ChIP-on-chip analysis reveals angiopoietin 2 (Ang2, ANGPT2) as a novel target of steroidogenic factor-1 (SF-1, NR5A1) in the human adrenal gland

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ABSTRACT **The nuclear receptor steroidogenic factor-1 (SF-1, *NR5A1*) is a key regulator of adrenal and gonadal biology. Disruption of SF-1 can lead to disorders of adrenal development, while increased SF-1 dosage has been associated with adrenocortical tumorigenesis. We aimed to identify a novel subset of SF-1 target genes in the adrenal by using chromatin immunoprecipitation (ChIP) microarrays (ChIP-on-chip) combined with systems analysis. SF-1 ChIP-on-chip was performed in NCI-H295R human adrenocortical cells using promoter tiling arrays, leading to the identification of 445 gene loci where SF-1-binding regions were located from 10 kb upstream to 3 kb downstream of a transcriptional start. Network analysis of genes identified as putative SF-1 targets revealed enrichment for angiogenic process networks. A 1.1-kb SF-1-binding region was identified in the angiopoietin 2 (Ang2, *ANGPT2*) promoter in a highly repetitive region, and SF-1-dependent activation was confirmed in luciferase assays. Angiogenesis is paramount in adrenal development and tumorigenesis, but until now a direct link between SF-1 and vascular remodeling has not been established. We have identified Ang2 as a potentially important novel target of SF-1 in the adrenal gland, indicating that regulation of angiogenesis might be an important additional mechanism by which SF-1 exerts its actions in the adrenal gland.—Ferraz-de-Souza, B., Lin, L., Shah, S., Jina, N., Hubank, M., Dattani, M. T., Achermann, J. C. ChIP-on-chip analysis reveals angiopoietin 2 (Ang2, ANGPT2) as a novel target of steroidogenic factor-1 (SF-1, NR5A1) in the human adrenal gland. *FASEB J.* 25, 1166–1175 (2011). www.fasebj.org**

Key Words: adrenal development · adrenal tumorigenesis · transcriptional regulation · angiogenesis · NCI-H295R adrenocortical cells

STEROIDOGENIC FACTOR-1 (SF-1, Ad4BP, encoded by *NR5A1*) is a nuclear receptor transcription factor that plays a central role in many aspects of adrenal and reproductive development and function (1, 2). SF-1 is expressed during early adrenal development in mice and humans (3, 4), and disruption of SF-1 can be associated with adrenal agenesis or hypoplasia in both species (5–10).

SF-1 is also emerging as a potentially important regulator of adrenal tumor development (11). Somatic duplication of the locus 9q33 that contains *NR5A1* has been reported in a high proportion of pediatric adrenal tumors on a background of *TP53* loss of heterozygosity (12). Overexpression of SF-1 transcript levels has been reported in pediatric adrenal carcinoma (13), and more recently, SF-1 protein expression was found to be associated with poor clinical outcome in a large cohort of adult adrenocortical carcinomas (14). These data are supported further by studies of overexpression of Sf-1 in the mouse, which results in adrenal cortical cell proliferation and adrenal tumorigenesis (15).

Although many SF-1 target genes already have been identified through detailed characterization of the promoter and enhancer regions of known key factors (1), it is likely that several other important SF-1 targets exist. By using chromatin immunoprecipitation (ChIP) microarrays (ChIP-on-chip) combined with systems analysis, we show that regulation of angiogenesis and vascular remodeling might be an important additional mechanism by which SF-1 exerts its actions in endocrine development and tumorigenesis.

MATERIALS AND METHODS

ChIP

ChIP assays were performed using the ChIP-IT Express kit (Active Motif, Rixensart, Belgium) following the manufac-

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turer's instructions. NCI-H295R human adrenocortical tumor cells were fixed with 1% formaldehyde and subsequently sonicated in eight 20-s pulses at 2-μm amplitude for 5 min (MSE Soniprep 150; Sanyo Gallenkamp, Loughborough, UK). Most resulting chromatin fragments ranged from 200 to 600 bp. Immunoprecipitation was performed in aliquots of sheared chromatin from $\sim 2 \times 10^6$ cells using 3 μg SF-1 antibody (07-618; Upstate Millipore UK Ltd., Watford, UK) or 2 μg negative control IgG (ChIP-IT Control Kit–Human; Active Motif; for ChIP-PCR only). Aliquots of sheared chromatin were separated to serve as input DNA control.

ChIP-on-chip analysis

Three independent ChIP assays were performed using Affymetrix Human Promoter 1.0R Microarrays (Affymetrix UK Ltd., High Wycombe, UK). SF-1-immunoprecipitated DNA (50 ng), and respective input DNA were amplified by ligation-mediated PCR, using a modified version of published protocols (16, 17). In brief, chromatin was blunt-ended by 1 h incubation at 37°C with T4 DNA Polymerase [New England Biolabs (NEB) UK Ltd., Hitchin, UK] and 0.1 mM deoxyribonucleotide triphosphate mix (dNTP; Bioline, London, UK). Long (5′-GCGGTGACCCGGGAGATCTGAATTC-3′) and short (5′-GAATTCAGATC-3′) unidirectional oligonucleotides were heated to 100°C in a 50 mM NaCl solution and slowly cooled to room temperature to generate double-stranded linkers, which were ligated to blunted chromatin overnight (16°C; NEB T4 DNA Ligase). Linker-ligated chromatin was amplified linearly by PCR using 0.1 U *Taq* polymerase, 3 μM long oligonucleotide, 1 M betaine, 2.5 mM $MgCl_2$, and 0.1 mM dNTP + dUTP mix (25% dATP, 25% dCTP, 25% dGTP, 20% dTTP, and 5% dUTP) and the following thermal cycling parameters: 1 cycle of 55°C for 2 min, 72°C for 5 min, and 95°C for 2 min; then 15 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 5 min; followed by a 4-min final extension at 72°C. Amplified DNA was fragmented and end-labeled using the GeneChip WT Double-Stranded DNA Terminal Labeling Kit, hybridized to GeneChip Human Promoter 1.0R arrays, and stained using the GeneChip Hybridization, Wash, and Stain Kit (all Affymetrix).

