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Contributions of Steroidogenic Factor 1 to the Transcription Landscape of Y1 Mouse Adrenocortical Tumor Cells

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Summary

The contribution of steroidogenic factor 1 (SF-1) to the gene expression profile of Y1 mouse adrenocortical cells was evaluated using short hairpin RNAs to knockdown SF-1. The reduced level of SF-1 RNA was associated with global changes that affected the accumulation of more than 2,000 transcripts. Among the down-regulated transcripts were several with functions in steroidogenesis that were affected to different degrees—*i.e.*, *Mc2r* > *Scarb1* > *Star* ≥ *Hsd3b1* > *Cyp11b1*. For *Star* and *Cyp11b1*, the different levels of expression correlated with the amount of residual SF-1 bound to the proximal promoter regions. The knockdown of SF-1 did not affect the accumulation of *Cyp11a1* transcripts even though the amount of SF-1 bound to the proximal promoter of the gene was reduced to background levels. Our results indicate that transcripts with functions in steroidogenesis vary in their dependence on SF-1 for constitutive expression. On a more global scale, SF-1 knockdown affects the accumulation of a large number of transcripts, most of which are not recognizably involved in steroid hormone biosynthesis.

Keywords

cDNA microarray; chromatin immunoprecipitation; genome-wide transcription profiling; short hairpin RNA (shRNA); steroidogenic factor-1; Y1 mouse adrenocortical tumor cells

1. Introduction

Steroidogenic factor 1 (SF-1) is considered to be a master transcription factor essential for the coordinate and tissue-specific expression of genes with functions in adrenal steroidogenesis (Schimmer and White, 2010). This role of SF-1 is based largely on reporter gene assays using limited promoter/regulatory DNA, which, as noted previously, may

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overemphasis the importance of SF-1 at the expense of more distal regulatory elements (Milstone et al., 1992). Indeed, manipulating SF-1 gene dosage *in vivo* raises some doubts about a master regulatory role for SF-1 in the expression of genes involved in adrenal steroidogenesis.

In SF-1^{+/-} heterozygous mice, the levels of SF-1 are reduced and the adrenal glands are hypoplastic; however, the adrenal cells from these animals paradoxically exhibit increased levels of Cyp11a1, Star and Mc2r—*i.e.*, transcripts putatively under SF-1 control (Bland et al., 2000b; Bland et al., 2004). In humans, heterozygous SF-1 mutations that disrupt its DNA binding activity sometimes cause adrenal insufficiency (Achermann et al., 1999; Biason-Lauber and Schoenle, 2000; Achermann et al., 2002); in other cases, however, they have no effect on adrenal function while at the same time impairing gonadal function (Lin et al., 2007; Kohler et al., 2008). Thus, the requirement for SF-1 in the expression of genes required for adrenal steroidogenesis appears not to be dosage sensitive either in mice or in humans.

Similarly, manipulating SF-1 dosage in H295R human adrenocortical tumor cells by over-expressing wild-type SF-1 (Doghman et al., 2007), by transformation with a dominant negative SF-1 mutant (Li et al., 2004) or by knocking down SF-1 with siRNA (Doghman et al., 2007; Ye et al., 2009) has little or no effect on key enzymes in steroidogenesis such as STAR and CYP11A1. Transcripts encoding HSD3B2 are only modestly reduced in the presence of dominant negative SF-1, but are not affected (Ye et al., 2009) or paradoxically decreased (Doghman et al., 2007) in cells over-expressing SF-1. Taken together, these results suggest that SF-1 may not be an obligatory regulator of adrenal steroidogenesis *in vivo*.

Given the concerns about the relative importance of SF-1 *in situ* discussed above, the present study was undertaken to determine the consequences of SF-1 knockdown on the steroidogenic potential of Y1 adrenocortical tumor cells. As reviewed elsewhere (Rainey et al., 2004), the Y1 mouse adrenocortical cell line has been used widely to study adrenal steroidogenesis and was used quite extensively in the reporter gene assays that gave rise to the hypothesis that SF-1 regulates the expression of genes with functions in steroidogenesis. We report that transcripts with functions in steroidogenesis vary in their dependence on SF-1 for constitutive expression. Transcripts encoding Cyp11a1 and Cyp11b1 are relatively resistant to SF-1 knockdown, whereas transcripts encoding StAR, Mc2r, Hsd3b1 and Scarb1 are considerably more sensitive. To determine if SF-1 has a more global role in the adrenal cortex extending beyond its role in steroidogenesis, we also have examined the genome-wide consequences of SF-1 knockdown in these cells. We observe that SF-1 knockdown alters the transcription landscape of Y1 adrenal cells by affecting the expression of slightly more than 2,000 transcripts, most of which are not recognizably involved in steroid hormone biosynthesis. These latter results strongly suggest that SF-1 has additional functions in the adrenal cortex.

