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POD-1 binding to the E-box sequence inhibits SF-1 and StAR expression in human adrenocortical tumor cells

Monica Malheiros França[a], **Bruno Ferraz-de-Souza**[b], **Mariza Gerdulo Santos**[c], **Antonio Marcondes Lerario**[c], **Maria Candida Barisson Villares Fragoso**[c], **Ana Claudia Latronico**[c], **Rork D. Kuick**[d], **Gary D. Hammer**[e], and **Claudimara F.P. Lotfi**[a,*]

[a]Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, São Paulo 05508-900, SP, Brazil

[b]Laboratory of Carbohydrates and Radioimmunoassays (LIM-18), School of Medicine, University of São Paulo, São Paulo 01246-903, SP, Brazil

[c]Laboratory of Hormones and Molecular Genetics (LIM-42), Division of Endocrinology, School of Medicine, University of São Paulo, São Paulo 01246-903, SP, Brazil

[d]Biostatistics Core of the Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI 48109-2200, USA

[e]Department of Internal Medicine, Metabolism, Endocrinology and Diabetes, University of Michigan, Ann Arbor, MI 48109-2200, USA

Abstract

Pod-1/*Tcf21* is expressed at epithelial-mesenchymal interaction sites during development of many organs. Different approaches have demonstrated that Pod-1 transcriptionally inhibits *Sf-1/NR5A1* during gonadal development. Disruption of Sf-1 can lead to disorders of adrenal development, while increased dosage of SF-1 has been related to increased adrenal cell proliferation and tumorigenesis. In this study, we analyzed whether POD-1 overexpression inhibits the endogenous Sf-1 expression in human and mouse adrenocortical tumor cells. Cells were transiently transfected with luciferase reporter gene under the control of *Sf-1* promoter and with an expression vector encoding Pod-1. Pod-1 construct inhibited the transcription of the *Sf1/Luc* reporter gene in a dose-dependent manner in mouse Y-1 adrenocortical carcinoma (ACC) cells, and inhibited endogenous *SF-1* expression in the human H295R and ACC-T36 adrenocortical carcinoma cells. These results were validated by chromatin immunoprecipitation assay with POD-1-transfected H295R cells using primers specific to E-box sequence in SF-1 promoter region, indicating that POD-1 binds to the *SF-1* E-box promoter. Moreover, POD-1 over-expression resulted in a decrease in expression of the SF-1 target gene, StAR (Steroidogenic Acute Regulatory Protein). Lastly, while the induced expression of POD-1 did not affect the cell viability of H295R/POD-1 or ACC-T36/POD-1 cells, the most significantly enriched KEGG pathways for genes negatively correlated to POD-1/TCF21 in 33 human ACCs were those associated with cell cycle genes.

*Corresponding author. Tel.: +55 11 3091 7492; fax: +55 11 3091 7366. clotfi@usp.br (C.F.P. Lotfi).

Keywords

POD-1; *TCF21*; *NR5A1*; SF-1; E-box; Human adrenocortical tumor cells

1. Introduction

Pod-1 (capsulin, epicardin, *Tcf21*) is a basic helix-loop-helix (bHLH) transcriptional regulatory protein expressed in mesenchymal cells at sites of epithelial-mesenchymal interactions in the developing urogenital, cardiovascular, respiratory, and gastrointestinal systems (Quaggin et al., 1998; Quaggin, 2002; Robb et al., 1998; Lu et al., 1998). Loss of Pod-1 leads to lung, kidney, and spleen abnormalities and neonatal lethality (Quaggin et al., 1999 and Lu et al., 2000).

Pod-1 is expressed in the urogenital ridge, which gives rise to gonadal and adrenal tissues later in embryonic development (Tamura et al., 2001). In adrenal glands, Pod-1 is expressed exclusively in the capsule as shown in mice with a lacZ reporter under the control of Pod-1 regulatory region (Kim et al., 2009). Different approaches have demonstrated that Pod-1 transcriptionally inhibits steroidogenic factor 1 (Sf-1/Ad4Bp/*Nr5a1*), an orphan nuclear receptor required for gonadal development (Tamura et al., 2001; Cui et al., 2004). During human and mouse development, SF-1 expression drives the differentiation of the steroidogenic lineages (Luo et al., 1994). Increased *SF-1* dosage can augment human adrenal cell proliferation, playing a critical role in adrenocortical tumorigenesis (Doghman et al., 2007). Indeed, SF-1 expression level in ACC is a prognostic factor for this type of cancer (Pianovski et al., 2006; Almeida et al., 2010; Sbiera et al., 2010). On the other hand, *POD-1* is downregulated in lung cancer and melanoma (Smith et al., 2006; Arab et al., 2011).

