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Teratocarcinoma-Derived Growth Factor-1 Is Upregulated in Aldosterone-Producing Adenomas and Increases Aldosterone Secretion and Inhibits Apoptosis In Vitro

Tracy A. Williams, Silvia Monticone, Fulvio Morello, Choong-Chin Liew, Giulio Mengozzi, Catia Pilon, Sofia Asioli, Anna Sapino, Franco Veglio, Paolo Mulatero

Abstract—Aldosterone-producing adenomas (APA) are a frequent cause of secondary hypertension characterized by autonomous hypersecretion of aldosterone. However, the molecular mechanisms involved in adrenal tumorigenesis and deregulated aldosterone secretion are currently unknown. To identify putative functional genes, a transcriptional screening was performed on 8 APA and 3 normal adrenals (NA) using oligonucleotide microarrays. Data were next validated on an expanded set of samples by real-time PCR (APA, $n=19$; NA, $n=10$). The epidermal growth factor–like teratocarcinoma-derived growth factor-1 (TDGF-1) was upregulated in APA compared with NA (14.7-fold and 21.4-fold by microarray and real-time PCR, respectively). In vitro studies and Western blot analysis using the NCI H295R adrenocortical cell line showed that TDGF-1 increased Akt phosphorylation on Thr308 and Ser473, consistent with activation of phosphatidylinositol 3-kinase/Akt signaling, and also demonstrated a concomitant inactivation of the Akt substrate glycogen synthesis kinase-3 β via Ser9 phosphorylation. Furthermore, TDGF-1 mediated a 3.8 ± 0.4 -fold increase in aldosterone secretion ($n=4$) that was specifically blocked by the phosphatidylinositol 3-kinase inhibitors wortmannin (50 nmol/L) and LY294002 (20 μ mol/L). Finally, TDGF-1 protected H295R cells from apoptosis induced by staurosporine, causing a decrease in caspase-3 activity, a reduction in the inactivation of poly(ADP-ribose) polymerase, and an inhibition of DNA fragmentation, detected by the TUNEL reaction and fluorescence microscopy that was blocked by LY294002. Taken together, our data suggest that TDGF-1, which is significantly upregulated in APA and mediates aldosterone hypersecretion and deregulated growth in adrenocortical cells in vitro, may represent a key player in the development and pathophysiology of primary aldosteronism. (*Hypertension*. 2010;55:00-00.)

Key Words: aldosterone-producing adenoma ■ aldosterone ■ NCI H295R cells ■ teratocarcinoma-derived growth factor ■ phosphatidylinositol 3-kinase/Akt signaling

Primary aldosteronism (PA) is the most frequent form of endocrine hypertension, accounting for up to 5% to 10% of all hypertensive patients,¹ and is characterized by the chronic, excessive, and autonomous secretion of aldosterone by the adrenal gland. The diagnosis of this form of hypertension is fundamental because, compared with essential hypertensives with similar risk profiles, patients with PA are more prone to stroke and myocardial infarction² and display an increase in cardiovascular damage and metabolic complications.³

Aldosterone-producing adenomas (APA) are a common underlying cause of PA and are found in 30% to 40% of PA patients, whereas bilateral adrenal hyperplasia is present in 60% to 70% of patients.⁴ Unilateral adrenalectomy normalizes, or at least markedly improves, the blood pressure in patients with APA, and therefore, APA is the most common,

specifically treatable, and potentially curable form of hypertension.^{4,5} However, the molecular mechanisms underlying the alterations in cell growth in the adrenal cortex and the hypersecretion of aldosterone have not yet been defined.

Previous genomic analyses^{6–9} have provided evidence for differential gene expression in APA compared with normal adrenals (NA). The present study was undertaken to better define upregulated gene transcripts in highly selected APA and to investigate the functional role of such genes specifically with regard to autonomous aldosterone secretion and deregulated cell growth. The APA used in this study were removed from patients studied in our hypertension unit who had been homogeneously selected following a rigorous diagnostic flowchart that includes adrenal venous sampling and postadrenalectomy evaluation. Furthermore, strict criteria were used for adrenal venous sampling, cannulation, and

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Table 1. Clinical and Biochemical Data of Patients Before and After Adrenalectomy

Parameter	Before Adrenalectomy	After Adrenalectomy
Age, years	49 (30 to 66)	
Sex, M/F	9/13	
SBP, mm Hg	178±18	130±7
DBP, mm Hg	108±10	82±5
PRA, ng ml ⁻¹ hour ⁻¹	0.26 (0.1 to 0.6)	2 (1 to 3.1)
PAC, ng/dL	54.7 (24.5 to 121.8)	9.3 (2.4 to 13.9)
ARR	321.5 (49 to 1218)	5 (2.5 to 11.7)
K ⁺ , mEq/L	3.4 (2.4 to 4.2)	4.7 (4.1 to 5.1)
Adenoma size, mm	12.8 (5 to 30)	

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PRA, plasma renin activity; PAC, plasma aldosterone concentration; ARR, aldosterone:renin ratio.

lateralization,¹⁰ and finally, NA and APA were removed under identical conditions; that is, in both cases, the same technique, surgical team, and anesthetic was used.

We demonstrate here, by microarray analysis and real-time PCR, that teratocarcinoma-derived growth factor-1 (TDGF-1, Cripto-1, CR-1), which belongs to the epidermal growth factor–Cripto-FRL1-Cryptic (CFC) protein family and plays a key role in early vertebrate development and carcinogenesis,^{11–13} is a highly upregulated gene in APA.¹¹ Furthermore, immunohistochemical studies show that this growth factor displays a distinct localization to APA nuclei, in contrast to the cytoplasmic expression observed in the surrounding adrenal cortex and in the zona glomerulosa of NA tissue. Functional studies of TDGF-1 in NCI H295R cells¹⁴ demonstrate that TDGF-1 mediates an activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and both an increase in aldosterone secretion and an inhibition of apoptosis *in vitro*. Furthermore, these latter 2 effects are specifically blocked by PI3K inhibitors. Therefore, this study identifies a gene transcript upregulated in APA that displays functional roles *in vitro* that are relevant to the aldosterone hypersecretion and deregulated cell growth typical of an APA.

Methods

An expanded Methods section is available online at <http://hyper.ahajournals.org>.

Patient Selection

Patients were studied following the procedure described in detail elsewhere.¹⁰ Clinical and biochemical data of the selected patients before and after adrenalectomy are shown in Table 1.

Microarray

RNA samples used for microarray experiments (n=12) were assayed for purity and quality using an Agilent Bioanalyzer. The gene expression profiling platform was oligonucleotide array Affymetrix GeneChip HG-U133 Plus 2.0. cRNA synthesis, labeling, hybridization, and chip scanning were performed following the manufacturer's protocols. CEL files were analyzed using dChip 2008 software.¹⁵

RNA Extraction From Adrenal Tissues

Adrenal tissue (≈100 mg) was homogenized (Ultra-Turrax T8 IKA tissue homogenizer, Staufen, Germany) in 1 mL of TRI-reagent

(Sigma-Aldrich Corp, St. Louis, Mo) and processed according to the manufacturer's instructions.

Gene Expression Assays

Reverse transcription was performed using 5 μg of total RNA, 250 ng of random primers, and SuperScript II reverse transcriptase (Invitrogen Corp, Carlsbad, Calif), as described by the manufacturer. Real-time PCR was performed in triplicate using TaqMan gene expression assays (Applied Biosystems, Foster City, Calif) on an Applied Biosystems ABI 7500 instrument following standard protocols. Gene expression levels were analyzed using the 2^{-ΔΔC_t} relative quantification method, using β-actin as the endogenous reference gene.