2. Materials and Methods

2.1 Oligonucleotides and plasmids

Gene-specific oligonucleotides were synthesized by Invitrogen Canada Inc. (Burlington, ON; Appendix A). pRNAT-CMV3.1/Neo vectors (Genscript Corp., Piscataway, NJ) expressing shRNAs targeted to three regions of the mouse SF-1 transcript (nucleotides 151–171, 565–585 and 1375–1395 respectively) were prepared as described previously (Rui et al., 2008). A control shRNA vector, with nucleotides 151 – 171 from SF-1 in scrambled order, was obtained from Genscript Corp. The SF-1 expression plasmid harboring a selectable Zeo^r gene was prepared by cloning a *Bam* HI/*Not* I cDNA fragment isolated from

a His-tagged mouse SF-1 expression vector (Frigeri et al., 2000) into the corresponding sites of pcDNA-Zeo+ (Invitrogen Canada, Inc). The resulting construct contained mouse SF-1 cDNA free of His- and other epitope tags.

2.2 Cells, cell culture and DNA-mediated gene transfer

The properties of the ACTH-responsive Y1 mouse adrenocortical tumor cell line used in these experiments and conditions for propagation have been described elsewhere (Rainey et al., 2004). Y1 clones were transfected with plasmids encoding SF-1 shRNAs (10 µg per plate) using a high-efficiency calcium phosphate precipitation technique (Ausubel et al., 2007). Cells were exposed to DNA-calcium phosphate precipitates for 24 h, rinsed, incubated for an additional 24 h in growth medium without antibiotics and then propagated in growth medium containing G418 (100 µg/ml) to select transformants. These latter SF-1 shRNA-transformed cells were transfected with the SF-1 Zeo^r expression plasmid (5 to 10 µg per plate) using calcium phosphate, as described above and then grown in culture medium containing G418 (100 µg/ml) and Zeocyn (200 µg/ml). G418 and Zeocyn were purchased from Invitrogen Canada, Inc. (Burlington, ON).

For experimental manipulations, Y1 cells were plated at a density of 4×10^5 cells per 100 mm dish in Alpha Minimal Essential Medium supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal bovine serum, penicillin G sodium (200 U/ml) and streptomycin sulfate (200 µg/ml); The culture medium also contained G418 and Zeocyn where required to maintain the transformed phenotype. Cells were cultured for three to four days until they reached approximately 40% confluence.

2.3 Steroid production

Cells were plated at a density of 5×10^4 per 60 mm tissue culture dish, propagated for 5-7 days and then incubated for 6 h in 2 ml of α -Minimal Essential Medium containing serum and antibiotics. Steroids were extracted from the incubation medium using methylene chloride and quantitated by fluorescence in acidic ethanol using corticosterone as a standard. This assay previously had been shown to measure the major steroid products of Y1 cells—*i.e.*, 20 α -hydroxyprogesterone and 11 β -20 α -dihydroxyprogesterone (Kowal and Fiedler, 1968).

2.4 RNA isolation and RT-PCR amplification

Total RNA was isolated using either RNeasy Mini (Qiagen Inc., Mississauga, ON) or Macherey-Nagel NucleoSpin RNA II (Clontech Laboratories, Inc. Mountain View, CA) kits. For larger-scale preparations, total RNA was extracted with guanidine thiocyanate and isolated by centrifugation through a cushion of 5.7 M CsCl (Chirgwin et al., 1979). Only the NucleoSpin RNA II Kit included a DNase treatment step; however, RNA prepared using either of the other methods was devoid of DNA contamination and so further treatment of these RNA samples with DNase was considered unnecessary.

Quantitative RT-PCR reactions were carried out as described previously (Schimmer et al., 2006). Essentially, total RNA (5 µg) was reverse transcribed in 20 µl reactions containing oligo-dT₁₈ primer (100 pmol) using Superscript IITM (Invitrogen Canada, Inc.). Aliquots of the RT reaction (1 µl) were amplified over 40 cycles in 25 µl reactions containing gene-specific forward and reverse primers (5 pmol each) using a Platinum SYBR Green qPCR SuperMix UDGTM kit (Invitrogen Canada, Inc.) in a 7300 Real Time PCR System with resident software (Applied Biosystems, Foster City CA). Each amplification cycle consisted of template denaturation at 95°C for 15 sec and primer annealing and extension at 60° C for 60 sec. In each experiment, transketolase mRNA was used as the reference standard to

normalize transcript levels among samples and changes in transcript concentrations were determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.5 Chromatin immunoprecipitation

For chromatin immunoprecipitation assays, cells were treated with 1% formaldehyde for 10 min to cross-link chromatin and isolated nuclei were lysed in 50 mM Tris-HCl (pH 8.1) containing 1% sodium dodecyl sulfate, 10 mM EDTA and a cocktail of protease inhibitors. The lysates were sonicated to shear DNA to an average length of 500 bp, centrifuged and immunocleared using a control IgG in the presence of salmon sperm DNA and protein A-agarose or protein G-agarose. Samples were then incubated overnight at 4 C in the presence of 1 μ g of the indicated IgG from Millipore Corp. (Billerica, MA); immune complexes were recovered by absorption onto protein A- or protein G-agarose beads. The beads were washed extensively, immune complexes were extracted using 1% sodium dodecyl sulfate and 0.1 M NaHCO₃ and the extracted complexes were heated for 4 h at 65 C to reverse cross-links (Winnay and Hammer, 2006). The resultant DNA fragments were purified by resin absorption and elution using a DNA purification kit from Qiagen Inc. and analyzed by quantitative PCR as described above using oligonucleotide primer pairs listed in Appendix A. In each experiment, samples were corrected for total DNA input and normalized to the signal obtained with a control IgG.