In this study, we examined the correlation of POD-1 expression to gene expression profiles in sporadic ACC and observed a significant decrease in expression in ACC versus benign adrenocortical adenomas (ACAs) and normal adrenocortical tissue. To determine whether a down-regulation of POD-1 expression might contribute to the elevated expression of SF-1 in a subset of ACC samples, we over-expressed POD-1 in two different human adrenocortical carcinoma cell lines, NCI-H295R and ACC-T36. POD-1 overexpression inhibited endogenous *SF-1* expression through binding to the E-box sequence of *SF-1* promoter in adrenocortical tumor cells. Moreover, POD-1 levels in human ACC samples inversely correlate with a number of genes associated with the cell cycle, including SF-1, supporting a regulatory role for of POD-1 in gene expression in ACC.

2. Materials and methods

2.1. Cell cultures

Y1 mouse adrenocortical tumor cell line (Yasumura et al., 1966) and TM3 mouse Leydig cell line (Mather, 1980) were grown, respectively, in DMEM supplemented with 12.5% horse serum and 2.5% fetal bovine serum, and 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5% horse serum and 2.5% bovine serum. The H295R human adrenocortical tumor cell line (Gazdar et al., 1990) was cultured in RPMI (Gibco, USA)

supplemented with 2% fetal bovine serum (Gibco, USA) and 1% Insulin-Transferrin-Selenium (Gibco, USA). ACC-T36 cells were generated by serial passage (ten passages) of a primary culture of an adrenocortical carcinoma presenting in a 38 year-old woman. Adrenal tumor fragments were obtained in the Clinical Medicine Department of Medicine School, Hospital das Clínicas from University of São Paulo. The tumor fragments were transported in sterile ice-cold DMEM (Dulbeco's Modified Eagle's Medium – Gibco) with antibiotics (25 mg/l of ampicillin and 100 mg/l of streptomycin - Sigma) washed several times in Phosphate Buffer Saline (PBS) at 37 °C and dissected from fat and necrotic tissues in a sterile environment. After digestion by sequential 4 mg/ml collagenase plus 1 μg/ml DNaseI (Gibco) at 37 °C in a humidified atmosphere of 95% air/5% CO_2, cells were mechanical disaggregated with gentile movements in a volumetric pipette. The digested material was then filtered in a 100 μm nylon filter, to retain non-digested material and pelleted at 700 rpm for 10 min. Dispersed cells were suspended in DMEM containing 10% FBS (fetal bovine serum), 25 mg/l of ampicillin and 100 mg/l of streptomycin, and then plated in 12.5 cm^2 Falcon flasks (Becton Dickinson, Franklin Lakes, NJ, USA). Medium renewal or subculturing was carried every 2 or 3-days. The protocol was approved by the Ethics Committee for Research Projects Analysis (CAPPesq) from Medicine School and by Ethics Committee for Research in Humans of Institute of Biomedical Sciences of University of São Paulo with protocol number 072/06. All cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2. Transfection assay

Y1 and TM3 cells were plated and transfected using 2 μg pcDNA3/Pod-1 or pcDNA3 in combination with Fulgene HD (Roche, Germany). NCI-H295R cell line and ACC cells were transiently transfected with pCMVMyc-Pod1, kindly provided by Dr. Masataka Nakamura (Tokyo Medical University, Japan) as described earlier (Funato et al., 2003) or with pcDNA3Pod1. 1.5×10^5 cells were plated and transfected with 2 μg of plasmid DNA in combination with 2 μl X-tremeGENE HP-DNA transfection reagent® (Roche, Germany). After 24 or 48 h of transfection, total RNA was extracted using Trizol® reagent (Invitrogen, USA) and treated with TURBO DNA-free™ (Ambion) before obtaining cDNA.

2.3. Luciferase reporter gene assays

Y1 and TM3 cells were plated in triplicate and 24 h after plating were transfected with 0.5 μg or 1μg of pDNA3/Pod-1 and 1 μg pDNA3Sf-1/Luc in combination with Fulgene 6 (Roche, Germany). Cells were lysed and assayed for both Firefly and Renilla Luciferase using the Dual-Luciferase Reporter Assay System (Promega).

2.4. RT-qPCR

After RNA extraction with Trizol® reagent (Invitrogen, USA), cDNA was generated from 1 μg of total RNA using Oligo dT, RNase-OUT, and M-MLV Reverse Transcriptase (Invitrogen, USA). RT-qPCR was performed on a RotorGene6000 Corbett (Quiagen, USA) sequence detector using Platinum SYBR qPCR SuperMix-UDG (Invitrogen, USA). A cycle threshold (Ct) value in log range of amplification was selected for each sample in triplicate and normalized to β-actin expression levels. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Chromatin immunoprecipitation (ChIP)

H295R cells transfected with pCMVMyc-Pod1 were fixed with 1% formaldehyde for 10 min. ChIP assays were performed using ChIP-IT Express kit (Active Motif, Rixensart, Belgium) following the manufacturer's instructions. Chromatin was fragmented by sonication with six 10 s pulses at 25 μm amplitude in a VCX130 PB ultrasonic processor (Sonics & Materials, CT, USA). Most resulting chromatin fragments ranged from 200 bp to 600 bp. Sheared chromatin was incubated with 3 μg anti-MYC (Clontech) or with 1 μg IgG as negative control (ChIP-IT control Kit-Human, Active Motif).