Quality control of microarray data (3 SF-1-IP arrays, 3 input DNA arrays) was performed using Affymetrix Tiling Analysis Software and R/Bioconductor. Plotted histograms of the raw data intensity suggested that enrichment by SF-1 IP was suboptimal in one of the experiments, which was excluded from final analysis.

The ChIP-on-chip peak detection tool cisGenome (18–20) was used to define potential protein-binding regions. Quantile normalization was applied prior to analysis, and a moving average (MA) statistic was computed for each probe, based on a half-window size of 300 bp or 5 probes. To increase stringency, probes with the MA statistic 3.5 SD away from the global mean were used to define protein-binding regions. Peaks were discarded if they contained <5 probes or were <100 bp in width. Peaks that were separated by <300 bp or 5 probes were merged. Peaks that had a left-tail false discovery rate >5% were discounted (18–20). The genomic coordinates of all regions were converted into coordinates based on U.S. National Center for Biotechnology Information (NCBI) genome build 36 (hg18) and mapped to neighboring transcriptional start sites (TSSs). Identified peaks were visualized using the Integrated Genome Browser (IGB; ref. 21).

Confirmation of chromatin enrichment by PCR (ChIP-PCR)

SF-1-IP, IgG-IP, and 0.2% input DNA samples were used as templates for PCR amplification of the promoters of the known SF-1 targets *CYP11A1* (22–25) and *CITED2* (26, 27). Forward and reverse primers were designed by Primer3 (28): *CYP11A1* proximal promoter (100 bp upstream of TSS), forward 5′-AGAAATTCCAGACTGAACCTTCATA-3′ and reverse 5′-CTGTGACTGTACCTGCTCCACTTC-3′ (198-bp long amplicon); and *CITED2* distal promoter (3.0 kb upstream of TSS), forward 5′-CGGGAAACCACCAAAAGC-3′ and reverse 5′-AAGCAATGGCGAAAACTGTAA-3′ (200-bp long amplicon). As a negative control for SF-1 binding, primers amplifying exon 2 of *PBX1* were used: forward 5′-TGTTTTCACCCTGTGCATTATC-3′ and reverse 5′-TAGATTTGTGACTGCTGGTTAAG-3′ (223-bp long amplicon). PCR reactions were performed with 0.4 μM primers and MegaMix (Microzone Ltd., Haywards Heath, UK) for 35 cycles of amplification with an annealing temperature of 55°C.

Promoter constructs

The 5.0-kb 5′-upstream sequences of *CITED2* (transcript ENST00000367651, ENSEMBL release 54; NCBI build 36) and *ANGPT2* (transcript ENST00000325203, ENSEMBL release 54; NCBI build 36) were analyzed for putative SF-1 binding sites using MatInspector software (http://www.genomatix.de; ref. 29). Based on the position of predicted sites, the 3.3-kb, 900-bp, and 600-bp upstream sequences of the *CITED2* promoter and the 4.5-, 1.9-, and 1.1-kb upstream sequences of the *ANGPT2* promoter were PCR amplified and individually cloned into a pGL4.10[luc2] luciferase reporter vector (Promega UK Ltd., Southampton, UK). For PCR amplification of these regions from genomic DNA, a 1:10 mixture of HotStarTaq DNA polymerase (Qiagen Ltd., Crawley, UK) and Platinum *Pfx* DNA polymerase (Invitrogen Ltd., Paisley, UK) was used, along with the following thermal cycling parameters: initial denaturation at 95°C for 15 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min/kb amplicon; and final extension at 68°C for 10 min. Amplicons were TA cloned using the TOPO XL PCR cloning kit (Invitrogen) and digested from pCR-XL-TOPO vectors using *KpnI* and *XhoI* (NEB) to allow directional cloning into pGL4.l0[luc2] vectors (ligation performed overnight at 16°C with T4 DNA ligase; NEB). Expression vectors (pCMX) containing SF-1 (*NR5A1*) [wild-type (WT) and mutant G35E] cDNAs have been described previously (8, 22).

Transient gene expression assays

Transient gene expression assays were performed in 96-well plates using NCI-H295R cells, Lipofectamine 2000 (Invitrogen), and a dual-luciferase reporter assay system (Promega) with cotransfection of pRLSV40 *Renilla* luciferase (7.5 ng/well; Promega) as a marker of transfection efficiency. To analyze the effects of SF-1 on *CITED2* and *ANGPT2* regulation according to promoter length, individual pGL4.10-luc reporter constructs (100 ng/well) were cotransfected with pCMXWT or mutant SF-1 expression vectors (50 ng/well). Activation of the 4.5 kb *ANGPT2* promoter by SF-1 was studied further with increasing amounts of pCMXWT or mutant SF-1 expression vectors (10, 50, and 100 ng/well). Total DNA transfected was kept constant. In all studies, cells were lysed 24 h after transfection and luciferase assays performed using a FLUOstar Optima fluorescence microplate reader (BMG Labtech, Aylesbury, UK). All data were standardized for *Renilla* coexpression. Results are shown as means ± SE of ≥3 independent experiments, each performed in triplicate. Statistical analysis was performed using Student's *t* tests.