Semiquantitative RT-PCR

Total RNA was extracted from 100-mm-diameter plates of subconfluent cells (RNeasy mini kit, Qiagen, Valencia, Calif). First-strand cDNA were generated from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and used as templates for PCR amplification of TDGF-1 or GAPDH cDNA fragments.

PCR Cloning of TDGF-1

The full-length coding region of the TDGF-1 cDNA was PCR amplified from first-strand cDNA using *pfu* polymerase (Promega, Madison, Wis) from an APA expressing the highest level of the TDGF-1 transcript and inserted into pcDNA3.1. A control plasmid was constructed (pcDNA3.1/control) comprising TDGF-1 cDNA inserted in the antisense direction with respect to the cytomegalovirus promoter.

Immunohistochemistry

Frozen sections of APA or adjacent normal cortical adrenals (4 μm) were incubated for 1 hour with antihuman TDGF-1 polyclonal antibody (ab19917, Abcam, Cambridge, United Kingdom; 200 μL, 1:20 dilution). Bound primary antibody was visualized using the Envision system horseradish peroxidase-labeled polymer (Dako, Carpinteria, Calif) conjugated with anti-rabbit secondary antibody and staining completed with 3,3'-diaminobenzidine substrate-chromogen. Nuclei were counterstained with hemalum.

Cell Culture

NCI H295R human adrenocortical carcinoma cells¹⁴ were cultured in RPMI 1640 medium without phenol red (Gibco, Billings, Mont) supplemented with 2% FBS, insulin-transferrin-selenium A, L-glutamine (292 μg/mL), streptomycin (100 μg/mL), and penicillin (100 U/mL) (all from Gibco) and maintained at 37°C in a humidified 5% CO₂ incubator.

Transfection of H295R Cells

H295R cells (2×10⁶) were electroporated using 2 μg of plasmid in Nucleofector solution R (100 μL) with the Amaxa nucleofector (program P20, Amaxa Biosystems, Cologne, Germany).

Aldosterone Measurements

Culture medium was assayed for aldosterone in duplicate by radioimmunoassay as described previously.¹⁰ Aldosterone measurements were normalized using protein concentrations of cell lysates.¹⁶

Cell Proliferation Assay

Cell proliferation of transfected H295R cells was determined using the Cell Titer 96 Aqueous One solution cell proliferation assay kit (Promega).

Apoptosis Assays

Poly(ADP-Ribose) Polymerase Assay

Transfected H295R cells were incubated with or without staurosporine (STS; 500 nmol/L for 16 hours) and then washed, lysed, and sonicated in SDS-PAGE sample buffer (300 μL). Samples (15 μL)

were resolved by 10% SDS-PAGE, and poly(ADP-ribose) polymerase (PARP), was visualized by Western blotting.¹⁷

Caspase-3 Assay

Cells were transfected and apoptosis was induced as described for the PARP assay. Caspase-3 activity was quantified in cell extracts (50 μ g), using a colorimetric assay kit (CaspACE assay system, Promega) as described previously.¹⁷

TUNEL

Transfected cells were grown in Laboratory-Tek Chamber Slides (Sigma-Aldrich), and apoptosis was induced with STS (500 nmol/L) with or without LY294002 (20 μ mol/L). The TUNEL reaction was performed according to the manufacturer's protocol (DeadEnd fluorometric TUNEL system, Promega). Slides were mounted in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, Calif) and viewed by fluorescence microscopy (Zeiss, Axio Observer, Göttingen, Germany).

Western Blotting

A chemiluminescent substrate was used to visualize specific bands binding horseradish peroxidase-linked secondary antibodies (Pierce Biotechnology).

Statistical Analyses

SAS software, version 8 (SAS Institute Inc., Cary, NC), was used for statistical analyses. Data are expressed as mean \pm SD, and differences between variables were evaluated using a parametric test for repeated measures and a Bonferroni post hoc test. A probability of less than 0.05 was considered statistically significant.

Results

Microarray Analysis of APA Transcript Profiles and Upregulation of TDGF-1 in APA

Oligonucleotide microarrays were used to perform a transcriptional screening of 8 APA, compared with 3 NA. On the basis of the filtering algorithm, described in the Methods, 64 probe sets (corresponding to 53 genes) were found to be differentially expressed in APA compared with NA (33 upregulated, 20 downregulated), and *TDGF1* ranked as the top upregulated gene (average 14.7-fold upregulation). Supplemental Tables I and II list the upregulated and downregulated known genes, respectively. The hierarchical clustering of differentially expressed genes is presented in Figure 1. Interestingly, the transcriptional fingerprint of a single unilateral adrenal hyperplasia sample corresponded closely to that of NA but not to that of APA.

The expression level of *TDGF1* was further examined by real-time PCR on a broader set of adrenal samples (19 APA and 10 NA) comprising 5 APA and 2 NA already used for the microarray screening. *TDGF1* was also upregulated in this group of APA (Figure 2A) and was observed in 79% of the APA samples (n=15/19, 21.4-fold compared with NA, Table 2). The microarray data were also confirmed for 3 other selected genes (*TPD52*, *VSNL1*, and *IGSF4*), thus validating our gene expression platform (Figure 2B through 2D, Table 2). Finally, the expression of *TDGF1* was assayed on distinct pathological adrenal samples, including cortisol-producing adenomas from patients with Cushing syndrome (n=3), adrenal incidentalomas (n=3), an aldosterone-producing carcinoma, and a primary adrenal hyperplasia. In all these samples, *TDGF1* expression levels were comparable to those present in NA (data not shown). Thus, the upregulation of

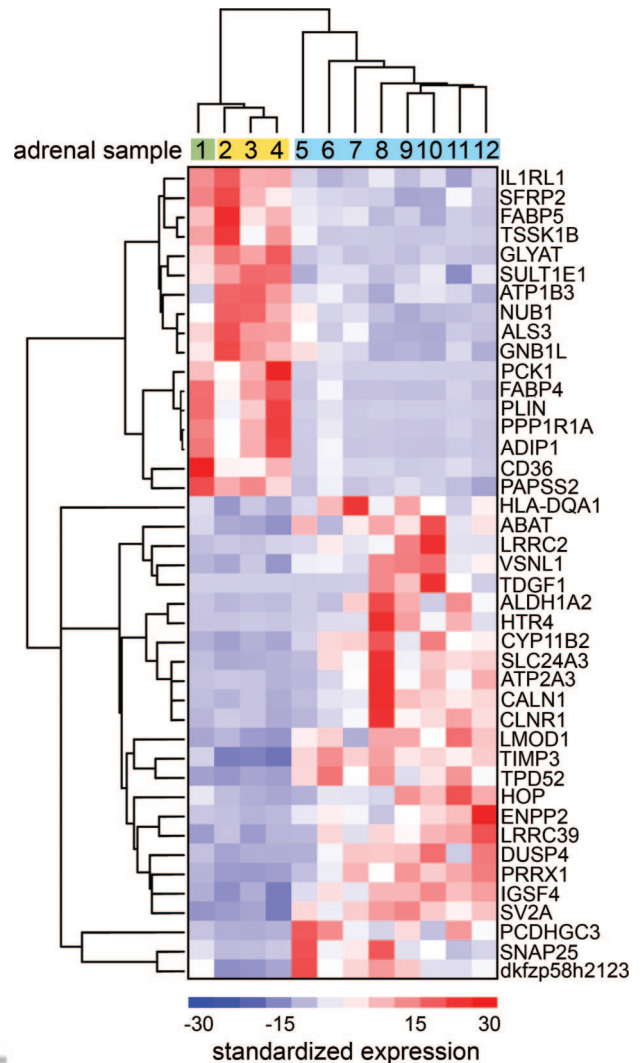


Figure 1. Hierarchical cluster analysis of adrenal samples. Shown is a hierarchical cluster analysis of differentially expressed known genes (n=42) and tissue samples. Transcript upregulation is represented in red and downregulation in blue. The 42 transcripts are represented on the lines and labeled on the right. Tissue samples are represented in the columns and labeled on top (1 to 12: 1, unilateral hyperplasia; 2 to 4, NA; 5 to 12, APA). The log color scale of gene expression is shown at the bottom of the figure.