2.6. ChIP-PCR

The 5.0-kb upstream sequences of human steroidogenic factor-1 (*NR5A1, transcript ENST00000373588, Ensembl release 64, GRCh37*) and androgen receptor (*AR, transcript ENST00000374690*) were analyzed for putative E-box binding sites using MatInspector (Cartharius et al., 2005). Based on this analysis and on the location of predicted E-box sites in mice (Funato et al., 2003; Hong et al., 2005), the following primers were designed using Primer3 (Untergasser et al., 2007): AR E-box forward 5′-CTCTGA TTCTTGGGGCTGAG-3′, and reverse 5′CATGACCAAGCCAGCAGATA 3′ (113 bp amplicon); SF-1 E-box-552, forward 5′CCCACAGCAGATG AGGACAT 3′, and reverse 5′TGCTGCTTCCACCTCTTTTT 3′ (132 bp amplicon); SF-1 E-box-55, forward 5′-ACCAACAAAGAAGGCGAGAG-3′, and reverse 5′-CTGTCCGTCCGTCCTCCT-3′ (109 bp amplicon). As a negative control for POD-1 binding, primers amplifying sequences in intron 1–2 of *AR* (forward 5′-TTGTCAAAGTCTTTTCCAGTTAATTT-3′ and reverse 5′-TTAACCCTACCAAGTAAATTTGTTC-3′, 114 bp amplicon) and 50-kb downstream of NR5A1 (forward 5′TGGAAGGGGAAGAATGAATG-3′and reverse 5′-CTGACGTCACACA TCCCATC-3′, 135 bp amplicon) were used.

Anti-MYC IP, IgG-IP, and 0.1% input DNA samples were used as templates for PCR amplification. PCR reactions were performed using 5 U/μl Platinum Taq DNA Polymerase (Invitrogen) and 5 μM primers for 35 cycles of amplification with an annealing temperature of 60 °C.

2.7. MTS assay

10^4 Cells/well were plated into 96-well plates and after 24 h were transfected with pCMVMycPod1 or pcDNA3Pod1. After 48 h, 20 μl of tetrazolium compound (MTS) was added for 4 h at 37 °C. After transfection, cells were analyzed daily during 5 days. The optical density was read at 490 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.8. Functional enrichment analysis

We collapsed probe-sets to distinct genes using Entrez gene identifiers, and calling a gene negatively correlated to TCF21 if any probe-set for the gene was negatively correlated to TCF21 with $p < 0.01$. We tested for enrichment of Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) using one-sided Fisher's exact tests. We estimated false discovery rates by permuting the gene labels 100 times.

2.9. Statistic analysis

Data were presented as the mean ± standard deviation (SD). Statistical significance was determined using paired *T*-tests on log-transformed data except where otherwise noted. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Pod-1 construct inhibited a luciferase reporter gene under the control of Sf-1 promoter, in a dose-dependent manner

Previous study indicated that Pod-1 repressed the transcription from the Sf-1 reporter gene in I-10 cell line, which was derived from the Leydig cells (Tamura et al., 2001). We examined this possibility in Y1 mouse adrenocortical tumor cell line and TM3 mouse Leydig cell line transiently transfected with luciferase reporter gene under the control of *Sf-1* promoter (pDNA3/Sf1Luc) and with an expression vector encoding Pod-1 (pPod1). As shown in Fig. 1, transfected Pod-1 decreased expression of the Sf-1 reporter construct in Y1 cells in a dose-dependent manner [0.5 mcg pDNA3/Pod-1: 0.77 ± 0.02 or 22% repression ($p = 0.003$); 1.0 mcg pDNA3/Pod-1: 0.55 ± 0.04 or 45% repression ($p = 0.005$)]. Similar results were obtaining in the TM3 mouse Leydig cell line [0.5 mcg pDNA3/Pod-1: 0.73 ± 0.19 or 27% repression ($p = 0.16$); 1.0 mcg pDNA3/Pod-1: 0.44 ± 0.08 or 56% repression ($p = 0.015$). Thus, Pod-1 could specifically repress expression of the reporter gene, which carried the regulatory region of the Sf-1 gene.