Assessment of gene function and network analysis

MetaCore software (GeneGo Inc., St. Joseph, MI, USA; http://www.genego.com) was used to investigate the func-

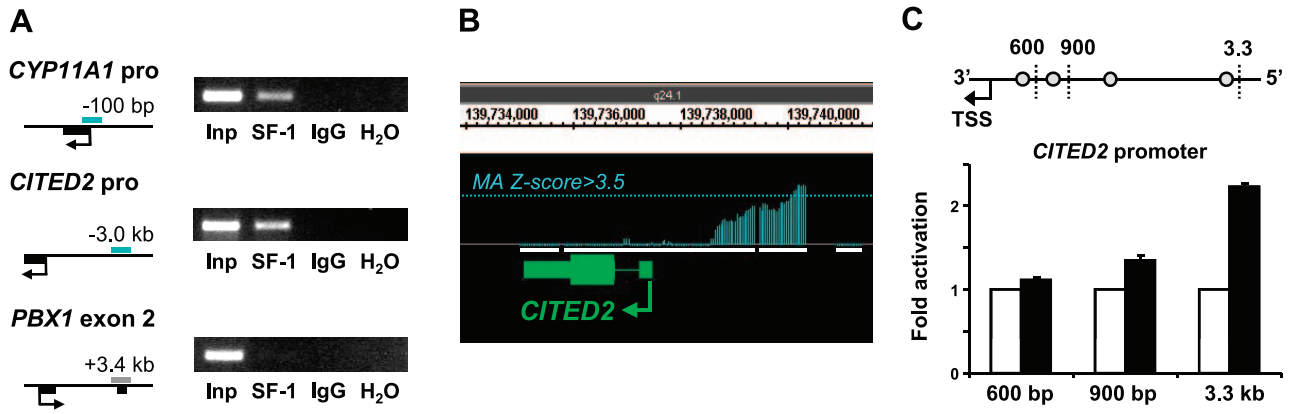

Figure 1. Validation of chromatin enrichment and correlation to transactivation studies. *A*) Chromatin enrichment was confirmed by PCR amplification of the promoters of known SF-1 targets, *CYP11A1* and *CITED2*, from anti-SF-1 immunoprecipitated DNA (SF-1) but not from anti-IgG immunoprecipitation (IgG). Exon 2 of *PBX1*, which is not expected to bear SF-1-binding sites, was used as negative control. Cartoons show position of amplicons in relation to TSSs, which are represented by arrows indicating start and direction of transcription (black bars represent exons). Inp, 0.2% input DNA. *B*) A SF-1-binding region was identified by ChIP-on-chip in the *CITED2* promoter, peaking at ~2.9 kb upstream from the transcriptional start (visualized using IGB; blue dotted line represents threshold for MA $Z > 3.5$; white solid bar represents array coverage of *CITED2* promoter). *C*) Top panel: cartoon representation of putative SF-1-binding sites (gray circles) identified by MatInspector in the *CITED2* promoter and their relation to the different reporter constructs studied (600 bp, 900 bp, and 3.3 kb in length). Bottom panel: activation of different length *CITED2* promoter constructs by SF-1 was investigated using luciferase assays. Consistent with the prediction from ChIP-on-chip analysis, SF-1-dependent activation (black bars) of the 3.3-kb construct was greater than that of the 900- and 600-bp constructs ($P<0.001$). Open bars represent transfection with empty expression vector. Data are presented as means ± SE of ≥3 independent experiments, each performed in triplicate (3.3 kb>control, $P<0.001$; 900 bp>control, $P<0.05$; 600 bp, not significant).

tional annotations and potential network interactions of genes identified as putative novel SF-1 targets from the ChIP-on-chip analysis. Genes were selected based on the criteria for protein-binding regions described above and where the identified site was within 10 kb upstream and 3 kb downstream of a recognized TSS. Only genes annotated by the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC; http://www.genenames.org) were included, and data were uploaded into GeneGo using unique Entrez Gene ID identifiers. To determine which functional categories and biological processes were enriched in the experimental list of target genes, we used the Compare Experiments workflow in GeneGo. Experimental targets were mapped onto functional ontologies in MetaCore, in particular GeneGo Process Networks, a set of ~110 cellular and molecular processes where content is defined and annotated by GeneGo. Gene interaction networks were constructed using the Analyze Networks algorithm with default settings.

Immunohistochemistry of SF-1 and Ang2

Fetal adrenal tissue (fetal stage 1, 8 wk postconception) was obtained from the Human Developmental Biology Resource (HDBR, http://www.hdbr.org) with ethical review board approval and informed consent. Samples were embedded in optimal cutting temperature (O.C.T.) compound and rapidly frozen, and 14-μm sections were obtained using an OTF5000 cryostat (Bright Instrument Co. Ltd., Huntingdon, UK). Unfixed sections were air-dried for 2 h, immersed in ethanol (−20°C) for 10 min, and stored at −20°C. For immunostaining, slides were thawed to room temperature and fixed for 3 min in 4% paraformaldehyde in PBS to preserve tissue integrity with minimal increase in aldehyde-induced autofluorescence. Samples were washed in 1% Tween 20 in Tris-buffered saline (TBST), then blocked by 1 h incubation in 10% lamb serum in TBST. Simultaneous overnight incubation at 4°C was performed with the following primary antibodies: mouse anti-human SF-1 (434200; Invitrogen; 1:200 dilution) and rabbit anti-human angiopoietin-2 (Ang2, ANGPT2; ab65835; Abcam; 1:200 dilution). Slides were washed and incubated overnight at 4°C in 10% lamb serum in TBST with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml), anti-mouse secondary antibody (Alexa647 goat anti-mouse, A21235; Invitrogen; 1:400 dilution), and anti-rabbit secondary antibody (Alexa555 goat anti-rabbit; A21429; Invitrogen; 1:400 dilution). After washing, slides were mounted in Vectashield (Vector Labs, Peterborough, UK) and imaged on a LSM 710 confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