TDGF1 appeared as a primary feature of APA and not of other adrenal diseases.

TDGF-1 Is Localized to the Zona Glomerulosa in NA Glands

Immunohistochemical staining of frozen tissue sections demonstrated that TDGF-1 was expressed in the cytoplasm of NA restricted to the zona glomerulosa (Figure 3A); in contrast to the immunoreactivity observed in APA, which was localized to nuclei but cytoplasmic in the surrounding adrenal cortex (Figure 3B). Nuclear and cytoplasmic immunoreactivity was confirmed in the pancreas control specimen that was undetectable in the negative control (data not shown).

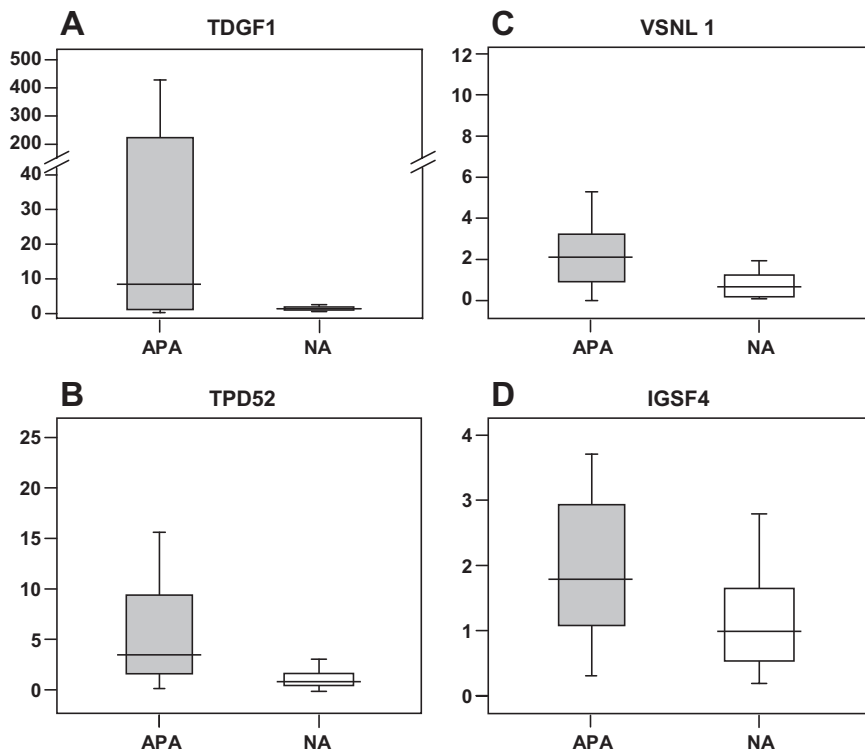


Figure 2. Validation of gene overexpression by real-time PCR. TaqMan gene expression assays were used in real-time PCR, performed in triplicate, to determine fold changes in expression levels in individual adrenal samples for each gene, using actin B as the endogenous reference gene. The genes studied were *TDGF1* (A), tumor protein D52 (*TPD52*) (B), visinin-like 1 (*VSNL1*) (C), and immunoglobulin superfamily member 4 (*IGSF4*) (D). A through D, Box plots of fold changes in expression of the indicated gene in APA (n=19) compared with NA (n=10). The horizontal line indicates the median, and the box and the bar represent the 25th to 75th and 5th to 95th percentiles, respectively. * $P < 0.05$, ** $P < 0.01$.

Human Adrenocortical Cells (NCI H295R) as a Model Cell Line to Study the Function of TDGF-1

TDGF-1 was undetectable in H295R cells by semiquantitative RT-PCR and in a pooled sample of first-strand cDNA from NA, whereas a TDGF-1-specific fragment was amplified from a pooled sample of first-strand cDNA from APA (Figure 4); the absence or low level expression of TDGF-1 gene transcription indicates the suitability of this cell line to study TDGF-1 function in vitro.

TDGF-1 Activates the Akt Signaling Pathway in H295R Cells

TDGF-1 has been reported previously to activate PI3K/Akt signaling in diverse cell types.^{11,18,19} H295R cells were transfected with either pcDNA3.1/TDGF-1 or pcDNA3.1/

control, and Western blot analysis demonstrated a greater activation of Akt, revealed by increased phosphorylation of Akt residues Ser473 and Thr308, in cell lysates from TDGF-1-transfected H295R cells compared with controls (Figure 5). Furthermore, increased phosphorylation and therefore inactivation of the Akt substrate glycogen synthase kinase-3 β on residue Ser9 was observed in TDGF-1-transfected H295R cells (Figure 5).

TDGF-1 Mediates an Increase in Aldosterone Secretion From H295R Cells That Is Specifically Blocked by PI3K Inhibitors

H295R cells were transfected with pcDNA3.1/TDGF-1 and basal aldosterone secretion was measured by radioimmuno-

Table 2. Upregulation of Genes in APA

Gene	Fold Change	Range	No. of APAs Overexpressing Gene
<i>TDGF1</i>	21.4	0.1 to 461.4	15/19 (79%)
<i>TPD52</i>	4.8	0.4 to 21.7	14/19 (74%)
<i>VSNL1</i>	4.1	0.1 to 11.5	10/19 (53%)
<i>IGSF4</i>	1.9	0.3 to 3.8	8/19 (42%)

TaqMan gene expression assays were used in real-time PCR to determine fold changes in expression levels in individual adrenal samples for each gene relative to the endogenous reference gene, actin B, performed in triplicate. The overall fold change in expression of each gene in APA (n=19) was calculated using the $2^{-\Delta\Delta Ct}$ quantification method, compared with NA (n=10). The range refers to the spread of the individual fold changes (relative quantitation values) calculated for each APA sample subsequently used to calculate the overall fold change. Genes were defined as upregulated when the expression of that gene was 2-fold greater than the average expression in normal adrenals. *TPD52* indicates tumor protein D52; *VSNL1*, visinin-like protein 1; *IGSF4*, immunoglobulin superfamily member 4.

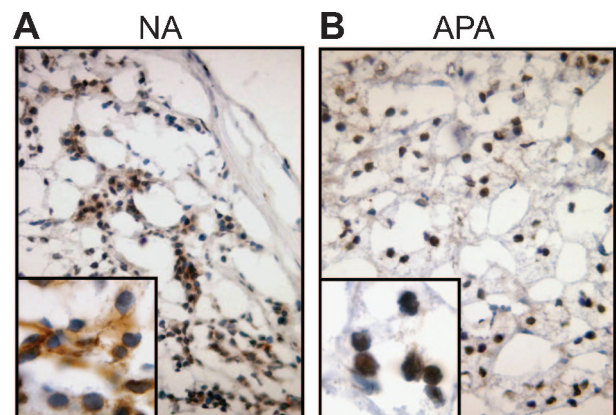


Figure 3. TDGF-1 expression in NA and APA. A, In frozen NA, TDGF-1 expression was evident in the cytoplasm (inset) and restricted to the zona glomerulosa. B, In contrast, in sections of frozen APA, TDGF-1 expression was observed predominantly in the nuclei of APA cells (inset).