3.2. POD-1 is markedly downregulated in adrenocortical carcinoma and negatively correlated to SF-1

We used our published array data from Gene Expression Omnibus (GEO) series GSE10927, consisting of mRNA abundance assays of 33 ACCs, 22 ACAs, and 10 normal adrenal cortex samples, that used Affymetrix HG-U133_Plus_2 arrays, which hold 54675 probe-sets representing approximately 20,000 genes (Giordano et al., 2009; Heaton et al., 2012). We used the log-transformed abundance estimates as given. The data set held two probe-sets measuring TCF21/POD-1 that were highly correlated ($r = 0.83$), which we averaged, and used to compute the correlation of POD-1 to every other probe-set, using just the 33 ACCs. The analysis of POD-1 in the cohort of adrenocortical tissues showed that this gene is expressed about 2-fold less in ACA vs NLs, and another 2-fold in ACCs (Fig. 2). We also found that the probe-set for SF-1/NR5A1 was negatively correlated to POD-1 in the 33 ACCs (Fig. 3). These results are consistent with POD-1 serving a repressive role in SF-1-mediated transcription and as a potential negative regulator of adrenocortical tumorigenesis.

3.3. pcDNA3Pod1 and pCMVMycPod1 transfection resulted in increased POD-1 expression in adrenocortical tumor cells

The transient transfection of pcDNA3Pod1 or pCMVMycPod1 in H295R and ACC-T36 cells resulted in an expected increased *POD-1* expression in both cell types. H295R and ACC-T36 cells transfected with POD-1 containing pcDNA3 vectors showed a 4.2-fold ($p = 0.04$) and 1.5-fold ($p = 0.004$), increase in *POD-1* expression, respectively (Fig. 4A). Similarly, transfection with POD-1-containing pCMVMyc promoted *a* 24.6×10^3-fold ($p =$

5×10^{-6}) and 6.4×10^{3}-fold ($p = 0.0006$) increase in *POD-1* expression in H295R and ACC-T36 cells, respectively (Fig. 4B).

3.4. Forced expression of POD-1 reduced the endogenous SF-1 expression in adrenal tumor cells

To investigate whether induced expression of POD-1 affects the endogenous expression of *SF-1,* we performed a RT-qPCR for the analysis of *SF-1* expression in cells transiently transfected with expression vectors pcDNA3Pod1 or pCMVMycPod1. pcDNA3Pod1 transfection in H295R and ACC-T36 cells showed *SF-1* reduction of 0.45 ($p = 0.042$) and 0.49 ($p = 0.025$) of control levels (Fig. 4A), whereas pCMVMycPod1 transfection reduced *SF-1* expression to 0.34 ($p = 0.009$) and 0.34 ($p = 0.017$) of controls, respectively (Fig. 4B). While the level of transfected POD-1 varied in each cell lines and did not correlate with degree of inhibition across the two cell lines, the data indicate that POD-1 inhibited the expression of *SF-1* in adrenal tumor cells.

3.5. Validation of chromatin enrichment and characterization of POD-1 binding to SF-1 E-box

In order to investigate whether the repressive effect of POD-1 overexpression on SF-1 expression was mediated through POD-1 binding to E-box elements in the SF-1 promoter region, chromatin immunoprecipitation (ChIP) assays were performed in H295R cells transfected with pCMVMycPod1 using an anti-Myc antibody (Fig. 5). To validate this approach, ChIP-PCR assays were performed using primers amplifying an E-box sequence in the promoter region of *AR*; it has been previously shown in mice that Pod-1 binds to an E-box element in the promoter of *Ar* to repress transcription (Hong et al., 2005). As shown in Fig. 5, immunoprecipitated chromatin was enriched for this region, whereas intron 1–2 of *AR*, used as a local negative control and not expected to bear E-box elements, failed to amplify. The 5.0-kb upstream sequence of human *SF-1* was analyzed for putative E-box elements using MatInspector (Cartharius et al., 2005) and two such elements were identified, located 552 and 55 base pairs upstream of the transcription start site, respectively. ChIP-PCR assays using specifically designed primers showed that both “E-box -552” and “E-box -55” sequences were amplified from Myc-immunoprecipitated DNA from NCI-H295R-pCMVMycPod1 cells, confirming chromatin enrichment by ChIP, while a negative control sequence, located 50 kb downstream of *SF-1,* failed to amplify (Fig. 5). Altogether, these results confirm POD-1-binding to E-box elements in the human *SF-1* promoter.

3.6. Decreased SF-1 expression induced by POD-1 overexpression affect the StAR expression of adrenal tumor cells

To investigate the effects of POD-1-mediated *SF1* repression on cell viability and steroidogenic function of adrenal tumor cells, colorimetric MTS assay and RT-qPCR for analysis of StAR expression was performed with NCI-H295R and ACC-T36 cells transiently transfected with pcCMVMycPod1. The viability of cells transfected with POD-1 was similar to that of cells transfected with empty vectors (Fig. 6). However, in both tumor cell types, *StAR* expression (Fig. 7) was downregulated following the transfection of POD-1, consistent with a decrease in SF-1-mediated StAR transcription.