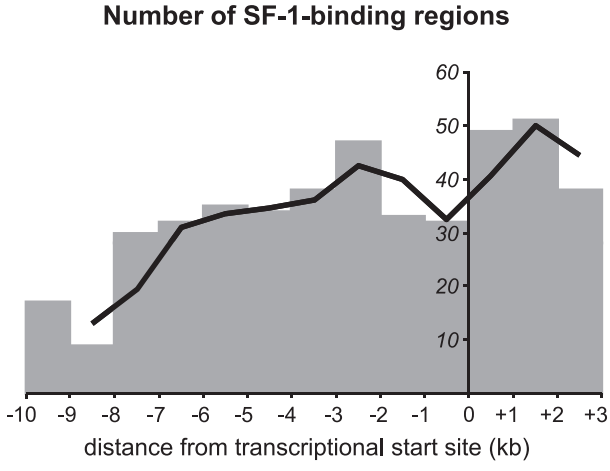

Figure 2. Distribution of SF-1-binding regions neighboring TSSs. A greater density of SF-1-binding sites is observed ~2.5 kb upstream (−) and 1.5 kb downstream (+) of TSSs, with peaks > +1 SD from the mean. Solid line represents a 2-point moving average for the data set.

TABLE 1. *Top-ranking SF-1-binding regions located from 10 kb upstream to 3 kb downstream of a TSS*

MA *Z* score	Chr	Start	End	Region length (bp)	Distance to TSS (bp)	Gene	Gene name	Selected GO biological process/molecular function
8.02	19	61052876	61053237	362	−2142	NLRP4	NLR family, pyrin domain containing 4	ATP binding; nucleotide binding; protein binding
7.26	4	606174	606996	823	−2787	PDE6B	Phosphodiesterase 6B, cGMP-specific, rod, β	Detection of light stimulus; response to stimulus; signal transduction
7.22	3	8671294	8671558	265	−2690	C3orf32	Chromosome 3 open reading frame 32	Mouse: protein folding; heat shock protein binding
7.14	13	113199316	113199973	658	−6621	DCUN1D2	DCN1, defective in cullin neddylation 1, domain containing 2 (*Saccharomyces cerevisiae*)	
6.81	9	99502264	99502676	413	−3011	XPA	Xeroderma pigmentosum, complementation group A	DNA damage removal; signal transduction resulting in induction of apoptosis; response to oxidative stress
6.51	15	20377626	20377881	256	−7189	TUBGCP5	Tubulin, γ complex associated protein 5	Microtubule cytoskeleton organization; microtubule nucleation
6.17	13	113367394	113368774	1381	−7583	ATP4B	ATPase, $H+/K^+$ exchanging, β polypeptide	Ion transport; ATP biosynthetic process; response to lipopolysaccharide
					−1513	GRK1	G-protein-coupled receptor kinase 1	Positive regulation of phosphorylation; signal transduction; apoptosis
6.12	3	8672091	8672356	266	−3487	C3orf32	Chromosome 3 open reading frame 32	Mouse: protein folding; heat shock protein binding
5.84	14	92455627	92456518	892	−3172	CHGA	Chromogranin A (parathyroid secretory protein 1)	Regulation of blood pressure; protein binding
5.78	19	3123496	3123904	409	−6065	S1PR4	Sphingosine-1-phosphate receptor 4	G-protein-coupled receptor protein signaling pathway; elevation of cytosolic calcium ion concentration
5.61	8	6411196	6412323	1128	−3588	ANGPT2	Angiopoietin 2	Angiogenesis; blood vessel morphogenesis; multicellular organismal development
5.49	13	113149179	113149920	742	1908	ADPRHL1	ADP-ribosylhydrolase like 1	Protein amino acid de-ADP-ribosylation; magnesium ion binding
5.49	19	40729390	40729965	576	1293	TMEM147	Transmembrane protein 147	Protein binding
5.36	2	183644509	183645199	691	−6877	DUSP19	Dual specificity phosphatase 19	Protein amino acid dephosphorylation; inactivation of MAPK activity; JNK cascade
5.30	7	43878032	43878592	561	−2643	MRPS24	Mitochondrial ribosomal protein S24	Translation; structural constituent of ribosome
5.24	9	110664647	110665656	1010	−7121	ACTL7B	Actin-like 7B	Protein binding; structural constituent of cytoskeleton
					728	ACTL7A	Actin-like 7A	Protein binding; structural constituent of cytoskeleton

(*continued on next page*)

TABLE 1. (*continued*)

MA *Z* score	Chr	Start	End	Region length (bp)	Distance to TSS (bp)	Gene	Gene name	Selected GO biological process/molecular function
5.21	22	22563867	22564131	265	−2565	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	Positive regulation of ERK1 and ERK2 cascade; negative regulation of apoptosis
5.19	21	44030889	44031745	857	−2541	RRP1	Ribosomal RNA processing 1 homolog (*S. cerevisiae*)	rRNA processing
5.14	9	130885372	130885644	273	2282	DOLPP1	Dolichyl pyrophosphate phosphatase 1	Protein amino acid N-linked glycosylation; catalytic activity
5.12	7	43881830	43882948	1119	−6720	MRPS24	Mitochondrial ribosomal protein S24	Translation; structural constituent of ribosome
5.08	2	121263371	121263617	247	−2832	GLI2	GLI family zinc finger 2	Mammary gland development; positive regulation of transcription; developmental growth; transcription factor activity
5.08	3	12804229	12804769	541	−8671	CAND2	Cullin-associated and neddylation-dissociated 2 (putative)	Regulation of transcription; transcription activator activity; TATA-binding protein binding
5.07	3	48450915	48451316	402	−9370	PLXNB1	Plexin B1	Multicellular organismal development; intracellular signaling pathway
					−5574	CCDC72	Coiled-coil domain containing 72	
5.07	9	139889204	139889711	508	−2604	CACNA1B	Calcium channel, voltage-dependent, N type, α 1B subunit	Calcium ion transport; transmembrane transport; voltage-gated calcium channel activity
5.06	15	38971991	38972237	247	−1805	VPS18	Vacuolar protein sorting 18 homolog (*S. cerevisiae*)	Protein transport; endosome organization
5.05	15	73417958	73418364	407	−8301	NEIL1	Nei endonuclease VIII-like 1 (*Escherichia coli*)	DNA repair; metabolic process; negative regulation of nuclease activity
					2735	COMMD4	COMM domain containing 4	Protein binding
5.04	7	43924834	43926219	1386	−7045	UBE2D4	Ubiquitin-conjugating enzyme E2D 4 (putative)	Regulation of protein metabolic process; post-translational protein modification