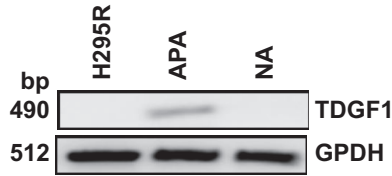


Figure 4. Semiquantitative RT-PCR of TDGF-1 in H295R cells and adrenal samples. Semiquantitative PCR amplification of a TDGF-1 fragment (490 bp) using templates of either first-strand cDNA reverse transcribed from total RNA isolated from H295R cells or pooled cDNAs produced from APA transcripts ($n=19$) or from NA ($n=10$). GAPDH was used as the endogenous control gene.

assay and compared with that of cells transfected with pcDNA3.1/control. Aldosterone secretion was significantly increased ($P<0.005$) from TDGF-1-transfected cells, displaying a 3.8 ± 0.4 -fold increase in secreted aldosterone after 72 hours (Figure 6A).

The increased aldosterone secretion was not due to an increase in cell proliferation (1.06 ± 0.07 -fold change in cell proliferation after 72 hours for cells transfected with TDGF-1/control plasmid, $n=4$, \pm SD).

We investigated the effect of angiotensin II (Ang II) and TDGF-1 on aldosterone secretion. Ang II (10 nM) produced a 13.7 ± 1.2 -fold increase in aldosterone secretion in control-transfected cells compared with unstimulated cells and a 15.8 ± 1.6 -fold increase in TDGF-1-transfected cells. TDGF-1 mediates a 3.8-fold increase in aldosterone secretion in unstimulated cells, and therefore, TDGF-1 does not potentiate the effect of Ang II in H295R.

The possible involvement of PI3K in TDGF-1-mediated aldosterone secretion was studied using specific inhibitors (50 nmol/L wortmannin and 20 μ mol/L LY294002), which decreased the TDGF-1-mediated increase in aldosterone secretion (Figure 6B) to levels similar to that of untreated control cells (1.1 ± 0.1 -fold and 1.3 ± 0.2 -fold increases in

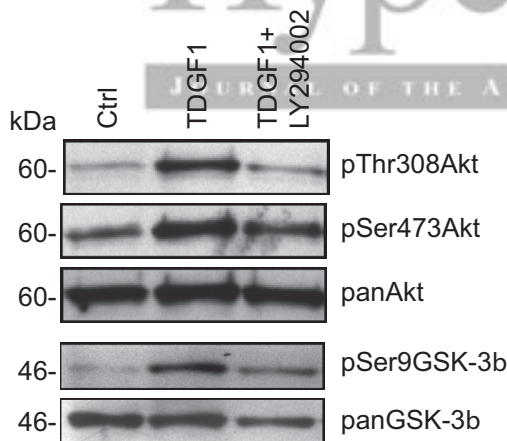


Figure 5. TDGF-1 activates the Akt signaling pathway in H295R cells. H295R cells were transfected with pcDNA3.1 carrying TDGF-1 or control cDNA. Proteins were analyzed by Western blotting using monoclonal antibodies specific for the protein forms indicated at the right. Nitrocellulose membranes were stripped using Restore stripping buffer (Pierce) at 37°C for 15 minutes before being reprobed with a different primary antibody. Representative blots are shown from experiments performed 3 times.

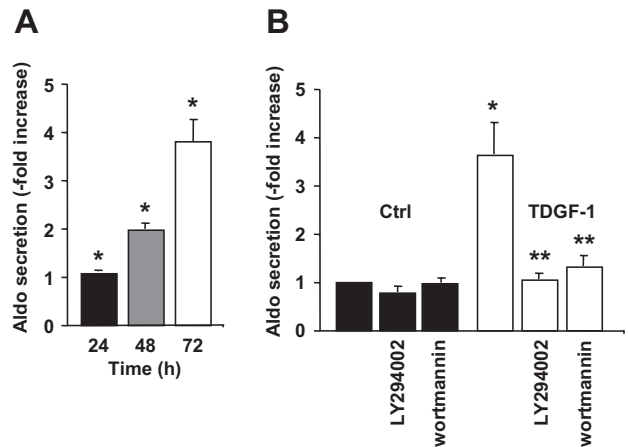


Figure 6. TDGF-1 mediates a PI3K-dependent increase in aldosterone secretion from H295R cells. H295R cells were transfected with TDGF-1 or control (Ctrl) plasmids, and secreted aldosterone was assayed in culture medium by radioimmunoassay. A, Data are shown as fold increases in aldosterone secretion from TDGF-1-transfected cells compared with controls and are the means of 4 independent experiments performed in duplicate \pm SD. $*P<0.005$ for all comparisons. B, Cells were transfected with Ctrl (black columns) or TDGF-1 (white columns) plasmids and incubated with or without wortmannin (50 nmol/L) or LY294002 (20 μ mol/L). Data are shown as fold increases in aldosterone secretion compared with controls and are the means of 4 independent experiments performed in duplicate \pm SD. $*P=0.002$, **nonsignificant difference compared with untreated control (left column).

aldosterone secretion in the presence of LY294002 and wortmannin, respectively; both values not significantly different from untreated control).

H295R cells transfected with TDGF-1 exhibited no significant difference in cell proliferation compared with control cells after 72 hours (0.99 ± 0.04 -fold change, $n=4$), and no significant differences in cell proliferation were detected in cells transfected with either TDGF-1 or the control plasmid after 72 hours in the presence of either wortmannin (50 nmol/L) or LY294002 (20 μ mol/L), 1.02 ± 0.06 and 0.97 ± 0.02 -fold change in cell proliferation in control cells in the presence of LY294002 and wortmannin, respectively, and 1.01 ± 0.04 and 0.97 ± 0.02 -fold change in TDGF-1-transfected cells in the presence of LY294002 and wortmannin, respectively ($n=4$).

TDGF-1 Protects H295R Cells From Apoptosis, and This Effect Is Blocked by a PI3K Inhibitor

TDGF-1 has been reported to function as a survival factor with an antiapoptotic effect in human cervical carcinoma cells via a PI3K-dependent signaling pathway.¹⁹ Induction of apoptosis by STS and specific detection of PARP by Western blotting demonstrated that cleavage of catalytically active PARP was relatively protected in TDGF-1-transfected cells: Figure 7A shows an increased ratio of the uncleaved:cleaved forms of PARP in these cells; thus, the inactive, cleaved form of PARP, a hallmark of apoptosis, is relatively more abundant in control cells.