2.6 Genome-wide transcription profiling

Transcription profiles were obtained by probing a NIA-15K mouse cDNA microarray with Cy3- and Cy5-labeled cDNA prepared from Y1 and shRNA-transfected clones respectively using a two-color dye hybridization protocol described previously (Schimmer et al., 2006). The microarrays were obtained from The University Health Network Microarray Centre (Toronto, ON, Canada); details are available at www.microarrays.ca. Briefly, total RNA (50 μ g) was reverse-transcribed using Superscript IITM (Invitrogen Canada, Burlington, ON) in a 40- μ l reaction that included oligo-dT₂₀-dVdN (150 pmol) as primer and Cy5-dCTP or Cy3-dCTP (25 μ M; Invitrogen Canada, Inc.) as labeling reagent for control and transfected cells respectively. The fluorescently labeled cDNAs from control and shRNA-transfected cells were combined, purified and applied to microarray slides in a cocktail containing DIG Easy HybTM solution (Roche Diagnostics, Laval, Que), yeast tRNA (Invitrogen Canada, Inc.) and salmon sperm DNA (Sigma/Aldrich Canada, Oakville, ON). Slides then were incubated overnight at 37° C in a sealed humidified chamber, washed and dried by centrifugation. Cy5 and Cy3 signals on the array were quantitated using an Axon GenePix 4000A laser scanner and Axon GenePixTM software (Molecular Devices, Downingtown, PA). On-slide data analyses, including the assignment of the Cy5 and Cy3 signals to specific cDNAs on each array and the application of the Lowess smoothing function to correct for global differences in Cy5 and Cy3 incorporation, were performed with GeneTrafficTM software (Stratagene, La Jolla, CA). Transcription profile differences between parent and SF-1 knockdown clones were analyzed using the SAM algorithm from Stanford University (Tusher et al., 2001) with a two-class (unpaired) analysis and false discovery rate ≤ 0.05 . The two-class analysis compares the ratios of Cy5 to Cy3 signals obtained using RNA from Y1 control cells and shRNA-transfected cells respectively to the ratios of signals obtained using RNA samples from a group of independent control cells separately labeled with Cy3 and Cy5. The two-class analysis controls for gene-specific variances in the data—*e.g.*, variances caused by preferential labeling of transcripts with either the Cy5 or Cy3 dye (Tseng et al., 2001). Annotations for the cDNAs spotted on the arrays were retrieved from the Stanford Online Universal Resource for Clones and ESTs (S.O.U.R.C.E.) database (<http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch>); only those changes affecting well-annotated transcripts are reported here.

The set of transcripts significantly affected by SF-1 knockdown in microarray experiments was tested for enrichment in specific categories relative to their representation on the NIA 15K mouse cDNA array using a false discovery rate ≤ 0.05 as described in detail previously (Schimmer et al., 2006).

3. Results

3.1 The levels of SF-1 and other transcripts with functions in steroidogenesis in shRNA-transformed clones

Ten G418-resistant clones, isolated from Y1 cells following transfection with plasmids encoding SF-1 shRNA, were screened at first passage for SF-1 transcripts by quantitative RT-PCR. Eight of these clones had transcript levels that were reduced by at least 75% (range from 9% to 23%) compared to untransfected cells (Fig. 1). These eight included isolates transformed with each of the three shRNAs tested, indicating that each vector was capable of knocking down SF-1. A control plasmid with a scrambled SF1 shRNA sequence did not affect SF-1 levels (data not shown), indicating that the knockdown of SF-1 was a specific effect of the SF-1 shRNA.

To evaluate the consequences of SF-1 knockdown in Y1 cells on steroidogenic potential, seven of the SF-1 shRNA transformants were examined by quantitative RT-PCR for transcripts derived from primary SF-1 targets in the adrenal cortex (reviewed in ref. Schimmer and White, 2010)—i.e., *Cyp11a1*, *Cyp11b1*, *Hsd3b1*, *Star*, *Mc2r* and *Scarb1*. Other targets of SF-1 in the adrenal cortex, such as *Cyp17*, *Cyp21* and *NR0B1* are not expressed in these cells (Parker et al., 1985; Lund et al., 1990; Xu et al., 2009) and thus were not examined. SF-1 knockdown was associated with a decrease in *Mc2r* transcripts to undetectable levels and with marked reductions in the levels of transcripts encoding *StAR* and *Hsd3b1* (73% and 77% decreases, respectively) and *Scarb1* (more than 90% reduction) (Fig. 2). Transcripts encoding *Cyp11b1* were reduced by only 30%, whereas *Cyp11a1* transcripts were unaffected by the decrease in SF-1 (Fig. 2). These effects of the SF-1 shRNA vector were specific and were not seen when Y1 cells were transfected with the scrambled SF-1 shRNA control vector (data not shown). In contrast, transfection of the SF-1 knockdown clones with an expression vector encoding mouse SF-1 increased SF-1 transcript accumulation and also increased the accumulation of transcripts for *StAR*, *Cyp11b1*, *Scarb1*, *Mc2r* and *Hsd3b1* (Fig. 3). *Cyp11a1*, a transcript not significantly affected by SF-1 knockdown (Fig. 2), also was not affected in the SF-1 transfected cells (Fig. 3). As shown in Table 1, SF-1 knockdown was accompanied by a more than 20-fold decrease in steroid output, whereas the restoration of SF-1 expression increased steroid output to levels seen in parental Y1 cells.