Chromosomal coordinates (NCBI human genome build 36) and length of anti-SF-1 immunoprecipitated regions meeting criteria of an MA *Z* score > 5.0 are shown. For each binding region, distances to the TSSs of neighboring genes are specified (negative if upstream of TSS; positive if downstream of TSS). Selected gene ontology (GO) annotation, when available, is also provided. Chr, chromosome.

RESULTS

Validation of chromatin enrichment and characterization of SF-1-dependent regulation of CITED2

ChIP with the anti-SF-1 antibody was performed in NCI-H295R human adrenocortical tumor cells. To validate chromatin enrichment by this anti-SF-1 antibody, ChIP-PCR assays were performed using primers located in the promoters of the known SF-1 targets, *CYP11A1* (22–25) and *CITED2* (refs. 26, 27 and **Fig. 1*A***). Primers located around exon 2 of *PBX1* were used as a negative control for SF-1 binding, as this region does not contain SF-1-binding sites. The promoter regions of *CYP11A1* and *CITED2* were amplified from SF-1-immunoprecipitated DNA, confirming chromatin enrichment by ChIP, whereas exon 2 of *PBX1* failed to amplify. In addition, specificity of immunoprecipitation with the anti-SF-1

TABLE 2. *Comparison of experimentally identified SF-1 targets to published adrenocortical tumor expression datasets*

MA *Z* score	SF-1 target	Giordano *et al.* (31)	West *et al.* (30)	Soon *et al.* (32)
5.61	*ANGPT2*	+	+	+
4.79	*SHMT2*	+		
4.58	*YKT6*	+	+	
3.84				
4.74	*TBRG4*	+		
4.18	*SLC31A2*		+	
4.07	*PCSK6*	+	+	
3.97	*SPARC*		+	
3.96	*RGS3*	+		
3.83	*KPNA4*	+		
3.76	*TSPAN17*	+		
3.73	*DOLK*		+	
3.70	*ATAD2*	+		
3.68	*CREB5*	+		
3.65	*LPCAT4*	+		
3.63	*NR6A1*	+		
3.55	*CDC20*	+		
3.54	*KIF3C*		+	

ChIP-identified SF-1 targets that have been found to be overexpressed in adrenocortical carcinomas in comparison to adenomas (31, 32) and in pediatric adrenocortical tumors in general (30). Two independent SF-1 binding regions were identified at the *YKT6* gene loci. Ang2 (*ANGPT2*) emerged as the common factor in all datasets.

antibody was confirmed by the absence of amplification of these promoters from immunoprecipitated chromatin when IgG was used for ChIP.

Following validation, SF-1 ChIP-on-chip analysis was performed using single-tiling promoter arrays (GeneChip Human Promoter 1.0R arrays) and genomic DNA (input chromatin) as hybridization control. ChIP-on-chip analysis identified a SF-1-binding region in the *CITED2* promoter (Chr6:139,739,907–139,740,330; NCBI genome build 36) peaking at ~2.9 kb upstream from the TSS (Chr6:139,737,478; NCBIv36) with an MA *Z* score of 4.44 (Fig. 1*B*). Using MatInspector software to analyze the 5.0-kb upstream sequence of *CITED2*, 4 putative SF-1 binding sites were identified at 3080, 1247, 665, and 458 bp upstream of the TSS (Fig. 1*C*). To verify SF-1-responsiveness of the binding site identified by ChIP-on-chip, reporter constructs containing different lengths of the CITED2 promoter were designed, and activation by SF-1 was studied in luciferase assays. Activation of a 3.3-kb *CITED2* promoter construct by SF-1 was greater than that of the 900- and 600-bp promoter constructs, in keeping with the ChIP-on-chip results (Fig. 1*C*).

Identification of novel SF-1-binding sites by ChIP-on-chip

Analysis of ChIP-on-chip experiments with CisGenome identified 738 SF-1-binding regions that met criteria of an MA score > 3.5 mean ± SD and a false discovery rate of <5%. Subsequent analysis focused on those regions that were located between 10 kb upstream and 3 kb downstream of the TSS of known genes (as defined by HGNC), in keeping with the design of the Human Promoter 1.0R arrays. Using this approach, binding regions were annotated to 445 gene loci (Supplemental Tables 1*A*, *B*). The distribution of immunoprecipitated sequences revealed greater density of SF-1-binding sites around 2.5 kb upstream and 1.5 kb downstream of TSSs (**Fig. 2**).

Binding regions surrounded the TSS of 397 unique genes. More than one SF-1-binding site was identified in the defined −10 kb < TSS < +3 kb region of 35 genes: of these, 27 genes had 2 binding sites, 4 genes had 3, 3 genes had 4, and 1 gene had 5 SF-1-binding sites. Top-ranking TSS-neighboring binding sites (MA *Z*>5.0) are detailed in **Table 1**.