3.7. Negatively correlated genes to POD-1 are enriched for cell cycle

We selected probe-sets on the arrays measuring human mRNA levels in ACCs that were correlated to POD-1 expression with *p*-values smaller than 0.01, which gave 732 positively and 527 negatively correlated probe-sets. To perform enrichment testing we collapsed the 54,675 probe-sets to 19,230 distinct genes, which now had 492 positively and 380 negatively correlated genes to POD-1. We tested 232 pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) obtained in May 2011, using one-sided Fisher Exact tests to ask if the gene intersections were significantly larger than expected by chance. The top three pathways for the negatively correlated genes are shown in Table 1, the most significant of which was for “Cell cycle”, suggesting that tumors with higher POD-1 expression have lower proliferation rates. Negatively correlated cell cycle genes selected include among others CDK1 (Cyclin-dependent kinase) and BUB1B (budding uninhibited by benzimidazoles 1 homolog beta). CDK1 is regulated by CKIs (cyclin-dependent kinase inhibitors) such as $p57^{Kip2}$, which is often downregulated coordinately with H19 in ACC (Gicquel et al., 1997; Heaton et al., 2012). BUB1B is overexpressed in ACC and the difference in the expression of BUB1B and PINK1 (PTEN-induced putative kinase 1) was strong predictor of overall survival in a group of adult ACCs (de Reynies et al., 2009; Fragoso et al., 2012).

4. Discussion

In this study, we found that POD-1/*TCF21* acts as a transcriptional suppressor of *SF-1* gene expression in adrenocortical carcinoma cells. A possible mechanism involved in the repression of *SF-1* expression is through POD-1 binding to the E-box sequence in the *SF-1* promoter region.

In Pod-1 KO embryos, Sf1 expression was increased at the boundary between the gonad and mesonephros, regions where Pod-1 is normally expressed (Quaggin et al., 1999). Also, Sf1 was co-expressed with the β-galactosidase reporter that replaced the first exon of the *Pod1* gene, showing ectopic expression of Sf1 in *Pod1*-expressing cells. These results support the model that Pod-1 normally represses SF1 expression in these development sites (Cui et al., 2004). Consistent with this model, our results show that induced expression of Pod-1 inhibited, in a dose-dependent manner, the *Sf-1* promoter activity in mouse Y1 and TM3 sterodoigenic cell lines. Indeed, induced expression of Pod-1 has been shown to inhibit the expression of *Sf-1* in the Leydig cell-derived I-10 cells (Tamura et al., 2001). Also, Pod-1 suppressed *Sf-1* expression by inhibiting the binding of *Usf1*, an activator of Sf-1 expression (Daggett et al., 2000), to the *Sf-1* E-box during gonadogenesis. Moreover, *Pod-1* knockout mice showed enhanced expression of Sf-1 in Leydig cells (Cui et al., 2004).

SF-1 is a key regulator of adrenal tumorigenesis and has been shown to be over-expressed in adrenocortical tumors (Figueiredo et al., 2005; Pianovski et al., 2006; Almeida et al., 2010). On the other hand *POD-1* is markedly downregulated in adrenocortical carcinoma (ACC) in comparison to adrenocortical adenoma (ACA) and normal adrenal tissue. Abnormal expression of POD-1 has been demonstrated in other types of tumors. POD-1 is aberrantly methylated and downregulated in head and neck carcinomas and in non-small-cell lung cancer (Smith et al., 2006). In melanoma, POD-1 has been suggested to be functionally

involved in tumor progression (Arab et al., 2011). In this study, we show that POD-1 is able to decrease endogenous *SF-1* expression in adrenocortical tumor cells, providing insight into the role of POD-1 in adrenal tumors. Furthermore, our results suggest that POD-1 binds directly to the *SF-1* promoter, inhibiting its activity. While such interaction did not affect the proliferation of adrenocortical tumor cells in culture, POD-1 levels inversely correlate with gene involved in cell cycle progression. Moreover, POD-1 over-expression decreases StAR expression, consistent with a down-regulation of SF-1 mediated steroidogenesis.

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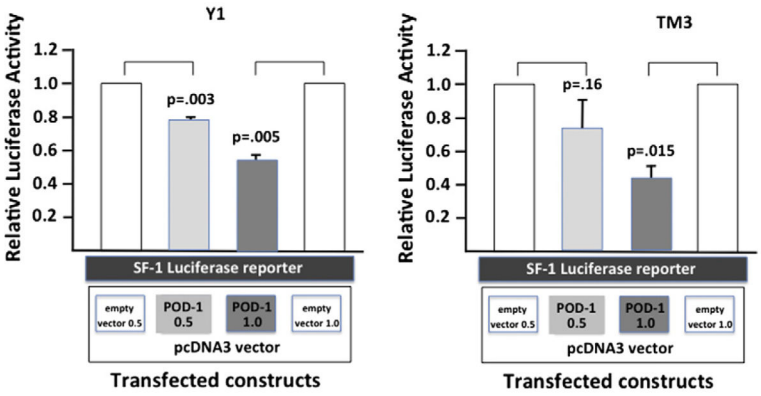

Fig. 1.