In addition, the increase in caspase-3 activity in response to STS was markedly reduced in cells transfected with TDGF-1: a 19.7 ± 3.7 -fold increase in caspase-3 activity in control cells (caspase-3 activity in STS-stimulated/unstimulated cells)

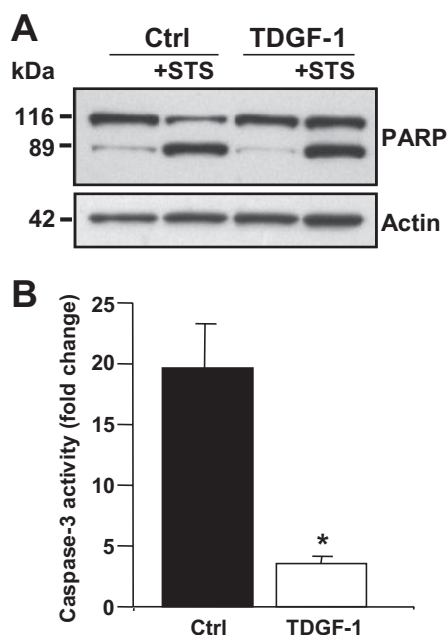


Figure 7. TDGF-1 confers reduced sensitivity to apoptosis in H295R cells. **A**, The hydrolysis of full-length PARP (116 kDa) to the inactive form (89 kDa) analyzed by Western blotting of lysates of TDGF-1 or control (Ctrl) plasmid-transfected cells after incubation with or without STS (500 nmol/L). A representative blot is shown from an experiment performed 3 times. **B**, Caspase-3 was assayed in cell lysates of TDGF-1 or Ctrl-transfected cells after incubation with or without STS (500 nmol/L) for 16 hours. Data are shown as fold changes in caspase-3 activity in the presence of STS compared with cells incubated in the absence of STS and are the means of 3 independent experiments performed in duplicate \pm SD. * $P=0.01$.

compared with a 3.6 ± 0.6 -fold increase in caspase-3 activity in TDGF-1-transfected cells (Figure 7B).

The possible involvement of PI3K in TDGF-1-mediated decreased sensitivity to apoptotic stimuli in H295R cells was studied using the TUNEL assay. In agreement with an antiapoptotic effect of TDGF-1, the strong fluorescent labeling observed in the nuclei of control cells was greatly decreased in TDGF-1-transfected cells. Furthermore, in the presence of the PI3K inhibitor LY294002 (20 μ mol/L), this

protective effect of TDGF-1 toward apoptotic stimuli was partially blocked (Figure 8).

Discussion

PA is the most common form of endocrine hypertension, and APA comprises a frequent subtype of this disease (30% to 40% of the total). The removal of the adenoma in patients with APA normalizes the excessive aldosterone secretion, characteristic of this condition, and, as a consequence, cures or markedly improves the hypertensive status.

This work defines TDGF-1 as a highly upregulated gene in APA compared with NA tissue. In agreement with previous transcriptional screens, we observed an increased expression of both aldosterone synthase (CYP11B2) and the 5-hydroxytryptamine receptor 4 (HTR4)^{6,7,9}; however, we did not find evidence for elevated expression of either the luteinizing hormone receptor⁸ or the Ca^{2+} /calmodulin-dependent protein kinases.⁹ This could be due to heterogeneity of the APA phenotype or to differences in normal subjects and patient selection: for example, in this study, APA were used from patients with florid forms of PA, adrenalectomized after performing adrenal venous sampling using particularly strict criteria both for successful cannulation and lateralization.

This study assigns a functional role for TDGF-1 in aldosterone secretion and in the protection from apoptosis in human adrenocortical carcinoma cells (NCI H295R). We show that inactivating PI3K with specific inhibitors blocks the increase in aldosterone secretion mediated by TDGF-1 in H295R cells and at least partially blocks the reduction in sensitivity to apoptosis. PI3K activation is followed by Akt phosphorylation, a key effector of PI3K-mediated cell survival.^{20,21} The present study demonstrates that the cell survival effect and the increase in aldosterone secretion are associated with the activation of Akt by increased phosphorylation in the C-terminal regulatory domain on residue Ser473 and in the catalytic domain on residue Thr308. Consistently, an increase in Akt signaling was demonstrated further by the enhanced Ser9 phosphorylation of the Akt substrate glycogen synthase kinase-3 β .

The antiapoptotic effect of TDGF-1 associated with Akt activation is consistent with the known effect of Akt in

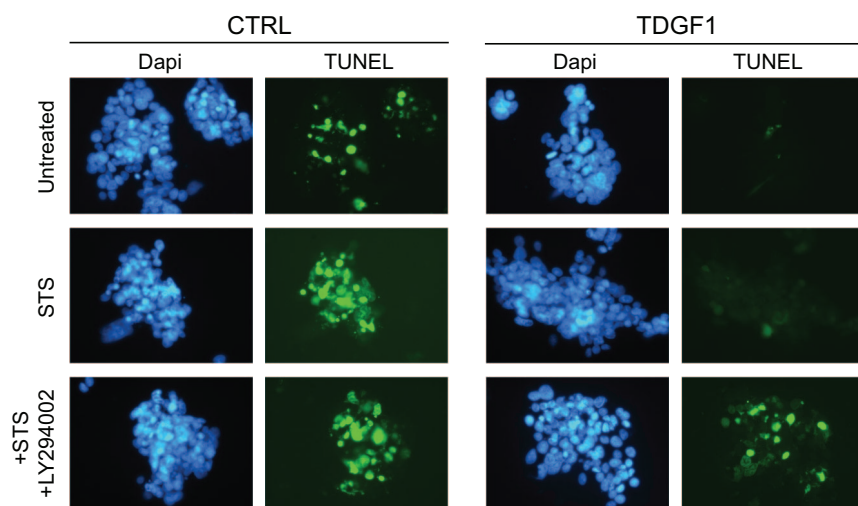


Figure 8. The antiapoptotic effect of TDGF-1 in H295R cells is blocked by PI3K inhibition. DNA fragmentation as a marker for apoptosis was detected by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and fluorescent microscopy in cells transfected with TDGF-1 or control plasmid and incubated with STS (500 nmol/L) for 2 hours with or without LY294002 (20 μ mol/L). Cells were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Dapi), and images were taken by fluorescent microscopy (taken at a $\times 40$ magnification) using appropriate filters. Representative fields are shown from experiments performed 3 times.

promoting cell survival via the phosphorylation and inactivation of the proapoptotic protein Bad.²² In agreement with the present study, other groups have demonstrated that TDGF-1 activates PI3K/Akt signaling, for example, in mouse mammary epithelial cells¹⁸ and in cervical carcinoma cells, leading to the phosphorylation and inactivation of glycogen synthase kinase-3 β ¹⁹; this latter study also demonstrated an antiapoptotic effect of TDGF-1 that was decreased by a specific PI3K inhibitor. Furthermore, the suppressed tumor growth in vivo, resulting from antibody blockade of TDGF-1,^{12,23} was related to increased apoptosis mediated by c-Jun-N-terminal kinase and inhibition of Akt phosphorylation.²³

Other factors have been identified that increase aldosterone secretion from adrenocortical cell lines or from cells isolated from the zona glomerulosa: for example, sphingosine-1-phosphate stimulates aldosterone secretion via the PI3K/Akt and mitogen-activated protein/extracellular signal-regulated kinase pathways²⁴; an adipokine, CTRP1, expressed specifically in the zona glomerulosa of the adrenal cortex, increased aldosterone production in H295R cells.²⁵ Furthermore, the phosphoprotein Disabled-2, expressed in the zona glomerulosa and zona intermedia of the rat adrenal gland, increased aldosterone secretion, although under conditions of Ang II stimulation.²⁶ However, to our knowledge, this is the first study that in addition to identifying a gene transcript specifically overexpressed in APA, also demonstrates potentially relevant functional roles in the adrenal cell model, NCI H295R, and provides evidence for the underlying mechanism via PI3K/Akt signaling.

