al., 1996). Twenty-one-day-old immature

Table 2
Oligonucleotides used in this experiments.

Oligonucleotide name	Sequence
Real time-PCR	
Rat Fdx1-F	GGTGAAACGCTAACGACCAAG
Rat Fdx1-R	TCACACGCACCAAATCCATC
Rat 36B4-F	GGCGACCTGGAAGTCCAACT
Rat 36B4-R	GGATCTGCTGCATCTGCTTG
ChIP	
Human FDX1-5991F	AAACCAAAACGTTGGCAGCTA
Human FDX1-5921R	CCGACTTTCTGTGGTGTCTGAC
Human FDX1-3938F	GGATACTGGGAGAATGCTGTGA
Human FDX1-3815R	CCACACAGCCAATATGTCCCA
Human FDX1-1329F	GCTACAGGAGGGCTGAGAGAAGA
Human FDX1-1234R	CCAAACTTCCAAGGTCGGATG
Human FDX1-649F	GGAAACTCTGATCCTCCATGAGA
Human FDX1-526R	GTTCATATGTTGTGGGAAATGCA
Human FDX1-429F	GGTTTCATGTACTCAGCCAGCA
Human FDX1-373R	CAGGTAGCCATCCTAGACCTTGA
Human FDX1+806F	CTGGCAGTGACCTTCGCG
Human FDX1+848R	CCAGGACAGCAGGAGGGAA
EMSA	
EMSA_FDX1-1st_SF-1_WT_F	CTTTGGCCTTTAGCCTCTTT
EMSA_FDX1-1st_SF-1_WT_R	AAAGAGGCTAAAGGCCAAAG
EMSA_FDX1-1st_SF-1_Mut_F	CTTTGGaaTTTAGaaTCTTT
EMSA_FDX1-1st_SF-1_Mut_R	AAAGAttCTAAAttCCAAAG
EMSA_FDX1-2nd_SF-1_WT_F	TTTTTCCAAGGTCTCAGATC
EMSA_FDX1-2nd_SF-1_WT_R	GATCTGAGACCTTGGAAAAA
EMSA_FDX1-2nd_SF-1_Mut_F	TTTTTCCAAttTCTCAGATC
EMSA_FDX1-2nd_SF-1_Mut_R	GATCTGAGAaaTTGGAAAAA
EMSA_FDX1_CRE-like_WT_F	CCTGGTTTGTGACGCACGGTGTGCATTGCGTATTAGA
EMSA_FDX1_CRE-like_WT_R	TCTAATACGCAATGCACACCGTGCGTCACAAACCAGG
EMSA_FDX1_CRE-like_Mut_F	CCTGGTTTGTGtgGCACGGTGTGCATTGCGTATTAGA
EMSA_FDX1_CRE-like_Mut_R	TCTAATACGCAATGCACACCGTGCcaCACAAACCAGG