Luciferase reporter gene assay in Y1 adrenocortical tumor cell line and TM3 Leydig cells transfected with 0.5 μg or 1 μg of pDNA3/Pod-1 and 1 μg pDNA3Sf-1/Luc. Cells were assayed for both Firefly and Renilla by using Dual-Luciferase reporter assay system. Statistical significance was tested by paired *T* test on 3 pairs.

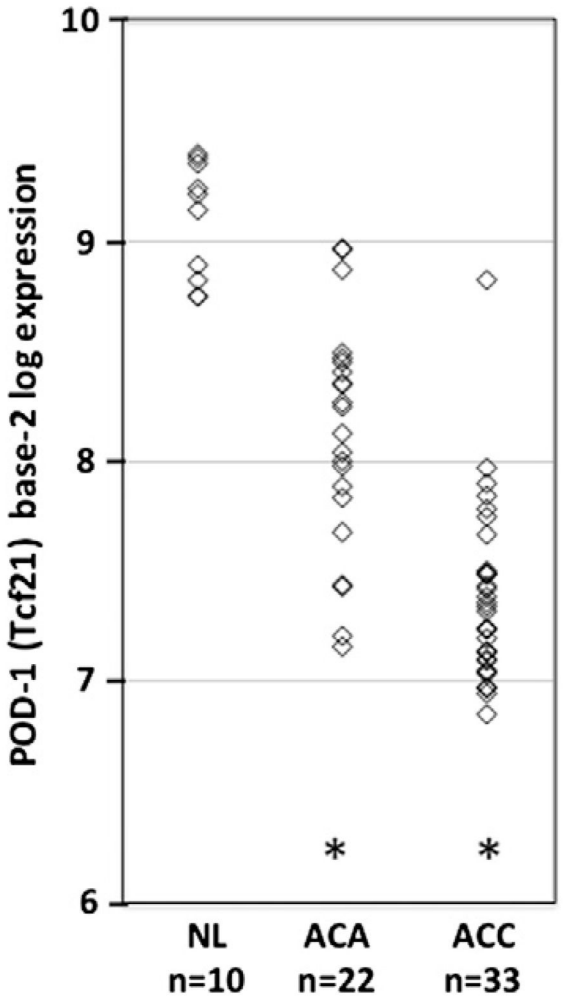

Fig. 2.

Expression data for POD-1 and SF-1 from arrays run on human adrenocortical tissues. POD-1 was lower in ACA than normal (NL, $p = 1 \times 10^{-7}$, average fold-change = 0.51) and even lower in ACC compared to ACA ($p = 3 \times 10^{-8}$, average fold-change = 0.60, both tests from a one-way ANOVA model fit to the three groups).

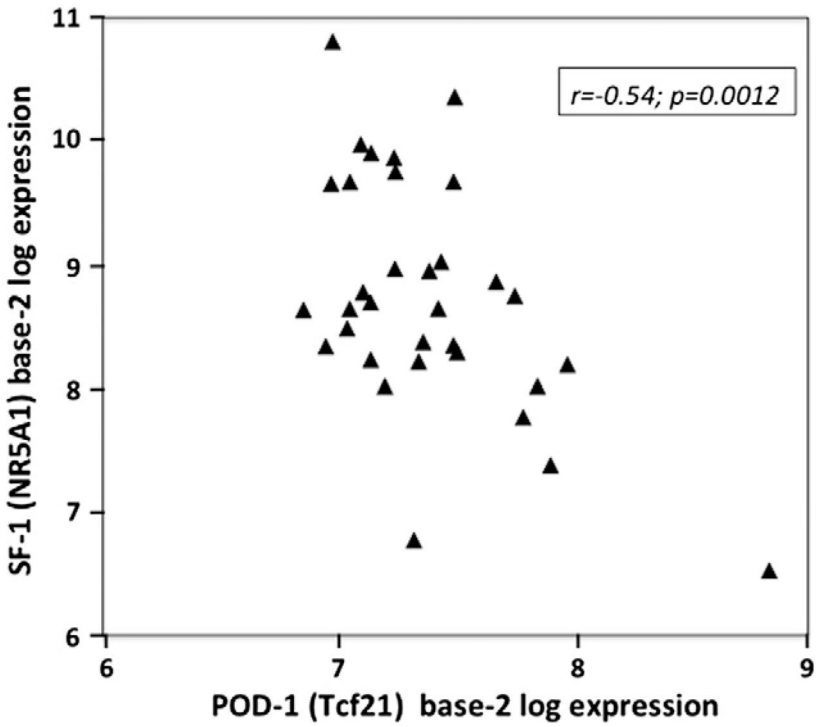

Fig. 3.