TDGF-1 functions as an essential cofactor for signaling by the transforming growth factor- β family ligand Nodal during early embryogenesis and belongs to the epidermal growth factor-CFC family of proteins that are present in diverse species such as zebrafish, *Xenopus*, and mouse, as well as humans.²⁷ TDGF-1 is overexpressed in many human cancers, such as breast, pancreatic, ovarian, and colon cancers. Furthermore, a functional role for TDGF-1 in tumor growth is suggested because its expression can transform some mammary cell lines; in addition, antibody blockade of TDGF-1 suppresses tumor growth in vivo,^{12,23} and antisense inhibition of TDGF-1 results in the loss of the transformed phenotype of colon carcinoma cells.²⁸ In normal adult tissues, TDGF-1 is primarily expressed in mammary glands during pregnancy and lactation; this is the first report of TDGF-1 expression in the NA gland and in APA. TDGF-1 is primarily distributed as a plasma membrane-bound protein but can also exist in a soluble form, with a dual function as both receptor and growth factor: however, we show here that, in NA cells, TDGF-1 has a cytoplasmic location, in contrast to the pathological state in APA, where TDGF-1 is localized exclusively to the nucleus. Nonetheless, a cytoplasmic subcellular distribution for TDGF-1 has indeed been reported previously.^{29,30} The reason for the nuclear versus cytoplasmic localization of TDGF-1 in APA or normal tissue, respectively, is unclear. It could be proposed that TDGF-1, in addition to its well-characterized role as a coreceptor for Nodal in early embryogenesis, may undergo nuclear translocation under certain conditions in adult tissues and subsequently regulate gene expression, thereby mediating other cellular processes. Such a precedent

has been described previously. For example, epidermal growth factor receptor family members, in addition to the traditional epidermal growth factor receptor pathway involving transduction of mitogenic signals via the activation of signaling cascades, can also translocate to the nucleus and regulate gene expression; a number of other receptor tyrosine kinases and cytokine receptors also undergo similar nuclear localization,³¹ but further studies would be required to establish whether this is the case for TDGF-1.

Future studies should address whether the upregulation of TDGF-1 is specific to a particular subtype of APA (eg, Ang II responsive/unresponsive). The number of Ang II-responsive patients in the present study was too small to adequately answer this question.

This work defines TDGF-1 as a highly upregulated gene in APA compared with NA cortex and assigns a functional role for TDGF-1 in aldosterone secretion and in the protection from apoptosis in human adrenocortical carcinoma cells via PI3K/Akt signaling. Changes in the level of TDGF-1 expression in specific cell populations of the adrenal gland, that is, in the zona glomerulosa, may constitute one of several changes that are important in the pathogenesis of an APA, in which the cumulative effect of a number of genetic alterations is required for the initiation of APA formation and subsequent autonomous hypersecretion of aldosterone.

Perspectives

The present study provides evidence for a potential role of TDGF-1 in the pathogenesis of APA and highlights a dual function for this epidermal growth factor–like protein in the inhibition of apoptosis and in increasing aldosterone secretion from adrenal cells via deregulated Akt signaling. In addition, we demonstrate significant upregulation or downregulation of a number of other genes compared with NA, thus providing the basis for future studies that address the role of genomic alterations in the pathophysiology of APA.

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Disclosures

None.

References

1. Mulatero P, Dluhy RG, Giacchetti G, Boscaro M, Veglio F, Stewart PM. Diagnosis of primary aldosteronism: from screening to subtype differentiation. *Trends Endocrinol Metab.* 2005;16:114–119.
2. Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *J Am Coll Cardiol.* 2005;45:1243–1248.
3. Mulatero P, Verhovez A, Morello F, Veglio F. Diagnosis and treatment of low-renin hypertension. *Clin Endocrinol.* 2007;67:324–334.
4. Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, Gomez-Sanchez CE, Veglio F, Young WF Jr. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab.* 2004;89:1045–1050.
5. Stowasser M, Gordon RD, Gunaekera TG, Cowley DC, Ward G, Archibald C, Smithers BM. High rate of detection of primary aldosteronism, including surgically treatable forms, after 'non-selective' screening of hypertensive patients. *J Hypertens.* 2003;21:2149–2157.

6. Assié G, Auzan C, Gasc J-M, Baviera E, Balaton A, Elalouf J-M, Jeunemaitre X, Plouin P-F, Corvol P, Clauser E. Steroidogenesis in aldosterone-producing adenoma revisited by transcriptome analysis. *J Clin Endocrinol Metab*. 2005;90:6638–6649.
7. Cartier D, Jégou S, Parmentier F, Lihmann I, Louiset E, Kuhn J-M, Bastard C, Plouin P-F, Godin M, Vaudry H, Lefebvre H. Expression profile of serotonin₄ (5-HT₄) receptors in adrenocortical aldosterone-producing adenomas. *Eur J Endocrinol*. 2005;153:939–947.
8. Saner-Amigh K, Mayhew BA, Mantero F, Schiavi F, White PC, Rao CV, Rainey WE. Elevated expression of luteinizing hormone receptor in aldosterone-producing adenomas. *J Clin Endocrinol Metab*. 2006;91:1136–1142.
9. Lenzini L, Seccia TM, Aldighieri E, Belloni AS, Bernante P, Giuliani L, Nussdorfer GG, Pessina AC, Rossi GP. Heterogeneity of aldosterone-producing adenomas revealed by a whole transcriptome analysis. *Hypertension*. 2007;50:1–8.
10. Mulatero P, Bertello C, Rossato D, Mengozzi G, Milan A, Garrone C, Giraudo G, Passarino G, Garaballo D, Verhovez A, Rabbia F, Veglio F. Roles of clinical criteria, computed tomography scan, and adrenal vein sampling in differential diagnosis of primary aldosteronism subtypes. *J Clin Endocrinol Metab*. 2008;93:1366–1371.
11. Adamson ED, Minchiotti G, Salomon DS. Cripto: a tumor growth factor and more. *J Cell Physiol*. 2002;190:267–278.
12. Adkins HB, Bianco C, Schiffer SG, Rayhorn P, Zafari M, Cheung AE, Orozco O, Olson D, De Luca A, Chen LL, Miatkowski K, Benjamin C, Normanno N, Williams KP, Jarpe M, LePage D, Salomon D, Sanicola M. Antibody blockade of the Cripto CFC domain suppresses tumor cell growth in vivo. *J Clin Invest*. 2003;112:575–587.
13. Sun Y, Strizzi L, Raafat A, Hirota M, Bianco C, Feigenbaum L, Kenney N, Wechselberger C, Callahan R, Salomon DS. Overexpression of human Cripto-1 in transgenic mice delays mammary gland development and differentiation and induces mammary tumorigenesis. *Am J Pathol*. 2005;167:585–597.
14. Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. *Mol Cell Endocrinol*. 2004;228:23–38.
15. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A*. 2001;98:31–36.
16. Bird IM, Hanley NA, Word RA, Mathis JM, McCarthy JL, Mason JJ, Rainey WE. Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. *Endocrinology*. 1993;133:1555–1561.
17. Williams TA, Verhovez A, Milan A, Veglio F, Mulatero P. Protective effect of spironolactone on endothelial cell apoptosis. *Endocrinology*. 2006;147:2496–2505.
18. Bianco C, Strizzi L, Rehman A, Normanno N, Wechselberger C, Sun Y, Khan N, Hirota M, Adkins H, Williams K, Margolis RU, Sanicola M, Salomon DS. A nodal- and ALK4-independent signaling pathway activated by Cripto-1 through glypican-1 and c-src. *Cancer Res*. 2003;63:1192–1197.
19. Ebert AD, Wechselberger C, Frank S, Wallace-Jones B, Seno M, Martinez-Lacaci I, Bianco C, De Santis M, Weitzel HK, Salomon DS. Cripto-1 induces phosphatidylinositol 3'-kinase-dependent phosphorylation of AKT and glycogen synthase kinase β in human cervical carcinoma cells. *Cancer Res*. 1999;59:4502–4505.
20. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*. 1997;275:661–665.
21. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell*. 1997;88:435–437.
22. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. 1997;91:231–241.
23. Hu XF, Li J, Yang E, Vandervalk S, Xing PX. Anti-Cripto Mab inhibit tumor growth and overcome MDR in a human leukaemia MDR cell line by inhibition of Akt and activation of JNK/SAPK and bad death pathways. *Br J Cancer*. 2007;96:918–927.
24. Brizuela L, Rábano M, Gangoiiti P, Narbona N, Macarulla JM, Trueba M, Gómez-Muñoz A. Sphingosine-1-phosphate stimulates aldosterone secretion through a mechanism involving the PI3K/PKB and MEK/ERK 1/2 pathways. *J Lipid Res*. 2007;48:2264–2274.
25. Jeon JH, Kim KY, Kim JH, Baek A, Cho H, Lee YH, Kim JW, Kim D, Han SH, Lim JS, Kim KI, Yoon do Y, Kim SH, Oh GT, Kim E, Yang Y. A novel adipokine CTRP1 stimulates aldosterone production. *FASEB J*. 2008;22:1502–1511.
26. Romero DG, Yanes LL, de Rodriguez AF, Plonczynski MW, Welsh BL, Reckelhoff JF, Gomez-Sanchez EP, Gomez-Sanchez CE. Disabled-2 is expressed in adrenal zona glomerulosa and is involved in aldosterone secretion. *Endocrinology*. 2007;148:2644–2652.
27. Shen MM, Schier AF. The EGF-CFC gene family in vertebrate development. *Trends Genet*. 2000;16:303–309.
28. De Luca A, Arra C, D'Antonio A, Casamassimi A, Losito S, Ferraro P, Ciardiello F, Salomon DS, Normanno N. Simultaneous blockage of different EGF-like growth factors results in efficient growth inhibition of human colon carcinoma xenografts. *Oncogene*. 2000;19:5863–5871.
29. Saeki T, Stromberg K, Qi CF, Gullick WJ, Tahara E, Normanno N, Ciardiello F, Kenney N, Johnson GR, Salomon DS. Differential immunohistochemical detection of amphiregulin and cripto in human normal colon and colorectal tumors. *Cancer Res*. 1992;52:3467–3473.
30. Qi CF, Liscia DS, Normanno N, Merlo G, Johnson GR, Gullick WJ, Ciardiello F, Saeki T, Brandt R, Kim N, Kenney N, Salomon DS. Expression of transforming growth factor alpha, amphiregulin and cripto-1 in human breast carcinomas. *Br J Cancer*. 1994;69:903–910.
31. Lo HW, Hung MC. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *Br J Cancer*. 2006;94:184–188.