3.2. Interactions of SF-1 with the proximal promoters of *StAR*, *Cyp11a1* and *Cyp11b1*

The very different effects of SF-1 knockdown on the accumulation of transcripts with functions in steroidogenesis (Fig. 2) suggested that the genes encoding these transcripts differed in their dependence upon SF-1 for expression. To explore the basis for this difference, we compared the binding of SF-1 to the proximal promoter regions of *Star*, *Cyp11a1* and *Cyp11b1* by chromatin immunoprecipitation. SF-1 antibodies precipitated greater amounts of promoter DNA from the *Star*, *Cyp11b1* and *Cyp11a1* genes in parent Y1 cells than did a control IgG (Fig. 4A), indicating the presence of SF-1 at these promoters. In the knockdown clones, the binding of SF-1 to the *Cyp11b1* promoter was reduced by only 40%, whereas the binding of SF-1 to the *Star* and *Cyp11a1* promoters were reduced to the background level obtained with the control IgG (Fig 4A). Thus, the residual levels of SF-1 in the knockdown clones were sufficient to bind to the *Cyp11b1* proximal promoter, but insufficient to bind to the *Star* and *Cyp11a1* promoters.

To assess the consequences of SF-1 knockdown on the overall transcriptional activity of these genes, we also examined the binding of RNA polymerase II to their proximal promoter regions, using an antibody directed towards the carboxyl-terminal repeat region of the enzyme's largest subunit (Palancade and Bensaude, 2003). As shown in Figure 4B, the RNA polymerase II antibodies precipitated appreciably greater amounts of promoter DNA from the *Star*, *Cyp11b1* and *Cyp11a1* genes in parent Y1 cells than did the control IgG. In the SF-1 knockdown clones, the binding of RNA polymerase II to the *Star* promoter was reduced by 70%, whereas the binding of RNA polymerase II to the *Cyp11a1* and to the *Cyp11b1* promoters were reduced by only 22% and 27%, respectively (Fig. 4B), reflecting the level of transcriptional activity at each promoter.

3.3. Transcription profiling of SF-1 knockdown clones

We next examined the effects of SF-1 knockdown on transcript accumulation in the Y1 adrenocortical cell line by microarray analysis. SF-1 knockdown was associated with an increase in 1033 transcripts and a decrease in 1002 transcripts, indicating global effects of SF-1 knockdown on the transcription landscape of Y1 adrenal cells (Appendix B). The list of down-regulated transcripts contained SF-1 (*Nr5a1*), *Star*, *Hsd3b1* and *Scarb1*, as expected from the qPCR studies above (Fig. 2). *Cyp11b1* was not represented on the arrays and variations in the signals for *mc2r* transcripts on the arrays resulted in changes that failed to reach statistical significance, despite the dramatic decrease in accumulation seen by RT-qPCR (Fig. 2).

We next examined the list of transcripts for sets that were enriched for specific biological processes, relative to their representation on the cDNA microarray, since such enrichment implies the coordinate expression of genes that is connotative of specific regulatory pathways (Tavazoie et al., 1999). The transcripts that increased in response to SF-1 knockdown were enriched only for those with functions in actin filament-based processes, including cytoskeleton organization and biogenesis, relative to their representation on the arrays; the transcripts that decreased in response to SF-1 knockdown were enriched only for those with functions in ribosome and ribonucleoprotein complex biogenesis and assembly, and translation elongation. These transcripts and their fold-changes after SF-1 knockdown are listed in Table 2. A total of 35 transcripts from Table 2—i.e., 16 from the up-regulated set and 19 from the down-regulated set—were selected for analysis by quantitative RT-PCR. Of these, 32 exhibited significant changes after SF-1 knockdown confirming the microarray results; three transcripts in this set exhibited small changes that failed to reach statistical significance, possibly due to limitations in the sensitivity of quantitative PCR (Table 3).

Twelve of the transcripts most affected by the SF-1 shRNA (six from the up-regulated group and six from the down-regulated group in Table 2) also were examined in Y1 cells stably transformed with a control shRNA vector and in SF-1 knockdown clones stably transformed with the SF-1 expression vector. Neither the control shRNA vector nor the SF-1 expression vector had any effect on the levels of these transcripts (data not shown), indicating that the changes in the levels of these transcripts resulted specifically from transfection with the SF-1 shRNA vector but were not reversed upon over-expression of SF-1 expression.