SF-1 was negatively correlated to POD-1 in public array data for 33 ACCs ($r = -0.54$; p = 0.0012). In ACAs the correlation was negative but not significant ($r = -0.25$; $p = 0.26$).

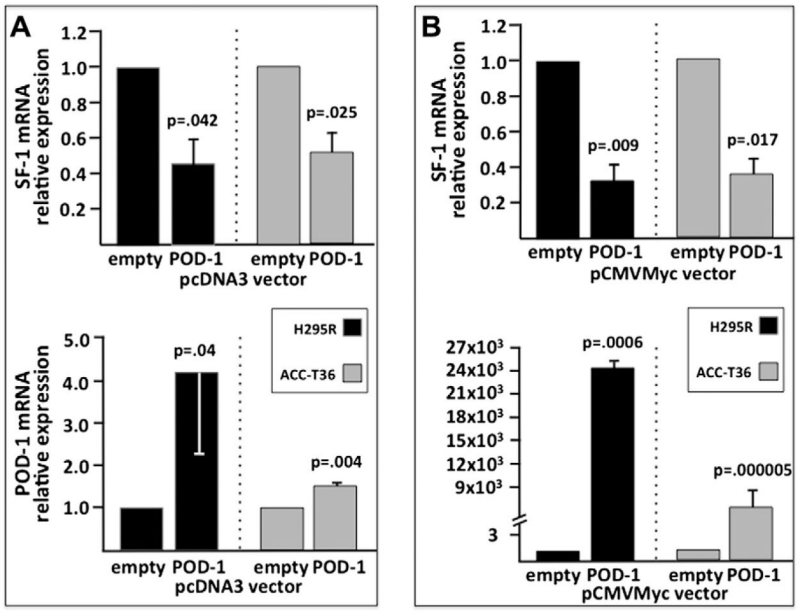

Fig. 4.

RT-qPCR analysis of relative gene expression of *POD-1* and *SF-1* in H295R and ACC-T36 cells transiently transfected with (A) empty vector pcDNA3 versus pcDNA3Pod1 and (B) empty vector pCMVMyc versus pCMVMycPod1. Statistical significance was tested by paired *T* test on 3 pairs.

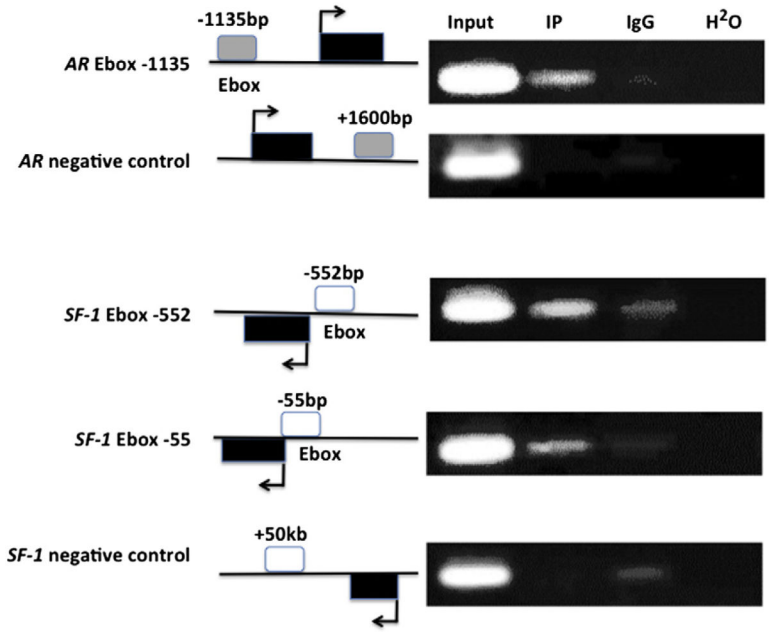

Fig. 5.

Chromatin enrichment was confirmed by PCR amplification of the E-box region of *SF-1* promoter from anti-Myc immunoprecipated H295pCMVMycPod1 DNA. The positions of the amplicons in relation to the Transcriptional Start Sites (TSSs – represented by arrows) are shown. Black and open bars represent, respectively, the exon and a different E-box sequence. Androgen Receptor (*AR*), Input (Inp) 0.1% DNA, Anti-MYC-IP NCIpCMVMycPod1 (IP), anti-IgG (IgG).