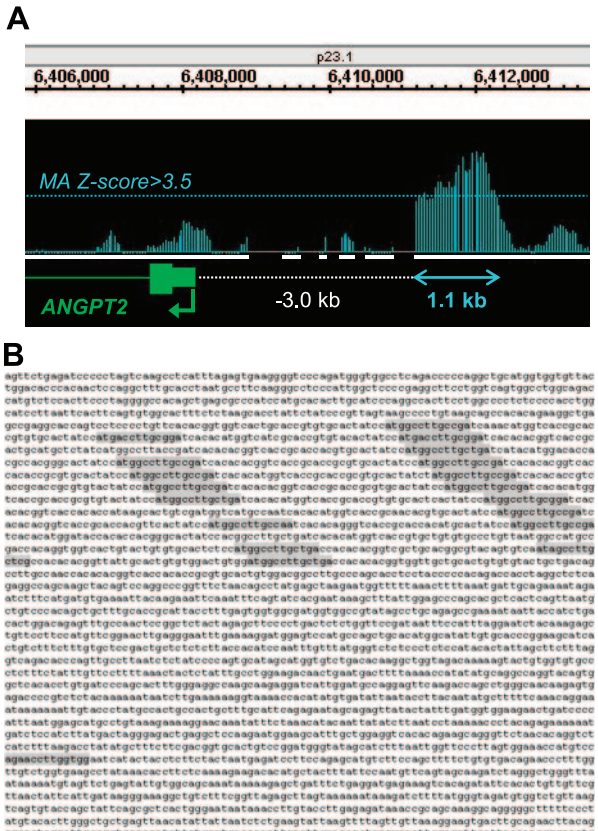

Figure 3. SF-1 binding on the *ANGPT2* promoter. *A*) ChIP-on-chip identified a 1.1-kb SF-1-binding region in the promoter region of *ANGPT2* (encoding Ang2) located from 3.0 to 4.1 kb upstream of the TSS (visualized using IGB; blue dotted line represents threshold for MA *Z* > 3.5; white solid bar represents array coverage of the *ANGPT2* promoter). *B*) Accordingly, *in silico* analysis of the upstream sequence of *ANGPT2* with MatInspector identified 20 putative SF-1 binding sites (highlighted in gray), 18 of which clustered in a highly repetitive region between 3.1 to 4.1 kb upstream of the TSS [transcribed sequence shown in bold and underscored; transcript ENST00000325203, ENSEMBL release 54; (NCBI build 36)].

Ang2 (ANGPT2) as a target of SF-1

To identify potential networks of target genes being regulated by SF-1, the gene loci dataset was analyzed using the MetaCore platform of GeneGo systems biology software. Although several potential gene interaction networks focused on established nuclear receptor systems (*e.g.*, Supplemental Figs. S1 and S2), enrichment analysis revealed that experimental targets mapped to process networks related to blood vessel morphogenesis ($P=0.002$) and regulation of angiogenesis ($P=0.027$). ChIP-on-chip-identified SF-1 targets involved in such process networks included *ADORA3*, *ANGPT2*, *CXCR4*, *GLI2*, *HTR1A*, *PLCD1*, *S100A7*, *SP1*, and *SPARC*, among others.

Considering that ChIP-on-chip experiments were performed on an adrenocortical tumor cell line and that SF-1 might play an important role in adrenal tumorigenesis, experimentally identified SF-1 targets were compared to published datasets of adrenocortical tumor gene expression in children (30) and adults (refs. 31, 32 and **Table 2**). Ang2 (encoded by *ANGPT2*; MIM 601922) emerged as a common factor in all these datasets and within a GeneGo angiogenic process network.

Characterization of ANGPT2 regulation

The SF-1-binding region identified in the *ANGPT2* promoter by ChIP-on-chip spans 1.1 kb from Chr8:6,411,196 to Chr8:6,412,323 (NCBIv36), with an MA *Z* score of 5.61 (**Fig. 3*A***). Since the TSS of *ANGPT2* is at Chr8:6,408,174 (in the reverse strand) in that genome assembly, ChIP-on-chip data predicted the binding region to be located from 3.0 to 4.1 kb upstream of the TSS. Analysis of the 5.0-kb upstream sequence of *ANGPT2* with MatInspector identified 20 putative SF-1 binding sites, 18 of which clustered in a highly repetitive region between 3.1 to 4.1 kb upstream of the TSS, in accordance with the experimental data (Fig. 3*B*). To confirm these findings, reporter vectors containing variable lengths of the *ANGPT2* promoter (1.1, 1.9, and 4.5 kb) were constructed, and activation by SF-1 in NCI-H295R human adrenal cells was assessed by luciferase assays. Shorter promoter fragments (1.1 and 1.9 kb) were not activated by WT SF-1 (**Fig. 4*A***), whereas dose-dependent activation of the 4.5 kb *ANGPT2* promoter construct was seen (Fig. 4*B*). This activation was lost when the functionally impaired G35E mutant SF-1 construct was used.

SF-1 and Ang2 expression during early human fetal adrenal development

To investigate the potential coexpression of SF-1 and Ang2 in a biologically relevant system, immunohistochemistry was performed using human fetal adrenal tissue (fetal stage 1, 8 wk postconception). This is a stage of rapid adrenal development and growth, as well as the onset of steroidogenesis (33). Strong nuclear expression of SF-1 was seen in the developing definitive zone, with less intense staining of a subset of cells within the fetal cortex (**Fig. 5**). Ang2 expression was observed in the cytoplasm of cells and extracellular space, predominantly in the definitive zone and subcapsular region and around areas of strong SF-1 expression.