4. Discussion

This study demonstrates that SF-1 shRNAs targeting the SF-1 transcript greatly diminished the accumulation of transcripts encoding *Mc2r* (the ACTH receptor), *Scarb1* (the HDL receptor that participates in cholesterol uptake), *Star* (a transport protein that carries cholesterol across mitochondrial membranes) and *Hsd3b1* (a steroid reductase that converts pregnenolone to progesterone) in Y1 mouse adrenal cells, while reducing the accumulation of SF-1 transcripts by at least 75% (Fig. 2). These effects were specifically related to the

knockdown of SF-1 since they were not duplicated by a scrambled SF-1 shRNA vector, they were reversed when SF-1 expression was restored (Fig. 3) and they translated into a reduction in steroid biosynthesis, as evidenced by a greatly diminished level of steroid output (Table 1). Nonetheless, SF-1 knockdown did not affect each of these steroidogenic transcripts in the same way. Transcripts encoding the *Mc2r* were the most affected, with only traces remaining after SF-1 knockdown, *Scarb1* was reduced to < 10% of its original level and *Star* and *Hsd3b1* were each reduced to approximately 25% of their original levels. In contrast, SF-1 shRNA had no effect on the accumulation of the transcripts encoding *Cyp11a1* (the cholesterol side chain cleavage enzyme) and only modestly diminished the accumulation of transcripts encoding *Cyp11b1* (i.e., the steroid 11 β -hydroxylase; Fig. 2). It is possible that these different responses to SF-1 knockdown reflected either the relative contributions of other transcription factors to promoter activity or different affinities of the corresponding promoters to SF-1. To test the latter possibility, the ability of the residual SF-1 in the knockdown clones to bind to the proximal promoter regions of *Star*, *Cyp11a1* and *Cyp11b1* was assessed by ChIP analysis (Fig. 4). The *Cyp11b1* proximal promoter was able to bind the residual SF-1 in the knockdown clones, suggesting that this level of SF-1 was sufficient to drive *Cyp11b1* expression. On the other hand, the proximal promoters of *Star* and *Cyp11a1* did not bind the residual SF-1, suggesting that the continued expression of these transcripts in the SF1 knockdown clones was due to the contribution of other transcription factors. Inasmuch as, the *Star* and *Cyp11a1* promoters have been shown to interact with other transcription factors, including GATA proteins, members of the *NR4A* family and CREB (Lavoie and King, 2009; Nakamura et al., 2009), these factors may have contributed, in varying degrees, to the expression of the latter steroidogenic transcripts in Y1 cells.

Whereas our finding of SF-1 independent *Cyp11a1* expression in Y1 mouse adrenal cells agrees with findings in H295R human adrenal cells, our finding that SF-1 is required for the optimal expression of *Star*, *Hsd3b1* and *Cyp11b1* differ (Doghman et al., 2007; Ye et al., 2009). These differences may reflect species differences among the gene promoters, intrinsic differences between Y1 and H295R cells or differences in serum and other growth factor supplements added to the cell culture medium. Our results also differ from studies of SF-1^{+/-} mice showing that the expression of *Star* and *Mc2r* are independent of SF-1 gene dosage (Bland et al., 2000b); however, the heterozygous animals used in the latter study appear to retain 80% of their original SF-1 levels (Bland et al., 2000a), which may be sufficient to support the expression of these genes.

It is clear that the functions of SF-1 in the adrenal cortex are not restricted to the regulation of steroidogenesis. SF-1 also plays essential roles in the differentiation and development of the adrenal gland as revealed through studies of SF-1 disrupted mice (Luo et al., 1994) and in adrenal proliferation as revealed in studies of increased SF-1 dosage in H295R adrenal cells (Doghman et al., 2007) and in mouse and human adrenal tumors (Pianovski et al., 2006; Doghman et al., 2009). As determined by gene expression profiling, increasing SF-1 dosage in H295R adrenal cells results in the enrichment of transcript clusters with functions in lipid and steroid metabolism, apoptosis and cell cycle regulation, cell adhesion and extracellular matrix (Doghman et al., 2007). In Y1 adrenal cells, SF-1 knockdown affected the accumulation of over 2000 transcripts, suggesting that SF-1 has a broad spectrum of actions (Appendix B); however the functional significance of these effects is unknown. While some of the affected transcripts have functions in steroidogenesis and cell proliferation, the numbers of transcripts affected in either category are insufficient to achieve enrichment relative to their representation on the microarray. This contrasts with ACTH-stimulated Y1 cells, which are enriched in transcripts with functions in steroidogenesis and cell proliferation (Schimmer et al., 2006). The absence of global changes in transcripts associated with cell proliferation following SF-1 knockdown is not

surprising. Inasmuch as the knockdown clones were selected on the basis of their ability to proliferate in the presence of G418, surviving colonies would have had to compensate for the anticipated growth inhibitory effects associated with SF-1 knockdown. The difference between ACTH stimulation and SF-1 knockdown with respect to steroidogenesis, at least in part, results from an ACTH-induced up-regulation of transcripts with functions in cholesterol biosynthesis (Schimmer et al., 2006); these transcripts are not affected in the SF-1 knockdown clones (Appendix B). Thus, ACTH appears to have a much broader role in regulating steroidogenesis than does SF-1.