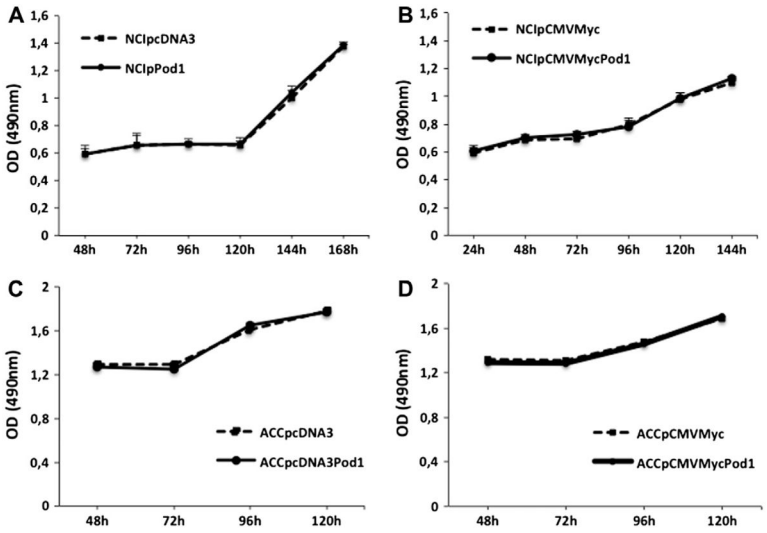

Fig. 6.

Viability assay of NCI-H295R cell line (A and B) and ACC-T36 adrenocortical cell culture (C and D) transiently transfected with pcDNA3Pod1 (A and C) or pCMVMycPod1 (B and D) versus respective empty vectors. The MTS assay was initiated 48 h after cell transfection. Results are expressed as mean ± SD. Statistical significance two-way ANOVA with Tukey (post-test); $n = 3$.

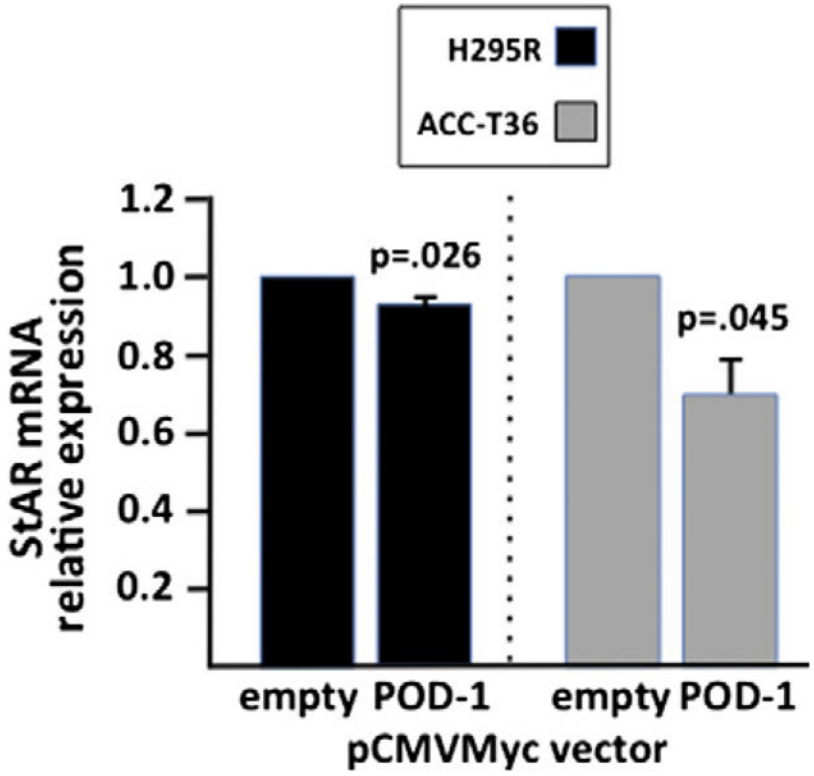

Fig. 7.

RT-qPCR analysis of relative gene expression of StAR in H295R and ACC-T36 cells transiently transfected with pCMVMycPod1 or with the empty vector pCMVMyc. Statistical significance was tested by paired *T* test on 3 pairs.

Table 1

Top 3 pathways found by enrichment testing 232 KEGG pathways using 380 distinct genes that were negatively correlated to POD-1 expression on arrays. Estimated false discovery rates were based on 100 random permutations of the gene labels.

KEGG pathway	Number of genes in pathway	Number negatively correlated to TCF21 (of 380)	*P*-value, one-sided Fisher's exact test	Estimated false discovery rate (*Q*-value)	Genes in intersection
Cell cycle	123	9	0.0008	0.050	YWHAQ, CHEK2, E2F2, ORC2, SKP2, BUB1B, CCNA2, CCNB2, CDK1
mRNA surveillance pathway	80	7	0.0010	0.045	HBS1L, PAPOLA, CSTF1, CPSF2, PPP2R5C, RNGTT, RBM8A
Glycosaminoglycan biosynthesis–heparan sulfate	26	4	0.0016	0.050	B3GALT6, EXTL1, EXTL2, HS2ST1