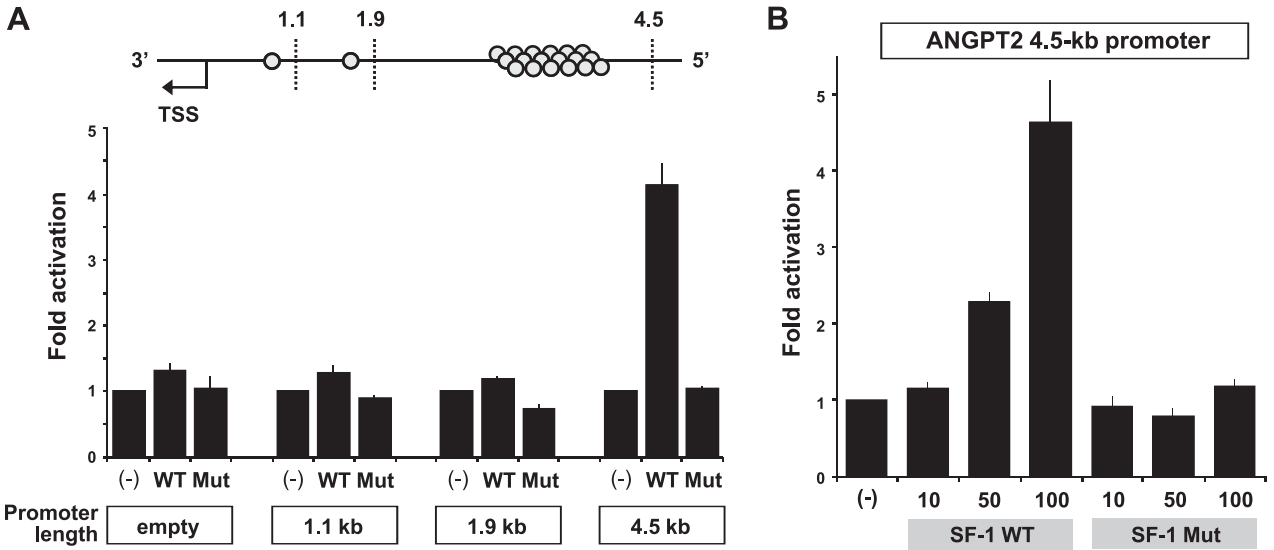

Figure 4. SF-1-dependent regulation of *ANGPT2*. *A*) Top panel: cartoon representation of putative SF-1-binding sites (gray circles) identified by MatInspector in the *ANGPT2* promoter in relation to the 3 different length reporter constructs studied (1.1, 1.9, and 4.5 kb). Bottom panel: pGL4 reporter vectors containing variable lengths of the *ANGPT2* promoter (or empty, for background assessment) were cotransfected into NCI-H295R human adrenal cells with either WT SF-1 (WT), mutant SF-1 (Mut) or empty (−) expression vectors 24 h prior to luciferase assays. While shorter promoter fragments (1.1 and 1.9 kb) failed to be activated by SF-1, the 4.5-kb *ANGPT2* promoter construct was activated by the WT protein ($P<0.05$ *vs.* empty). *B*) Dose-dependent activation of the 4.5-kb *ANGPT2* by WT SF-1 was not observed when the functionally impaired G35E mutant SF-1 construct was used (means ± SE of ≥3 independent experiments, each performed in triplicate).