Online Supplement

TDGF-1 IS UP-REGULATED IN ALDOSTERONE-PRODUCING ADENOMAS AND INCREASES ALDOSTERONE SECRETION AND INHIBITS APOPTOSIS *IN VITRO*

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Expanded Materials and Methods

Patient selection

Patients were studied following the procedure described in detail elsewhere (1). Briefly, patients were screened using the aldosterone (PAC)/plasma renin activity (PRA) ratio (ARR) and PA was confirmed using the intravenous saline load test. (2). All patients with confirmed PA underwent CT scanning and adrenal vein sampling (AVS). AVS was considered successful if the adrenal vein/inferior vena cava cortisol gradient was at least 3; lateralization was considered when the aldosterone/cortisol ratio (A/C) from one adrenal was at least 4 times that of the other adrenal gland (lateralization ratio, LR) with an A/C in the contralateral lower than the A/C in the peripheral vein. Finally, all patients with PA were screened for GRA using a long-PCR technique (3). A definitive diagnosis of APA was made as defined elsewhere (1). All patients with APA were adrenalectomized by laparoscopy. NA were obtained from normotensive individuals undergoing laparoscopic nephrectomy for localized renal carcinomas: in all cases, careful histological examination excluded the involvement of the adrenal in the tumor lesion. The study was approved by our institutional ethics committee and informed consent was obtained from all subjects.

Microarray

RNA samples used for microarray experiments (n=12) were assayed for purity and quality using an Agilent Bioanalyzer according to the manufacturer's instructions. The gene expression profiling platform was oligonucleotide array Affymetrix GeneChip HG-U133 Plus 2.0, that allows the study of more than 47 000 transcripts. cRNA synthesis, labeling, hybridization and chip scanning were performed following the manufacturer's protocols. CEL files were analyzed using dChip 2008 Software (4). Briefly, the data underwent invariant set and running median smoothing normalization in order to adjust for differences in overall array brightness. The expression levels of probe sets were then computed using the model-based expression indexes (MBEI). We retained for further analysis: (i) probe sets receiving a presence (P) call across all samples and (ii) probe sets receiving a P call only in case (APA, n=8) or only in control (normal adrenals, n=3) samples. Finally, filtered probe sets were ranked based on the ratio between their average expression level in case and control samples. Only transcripts with a lower 90% bound of fold change >2.0 or <-2.0 and an absolute signal intensity difference between case and control sets >100 were considered as differentially expressed. Hierarchical cluster analysis of genes and samples was performed applying correlative algorithms, after averaging of redundant probe sets.

RNA extraction from adrenal tissues

Adrenal tissue (approx. 100 mg) was homogenized (Ultra-Turrax T8 IKA tissue homogenizer, Staufen, Germany) in 1 mL TRI-reagent (Sigma-Aldrich Corp., St. Louis, MO) and processed according to the manufacturer's instructions. RNA purity and quality was analyzed by spectrophotometry (A260/280 nm) and agarose gel electrophoresis. For RNA positive controls the outer part of the cortex of fresh adrenal gland was dissected and processed similarly. The histology of parallel specimens were checked on frozen sections, confirming that the tissue used for the RNA analysis spanned across the *zona glomerulosa* and part of the *fasciculata* zone of the adrenal cortex. The ideal control tissue for expression studies with APA is unknown since adenoma cells display a histological phenotype of both *zona glomerulosa* and

zona fasciculata cells and often appear as hybrid cells; furthermore, it has been demonstrated that APA cells express genes that, in normal adrenal tissues, are exclusively expressed in either the *zona glomerulosa*, such as, CYP11B2, or in the *zona fasciculata*, such as, CYP17 (5, 6).

Gene expression assays

Quantitative real time-PCR (qRT-PCR) was performed in 2-steps. Reverse transcription was performed using 5 µg total RNA, 250 ng random primers (Invitrogen Corporation, Carlsbad, CA), 500 µmol/L dNTPs, 10 mmol/L DTT and SuperScript II reverse transcriptase (200 units, Invitrogen) in First-Strand Buffer in a 20 µL reaction volume, as described by the manufacturer. Quantitative PCR was subsequently performed in triplicate using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) on an Applied Biosystems ABI 7500 instrument following standard protocols. Gene expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ relative quantification method, using β -actin as the endogenous reference gene. Pre-designed Primer-TaqMan probe assays were purchased from Applied Biosystems.