Although the SF-1 knockdown clones exhibit a down-regulation of transcripts with functions in ribosome and ribonucleoprotein complex biogenesis and translation elongation and an up-regulation of transcripts with functions in actin filament-based processes (Table 2), we cannot be certain that these effects are a direct consequence of SF-1 knockdown, since they are not reversed when SF-1 levels are restored. It is unlikely that these changes result from non-specific effects of RNA-mediated RNA breakdown, since they are not mimicked by the scrambled SF-1 shRNA vector or by another shRNA vector targeted to an unrelated transcript (data not shown).

Whereas most studies of SF-1 function have focused on its role as an activator of transcription, its ability to form complexes with transcription corepressors (reviewed in ref. Schimmer and White, 2010) and inhibitory ligands (Urs et al., 2006) suggests that SF-1 also may have repressor activity in certain contexts. The best example of SF-1 dependent gene repression to date is in the regulation of *Adcy4* expression (Rui et al., 2008). In this example, disruption of a non-canonical SF-1 binding site in the proximal promoter region of mouse *Adcy4* enhances *Adcy4* promoter activity, over-expression of SF-1 inhibits *Adcy4* promoter activity and shRNA-mediated knockdown of SF-1 increases endogenous *Adcy4* expression. It is thus possible that the large number of up-regulated transcripts resulting from SF-1 knockdown (Appendix B) include a subset that is under negative control by SF-1.

In conclusion, our findings establish major regulatory roles for SF-1 in the expression of genes encoding key enzymes in adrenal steroidogenesis *in situ* and generally are consistent with conclusions based on the activities of promoter/regulatory DNA elements in reporter gene assays (reviewed in ref. Parker and Schimmer, 1997). The exception is *Cyp11a1*, which was not affected following SF-1 knockdown and thus seems not to require SF-1 for expression. SF-1 also has global effects on the adrenal transcription landscape that extend beyond the regulation of steroidogenesis. These global effects are evidenced by changes in over 2,000 transcripts, of which approximately half are up-regulated and half are down-regulated, following SF-1 knockdown. The precise roles of these transcripts in adrenal cell function and the roles of SF-1 in the regulation of these transcripts have yet to be determined. We speculate that some of these changes reflect direct actions of SF-1 on novel, non-steroidogenic targets to both activate and repress gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NIA National Institute of Aging

SF-1 steroidogenic factor 1
shRNA short hairpin RNA

Figure 1. shRNA-mediated knockdown of SF-1

Ten clones were randomly isolated from Y1 cells stably transformed with plasmids encoding SF-1 shRNA and screened for SF-1 transcripts by quantitative RT-PCR. Each clone contains a prefix that identifies the specific SF-1 nucleotides targeted by the knockdown vector as follows: #1-, SF-1 nucleotides 151-171; #2-, SF-1 nucleotides 565-585; #3-, SF-1 nucleotides 1375-1395. The levels of SF-1 RNA were normalized to the levels in parental Y1 cells using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Figure 2. Effects of SF-1 knockdown on transcripts with functions in steroidogenesis

The levels of transcripts encoding SF-1, Mc2r, StAR, Hsd3b1, Cyp11b1 and Cyp11a1 were measured by quantitative RT-PCR in seven SF-1 shRNA-transformed clones at first passage (*white bars*) and normalized to the levels in up to eight different preparations of parent Y1 cells (*black bars*) as described in Figure 1. The levels of Scarb1 RNA were determined in four SF-1 shRNA transformants after several passages and normalized to the levels in four different preparations of parent Y1 cells. In each case, results are presented as means $\pm$ S.E.M. Statistical significance was determined by 2-way ANOVA and Bonferroni post-hoc test (** $p < 0.001$; * $p < 0.05$).

Figure 3. Effects of SF-1 expression on the SF-1 knockdown phenotype

The levels of transcripts encoding SF-1, StAR, Cyp11a1, Cyp11b1, Scarb1, Mc2r and Hsd3b1 were measured by quantitative RT-PCR in a total of six isolates recovered from two different SF-1 shRNA-transformed clones transfected with an expression vector encoding SF-1 (*black bars*) and normalized to the levels of transcripts obtained in a total of four isolates recovered from the same SF-1 shRNA-transformed clones transfected with an empty expression vector (*white bars*). Results were calculated as described in Figure 1 and are presented as means $\pm$ S.E.M. Statistical significance was determined using the Bonferroni's Multiple Comparison Test (** $p < 0.001$; ns, not significant).

Figure 4. Chromatin immunoprecipitation of genomic DNA fragments bound to SF-1 and to RNA polymerase II

Chromatin was immunoprecipitated from two independent cultures of parent Y1 cells and two independent SF-1 knockdown clones using antibodies to SF-1 (*panel A*) or RNA polymerase II (*panel B*) and the recovered DNA fragments were analyzed by quantitative PCR as described in Materials and Methods. Results were normalized to the amount of product obtained with the control IgG as described in the legend to Figure 1.