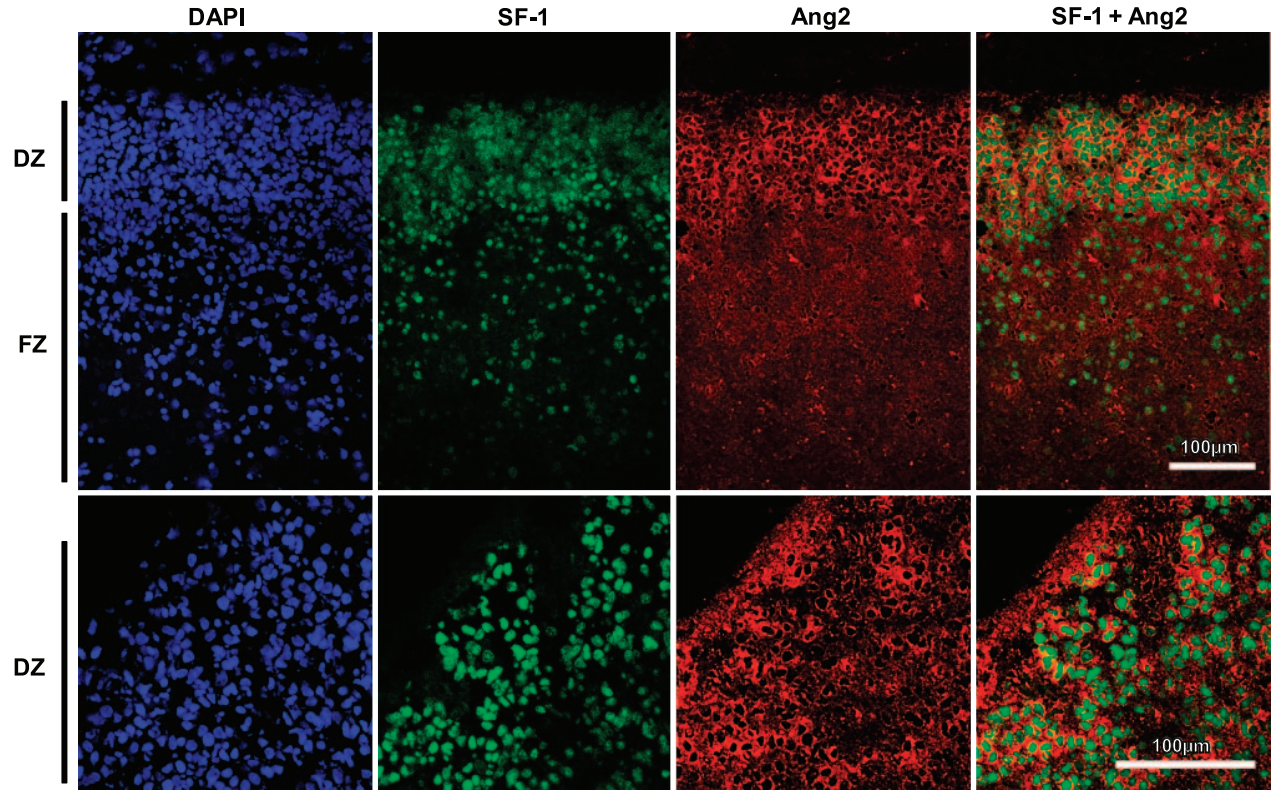

Figure 5. SF-1 and Ang2 expression during early human fetal adrenal development. Top panel: immunohistochemistry for SF-1 and Ang2 in the human fetal adrenal gland (fetal stage 1, 8 wk postconception) shows strongest expression of these factors in the developing definitive zone (DZ). FZ, fetal zone. Nuclear staining is shown with DAPI. Bottom panel: higher-power magnification shows coexpression of SF-1 and Ang2 in areas of the definitive zone.

DISCUSSION

SF-1 is a key regulator of adrenal development and cancer, and angiogenesis is important in these processes; however, until now a direct link between SF-1 and vascular remodeling has not been established. By using a combination of ChIP-on-chip, network analysis, expression data, and functional genomic studies, we have identified Ang2 (*ANGPT2*) as a potentially important novel target of SF-1 in the adrenal gland.

Angiopoietins are secreted ligands for the tyrosine receptor kinase Tie2 that act in concert with vascular endothelial growth factor (VEGF, mainly VEGF-A) to regulate angiogenic remodeling (34). While Ang1, the first identified Tie2 ligand, acts toward stabilizing vessel walls, Ang2 has a central role in destabilizing vasculature so that regression (in the absence of VEGF) or angiogenic sprouting (in presence of VEGF) can occur. Ang2 is widely expressed in the endothelium during developmental stages and in sites of active vascular remodeling such as the placenta, ovaries and uterus (34). VEGF and Ang2 are key regulators in tumor angiogenesis, facilitating tumor growth and metastasis, and overexpression of Ang2 has been documented in many tumor types (*e.g.*, liver, renal, gastric, breast, colon, pancreas, lung; refs. 35, 36). Consistent with this, a higher Ang2:Ang1 ratio correlates with worse prognosis for many cancers (36).

Angiogenesis and vascular remodeling are also important in the development of endocrine tissues, including the adrenal gland. Although these systems are not as well studied as tumorigenesis, recent work by Jaffe and colleagues (37, 38) has shown a preponderance of subcapsular angiogenesis during human fetal adrenal development (14 to 22 wk postconception) and has reported that adrenocorticotropin (ACTH)-driven zonal differential expression of Ang2 is an important part of this process. In those studies, it was proposed that local factors would mediate the angiogenic response to tropic stimuli during fetal adrenal development, and up-regulate *ANGPT2* in the definitive zone of the fetal gland. Our results now suggest a direct interaction of SF-1 in binding to and activating the *ANGPT2* promoter. Furthermore, we demonstrate that SF-1 and Ang2 are strongly coexpressed in the subcapsular region and developing definitive zone of the human fetal adrenal gland at a critical early stage of development, namely 8 wk postconception. Considering that the architectural arrangement of blood vessels in the adrenal cortex is such that nearly every adrenocortical cell is thought to lie adjacent to a vascular endothelial cell (39), SF-1-dependent Ang2 expression in adrenocortical cells could be postulated to act in a paracrine way and coordinate vascular remodeling in the developing adrenal. This is supported by findings of abnormal adrenocortical vasculature in adult *Sf-1* haploinsufficient mice (40).

Even though a role for SF-1 in regulating angiogenesis

has not yet been described, members of the related NR4A nuclear receptor subfamily have recently been recognized as important regulators of vascular gene expression, playing critical roles in many aspects of vascular biology (41). In particular, NR4A1 (Nur77, NGFI-B) has been shown to drive the transcription of the plasminogen activator inhibitor 1 (PAI-1) in human umbilical vein endothelial cells (42) and to act as a transcriptional mediator for VEGF-A-induced angiogenesis, with distinct proangiogenic effects *in vivo* (43). Interestingly, up-regulation of NR4A1 was proposed by Bland *et al.* (44) as a compensatory mechanism for maintaining transcriptional regulation of steroidogenesis in *Sf-1* haploinsufficient mice. Considering the crossover in the regulatory *spectra* of these related nuclear receptors, a role for SF-1 itself in the regulation of adrenal vascular biology is further supported.

The link between developmental processes and tumorigenesis is not novel and an increasing number of examples are being reported in the endocrine and genetic literature. In some cases, loss of function changes in key transcriptional regulators or signaling pathways results in hypoplasia of developing glands and hormone deficiency syndromes. Conversely, overactivity or increased expression of these factors and/or pathways has been associated with tumorigenesis. This is the case for the thyroid transcription factor-1 (TTF-1, encoded by *NKX2–1;* refs. 45, 46) and the thyrotropin receptor (TSHR; refs. 47, 48) in the thyroid and for the luteinizing hormone/choriogonadotropin receptor (LHCGR) in the testis (49, 50). While mitogenic processes might be the key drive in many of these events, the regulation of angiogenesis and vascular remodeling is now emerging as an important additional mechanism.

Taken together, our data provide insight into additional mechanisms by which SF-1 might regulate adrenal development during fetal life. Although these studies were performed predominantly in an adrenal carcinoma-derived cell line, our results might be relevant to the pathophysiological mechanisms promoting adrenal tumorigenesis following somatic SF-1 overexpression. FJ

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