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 endocrine development and tumorigenesis.—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. FASEBJ. 25, 1166–1175 (2011). www.fasebj.org

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

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 manufa-
sheared chromatin from UK). Most resulting chromatin fragments ranged from 200 to 800 bp. Immunoprecipitation was performed in aliquots of sheared chromatin from \( \sim 2 \times 10^6 \) cells using 3 \( \mu \)g SF-1 antibody (07-618; Upstate Millipore UK Ltd., Watford, UK) or 2 \( \mu \)g 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 chromatin (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, Biolabs (NEB) UK Ltd., Hitchin, UK) and 0.1 mM deoxyribose (50 ng), and respective input DNA were amplified by ligation-mediated PCR (New England Biolabs (NEB) UK Ltd., High Wycombe, UK). SF-1-IP, IgG-IP, and NCI-H295R human adrenocortical tumor cells were fixed with 1% formaldehyde and subsequently sonicated in eight 20-s pulses at 2-\( \mu \)m 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 \( \mu \)g SF-1 antibody (07-618; Upstate Millipore UK Ltd., Watford, UK) or 2 \( \mu \)g 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.

**Promoter constructs**

The 5.0-kb 5'-upstream sequences of *CITED2* (transcript ENST00000367561, 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 Pfu 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 ampiclon; 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 (Invitrogen) into pGL4.10[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 \( \pm \) SE of \( \geq 3 \) 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-
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 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 S2.9 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 ± sd 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).

**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 s.d from the mean. Solid line represents a 2-point moving average for the data set.
| MA Z score | Chr | Start  | End    | Region length (bp) | Distance to TSS (bp) | Gene    | Gene name                  | Gene name                  | Selected GO biological process/molecular function                                                                 |
|------------|-----|--------|--------|--------------------|----------------------|---------|----------------------------|----------------------------|-----------------------------------------------------------------------------------------------------------------|
| 8.02       | 19  | 61052867 | 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 | −1513 GRK1 G-protein-coupled receptor kinase 1 | Ion transport; ATP biosynthetic process; response to lipopolysaccharide receptor signaling pathway; elevation of cytosolic calcium ion concentration |
| 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 |                                |                                                                                                                  |
| 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)
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. 1A). 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 antibody.

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                | RRPI | Ribosomal RNA processing 1 homolog (S. cerevisiae) Protein 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   | 12126371 | 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 |
| 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 |
| 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.
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 score of 4.44 (Fig. 1B). 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. 1C). 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. 1C).

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 1A, 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.

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.

ChIP-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        | +                    |                 |                 |
| 3.85       | KPNAA4      | +                    |                 |                 |
| 3.76       | TSPAN17     | +                    |                 |                 |
| 3.73       | DOLK        | +                    |                 |                 |
| 3.70       | ATAD2       | +                    |                 |                 |
| 3.68       | CREB5       | +                    |                 |                 |
| 3.65       | LPCAT4      | +                    |                 |                 |
| 3.63       | NR6A1       | +                    |                 |                 |
| 3.55       | CDC20       | +                    |                 |                 |
| 3.54       | KIF3C       | +                    |                 |                 |

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

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. 3A). 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. 3B). 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. 4A), whereas dose-dependent activation of the 4.5 kb ANGPT2 promoter construct was seen (Fig. 4B). 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.
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 adrenocorticotropic hormone (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 (LH/CG) in the testis (49, 50). While mitogenic processes might be the key driver 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.

The authors are grateful to Rahul Parnaik for performing immunohistochemical studies. The authors thank P. S. H. Soon for making the supplemental material to ref. 32 available, Jacky Pallas and Catherine Riebro for useful discussions, and J. Larry Jameson (Northwestern University, Chicago, IL, USA) and Ronald Evans (Salk Institute, San Diego, CA, USA) for plasmids. Human fetal tissue was provided by the Medical Research Council/Wellcome Trust-funded Human Developmental Biology Resource (http://www.hdbr.org) (MRC G0700089; The Wellcome Trust 082557), with technical assistance from Dianne Gerrelli. This work was supported by a Capes scholarship for making the supplemental material to ref. 32 available, Jacky Jameson, J. L. (1999) A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. Nat. Genet. 22, 125–126. 9. Achermann, J. C., Oishi, G., Ito, M., Orum, U. A., Harmanek, C., Gurkan, B., and Jameson, J. L. (2002) Gonad development and adrenal development are regulated by the orphan nuclear receptor steroidalogenic factor-1, in a dose-dependent manner. J. Clin. Endocrinol. Metab. 87, 1829–1833. 10. Biasin-Lauber, A., and Schoenle, E. J. (2000) Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidalogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. Am. J. Hum. Genet. 67, 1563–1568. 11. Lalli, E. (2010) Adrenocortical development and cancer: focus on SF-1. J. Mol. Endocrinol. 44, 301–307. 12. Figueiredo, B. C., Cavalli, L. R., Pianovski, M. A., Lalli, E., Sandrini, R., Ribeiro, R. C., Zambetti, G., DeLacerda, L., Rodriguez, G. A., and Haddad, B. R. (2005) Amplification of the steroidogenic factor 1 gene in childhood adrenocortical tumours. J. Clin. Endocrinol. Metab. 90, 615–619. 13. Almeida, M. Q., Soares, I. C., Ribeiro, T. C., Fragoso, M. C., Marins, L. V., Wakamatsu, A., Rossio, R. A., Nishi, M. Y., Jorge, A. A., Lerario, A. M., Alves, V. A., Mendonca, B. B., and Latronico, A. C. (2010) Steroidogenic factor 1 overexpression and gene amplification are more frequent in adrenocortical tumours from children than from adults. J. Clin. Endocrinol. Metab. 95, 1458–1462. 14. Sibera, S., Schmull, S., Assis, G., Voelker, H. U., Kraus, L., Beyer, M., Ragazvon, B., Beuschein, F., Willenberg, H. S., Hahner, S., Sager, W., Bertherat, J., Allolio, B., and Fasnacht, M. (2010) High diagnostic and prognostic value of steroidogenic factor-1 expression in adrenal tumours. J. Clin. Endocrinol. Metab. 95, 161–171. 15. Doghmah, M., Karpova, T., Rodrigues, G. A., Arhatte, M., De Moura, J., Cavalli, L. R., Viroille, V., Barbry, P., Zambetti, G. P., Figueiredo, B. C., Heckert, L. L., and Lalli, E. (2007) Increased steroidogenic factor-1 dosage targets adrenocortical cell proliferation and Cancer. Mol. Endocrinol. 21, 2968–2987. 16. Ren, B., Robert, F., Wyrick, J. J., Aparicio, O., Jennings, E. G., Simon, L. Zeitlinger, J., Schreiber, J., Hannett, N., Kanin, E., Volkert, T. L., Wilson, C. J., Bell, S. P., and Young, R. A. (2000) Genome-wide location and function of DNA binding proteins. Science 290, 2306–2309. 17. Oberley, M. J., Tsao, J., Yau, P., and Farnham, P. J. (2004) High-throughput screening of chromatin immunoprecipitates using ChIP-chip and ChIP-seq data. Nat. Biotechnol. 26, 1293–1300.
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