Table 1
Effects of SF-1 knockdown on steroid output

Parent Y1 cells, one SF-1 shRNA transformant (#3-8) and the same transformant transfected with the SF-1 expression vector were assayed for steroid production under basal conditions as described in Materials and Methods. Results are expressed as means of three independent determinations $\pm$ S.E.M. Statistical significance was determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test

Cell line	Steroids	p value ^a
	(μ g/mg protein)	
Y1	29.04 $\pm$ 0.98	
+SF-1 shRNA	1.31 $\pm$ 0.2	0.001
+SF-1 shRNA + SF-1Zeo	23.47 $\pm$ 10.07	> 0.05

^a Versus steroid output in untransformed Y1 cells

Table 2
Transcripts affected by SF-1 knockdown and enriched for specific biological processes

Transcripts affected by SF-1 knockdown, as determined from microarray analysis (Appendix B), were assessed for enrichment in specific processes as described in Materials and Methods. Listed are the up-regulated transcripts with functions in actin filament-based processes and the down-regulated transcripts with functions in ribosome and ribonucleoprotein complex biogenesis and translation elongation (full gene names are provided in Appendix B). Included are the fold-changes in transcript accumulation following SF-1 knockdown and false discovery rates (q) as extracted from the Appendix B.

Symbol	Fold Change	q (%)	Symbol	Fold Change	q (%)	Symbol	Fold Change	q (%)
Actin filament-based processes								
Cav1	2.26	0.06	Arpc2	1.31	0.06	Rock1	1.31	1.58
Actn2	2.18	0.06	Llgl1	1.57	0.19	Cdc42	1.23	1.58
Sorbs1	2.14	0.06	Spna2	1.70	0.22	Trpm7	1.86	1.88
Itgb1	2.14	0.06	Ccnb1	1.46	0.22	Shc1	1.20	1.88
Tmod3	2.06	0.06	Arpc5	1.30	0.22	Lims1	1.16	1.88
Actn1	2.03	0.06	Rac1	1.30	0.40	Anxa6	1.33	2.33
Cnn2	1.98	0.06	Pard3	1.30	0.54	Ptkci	1.21	2.98
Fhod3	1.94	0.06	Myl6	1.27	0.63	Ube2e	1.13	2.98
Anh1	1.92	0.06	Prc1	1.20	0.63	Macf1	1.25	3.48
Cnn3	1.83	0.06	Nusap1	1.22	0.81	Myh9	1.18	3.48
Cald1	1.70	0.06	Ppp1cb	1.19	0.81	Rhoa	1.17	3.48
Tnnsb10	1.69	0.06	Tpm2	1.19	0.81	Dbn1	1.19	4.16
Spnb2	1.66	0.06	Actg1	1.27	1.02	Cks2	1.23	4.84
Dstn	1.50	0.06	Sept2	1.18	1.02	Dbn1	1.18	4.84
Tpm3	1.36	0.06	Svil	1.31	1.21	Arhgap6	1.14	4.84
Actn3	1.35	0.06	Abl2	1.16	1.21	Calr	1.10	4.84
Cttna1	1.35	0.06	Tbx20	1.50	1.58	Arg2	1.10	4.84
Pdgfra	1.35	0.06	Vil2	1.45	1.58			
Ctfl1	1.34	0.06	Arhgap8	1.33	1.58			
Ribosome and ribonucleoprotein complex biogenesis								
Ipo9	0.47	0.06	Rps6ka1	0.70	0.22	Snrpd1	0.85	1.58
Rps14	0.54	0.06	Mbnl1	0.71	0.22	Eif4e	0.79	2.98
Rpl26	0.55	0.06	Emg1	0.74	0.22	Bzw2	0.86	2.98
Rps8	0.55	0.06	Mphosph10	0.75	0.22	Rpl6	0.82	4.16

Symbol	Fold Change	q (%)	Symbol	Fold Change	q (%)	Symbol	Fold Change	q (%)
Rpl22	0.57	0.06	Nhp21l	0.77	0.22	Sfrs3	0.86	4.16
Rps7	0.58	0.06	Nola2	0.79	0.22	Rrs1	0.86	4.16
Rpl12	0.60	0.06	Dazl	0.81	0.33	Imp3	0.87	4.16
Rps29	0.62	0.06	Rps4x	0.82	0.33	Eif4g1	0.83	4.84
Rps5	0.64	0.06	Eif2s2	0.81	0.54	Exosc4	0.85	4.84
Snrpd2	0.66	0.06	Dkc1	0.83	0.54	Mrpl23	0.86	4.84
Rpl27	0.67	0.06	Utp11l	0.82	0.63	Mrpl10	0.87	4.84
Rpl29	0.69	0.06	Rpl37a	0.84	0.63	Utp18	0.88	4.84
Rps6	0.70	0.06	Fcfl	0.82	0.81	Translation elongation		
Dimt1	0.72	0.06	Nip7	0.83	1.02	Srp14	0.62	0.06
Rps24	0.75	0.06	Eif5	0.83	1.02	Tsfm	0.69	0.06
Pa2g4	0.75	0.06	Eif1a	0.83	1.58	Eef1d	0.78	0.06
Rps15	0.69	0.06	Nol5	0.85	1.58	Rplp2	0.66	0.06
Ddx1	0.73	0.06	Rps16	0.82	0.81	Rplp1	0.60	0.06
Rpl36a	0.74	0.06	Rpl7	0.83	1.02	Srp9	0.79	1.02
Rps3a	0.75	0.06	Eif5a2	0.77	1.58	Eef1g	0.88	2.98
Bxdc2	0.81	0.06	Eif1	0.85	1.58	Eef1b2	0.88	2.98

Table 3
Quantitative analysis of selected transcripts enriched for biological processes following SF-1 knockdown

Transcripts selected from Table 2 were analyzed by quantitative RT-PCR as described in Materials and Methods. Results are expressed as the levels of transcripts in the SF-1 knockdown clones (+SF-1 *shRNA*) relative to the levels in untransfected Y1 cells (-SF-1 *shRNA*), calculated as described in Figure 1. Statistical significance was determined by ANOVA, and the Newman-Keuls multiple comparison post hoc test (*^{***}, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

Gene ID	-SF-1 shRNA	+SF1 shRNA	
	Mean $\pm$ S.D.	N ^a	Mean $\pm$ S.D. N ^b p
<i>Up-regulated transcripts</i>			
Sorbs1	1.00 $\pm$ 0.03	4	4.21 $\pm$ 2.27 4 ***
Tmod3	1.00 $\pm$ 0.09	4	4.17 $\pm$ 0.38 4 ***
Cnn2	1.01 $\pm$ 0.15	4	3.58 $\pm$ 1.74 4 ***
Cav1	1.00 $\pm$ 0.09	4	3.32 $\pm$ 0.88 4 ***
Fhod3	1.00 $\pm$ 0.09	4	3.03 $\pm$ 0.72 4 ***
Pdgfra	1.01 $\pm$ 0.21	4	2.46 $\pm$ 0.31 4 ***
Ccnb1	1.14 $\pm$ 0.66	4	2.37 $\pm$ 0.48 4 ***
Igfb1	1.01 $\pm$ 0.12	4	2.32 $\pm$ 0.34 4 ***
Dstin	1.00 $\pm$ 0.07	4	2.23 $\pm$ 0.50 4 ***
Spnb2	1.02 $\pm$ 0.25	4	2.21 $\pm$ 0.23 3 ***
Anln	1.01 $\pm$ 0.19	4	2.12 $\pm$ 0.25 4 ***
Cnn3	1.00 $\pm$ 0.87	4	2.11 $\pm$ 0.86 4 ***
Cttna1	1.02 $\pm$ 0.24	4	1.77 $\pm$ 0.10 4 **
Spna2	1.00 $\pm$ 0.88	4	1.40 $\pm$ 0.75 4 *
Apc2	1.00 $\pm$ 0.91	4	1.32 $\pm$ 0.79 4 ns
Ctfl	1.00 $\pm$ 0.93	4	1.20 $\pm$ 0.76 4 ns
<i>Down-regulated transcripts</i>			
Rps14	1.01 $\pm$ 0.16	4	0.43 $\pm$ 0.20 3 ***
Bxdc2	1.00 $\pm$ 0.08	4	0.5 $\pm$ 0.07 4 ***
Rps7	1.00 $\pm$ 0.04	4	0.58 $\pm$ 0.05 3 ***
Rpl12	1.00 $\pm$ 0.11	4	0.60 $\pm$ 0.07 3 ***
Rps8	1.00 $\pm$ 0.08	4	0.61 $\pm$ 0.07 3 ***

Gene ID	-SF-1 shRNA		+ SF1 shRNA		p
	Mean ± S.D.	N ^a	Mean ± S.D.	N ^b	
Rpl36a	1.00 ± 0.05	4	0.65 ± 0.05	4	***
Rps15	1.00 ± 0.11	4	0.68 ± 0.04	4	***
Rps5	1.00 ± 0.03	4	0.69 ± 0.04	3	**
Rplp1	1.00 ± 0.08	4	0.69 ± 0.05	3	***
Rpl27	1.01 ± 0.17	4	0.69 ± 0.06	4	***
Srp14	1.00 ± 0.08	4	0.69 ± 0.09	3	***
Tsfn	1.00 ± 0.11	4	0.70 ± 0.06	4	***
Rps6	1.00 ± 0.04	4	0.74 ± 0.04	4	**
Rps29	1.00 ± 0.04	4	0.74 ± 0.04	3	**
Rplp2	1.00 ± 0.03	4	0.74 ± 0.08	3	**
Rps3a	1.01 ± 0.12	4	0.75 ± 0.09	4	**
Snrpd2	1.00 ± 0.09	4	0.78 ± 0.11	4	**
Pa2g4	1.00 ± 0.10	4	0.80 ± 0.05	4	**
Eef1d	1.00 ± 0.02	4	0.91 ± 0.07	4	ns

^aThe number of independent samples of Y1 adrenal cells analyzed

^bThe number of independent clones evaluated