Semi-quantitative RT-PCR

Total RNA was extracted from 100 mm diam. plates of sub-confluent cells (RNeasy mini kit, Qiagen, Stanford Valencia, CA). First strand cDNA were generated from 5 µg total RNA using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions and the cDNA was used as template for PCR amplification of a TDGF-1 fragment (forward primer, 5'cgatgctaacgcctcttttc3'; reverse primer, 5'catcacagccgggtagaaat3'): 95°C, 5 min; 35 cycles (95°C, 20 sec; 55°C, 20 sec; 72°C, 1 min); 72°C, 10 min. Pooled samples of APA cDNA (n=19, equal volume of each cDNA) or NA cDNA (n=10), reverse transcribed from total RNA, were used similarly as templates for PCR amplification of TDGF-1, and GAPDH was used as the endogenous reference gene and amplified similarly but using 25 PCR cycles (forward primer, 5'gagtcaacggatttggtcgt3'; reverse primer, 5'tgtggtcatgagtcctcca3').

PCR cloning of TDGF-1

The full-length coding region of the TDGF-1 cDNA was PCR amplified from first strand cDNA using *pfu* polymerase (Promega, Madison, WI) from a subject expressing the highest level of the TDGF-1 transcript, as determined by qRT-PCR. The primers 5'gcctttttccctaattg3' (forward) and 5'agcagcagcctttactggct3' (reverse) were used to amplify a TDGF-1 669 bp cDNA fragment that was subcloned into pCDNA3.1 and the PCR-amplified region was subsequently sequenced to verify the absence of mutations.

Immunohistochemistry

Frozen sections of APA or adjacent normal cortical adrenals were cut (4 µm), quenched for 5 min with DAKO Peroxidase Block (Dako, Carpinteria, CA, USA) and incubated for 1 h at RT with anti-human TDGF-1 polyclonal antibody (ab19917, Abcam plc, Cambridge, UK, 200 µL, 1:20 dilution). Sections were washed and incubated for 30 min with Envision system-HRP labeled polymer (Dako) conjugated with anti-rabbit secondary antibody. Staining was completed by a 5 min incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen and nuclei were counterstained with hemalum for 5 min. Sections from pancreatic tissue known to express TDGF-1 were used as a positive control (7). The primary antibody was omitted as a negative control.

Cell culture

NCI H295R human adrenocortical carcinoma cells (8) were cultured in RPMI 1640 medium without phenol red (Gibco, Billings, MT) supplemented with 2% FBS, insulin-transferrin-selenium A, L-glutamine (292 µg/mL), streptomycin (100 µg/mL) and penicillin (100 U/mL) (all from Gibco) and maintained at 37°C in a humidified 5% CO₂ incubator.

Transfection of H295R cells

Sub-confluent cells were split 1:2, 24 h before electroporation with the Amaxa nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were harvested and resuspended (2×10^6 cells) in Nucleofector solution R (100 µL), and electroporated with 2 µg of plasmid (programme P20). Resuspended cells were cultured in 6-well plates and medium was changed after 24 h (1.5 mL). Cell viability assays (Trypan blue exclusion), performed before and after electroporation, indicated 38-50% cell viability. Transfection efficiency was routinely checked by electroporation of the pmaxGFP plasmid and fluorescence microscopy that demonstrated at least 60% transfection efficiency up to 96 h post-transfection (data not shown).

Aldosterone measurements

H295R cells were transfected by Amaxa electroporation with pcDNA3.1/TDGF-1 or control plasmid, pcDNA3.1/Ctrl (TDGF-1 cDNA inserted in the antisense direction with respect to the pCMV promoter). Some cells were incubated with PI3K inhibitors wortmannin (50 nmol/L) or LY294002 (20 µmol/L). Culture medium was assayed for aldosterone in duplicate by radioimmunoassay as described previously (1). Aldosterone measurements were normalized by protein concentrations of cell lysates (9).

Cell proliferation assay

H295R cells were transfected, viable cells (0.2% Trypan blue exclusion) were counted and the viable cell density was adjusted to 10^4 cells/100µL medium. Cells were seeded in 96-well plates (10^4 cells/well) and after 24 h medium was replaced (100 µL) and cells incubated a further 72 h. Cell Titer solution (20 µL) was added (Cell Titer 96 aqueous one solution cell proliferation assay kit, Promega, Madison, WI), and cell proliferation quantified following the manufacturer's protocol.

Apoptosis assays

PARP assay- H295R cells were transfected, after 24 h they were incubated for 16 h ± staurosporine (500 nmol/L, Sigma-Aldrich) to induce apoptosis. Cells were washed with PBS, lysed and sonicated directly in SDS-PAGE sample buffer (300 µL). Samples (15 µL) were resolved by 10% SDS-PAGE and the inactivation of the 116 kDa DNA repairing enzyme Poly (ADP-ribose) polymerase (PARP) to the 89 KDa fragment, was visualised by Western blotting using an anti-PARP monoclonal antibody, as described previously (10).

Caspase-3 assay- Cells were transfected and apoptosis was induced as described for the PARP-assay. Caspase-3 activity was quantified in cell extracts (50 µg), using a colorimetric assay kit (CaspACE assay system, Promega) that employed the substrate *N*-acetyl-DEVD-p-nitroanilide as described previously (10).

TUNEL- H295R cells were transfected as described above. Cells were added to single-well Lab-Tek Chamber Slides (Sigma-Aldrich) containing 4 mL medium. After 24 h, medium was changed (1.5 mL) and cells grown a further 24 h. Apoptosis was induced for 2 h with staurosporine (500 nmol/L) and incubated \pm the PI3K inhibitor LY294002 (20 μ mol/L, cells pre-incubated 30 min), and the TUNEL reaction was performed according to the manufacturer's protocol (The DeadEnd fluorimetric TUNEL system, Promega). Slides were mounted in Vectashield with Dapi (Vector Laboratories, Burlingame, CA) and viewed by fluorescence microscopy.

Western Blotting

The primary antibodies used were mouse monoclonal anti-PARP (F-2, 1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-actin (1:5000 dilution, Sigma-Aldrich) and rabbit monoclonal antibodies to phospho Ser473 Akt (D9E), phospho Thr308 Akt (C31E5) and pan Akt (C67E7), as well as phospho-Ser9-GSK-3 β (5B3) and pan GSK-3 β (27C10) (all used at 1:1000 dilution, Cell Signaling Technology Inc., Beverly, MA). Nitrocellulose membranes were stripped of primary and secondary antibodies by incubating in Restore Western Blot Stripping buffer (Pierce Biotechnology, Rockford, IL) for 15 min at 37°C, washed, blocked and incubated with the subsequent primary antibody. A chemiluminescent substrate (ECL) was used to visualize specific bands binding HRP-linked secondary antibodies (Pierce Biotechnology).

Statistical analyses

SAS V8 software (SAS Institute Inc., Cary, NC) was used for statistical analyses. Data are expressed as mean \pm s.d. and differences between variables were evaluated using a parametric test for repeated measures and a Bonferroni post-hoc test. A probability of less than 0.05 was considered statistically significant.

References

- 1- Mulatero P, Bertello C, Rossato D, Mengozzi G, Milan A, Garrone C, Giraudo G, Passarino G, Garaballo D, Verhovez A, Rabbia F, Veglio F. Roles of clinical criteria, computed tomography scan, and adrenal vein sampling in differential diagnosis of primary aldosteronism subtypes. *J Clin Endocrinol Metab.* 2008;93:1366-1371.
- 2- Mulatero P, Milan A, Fallo F, Regolisti G, Pizzolo F, Fardella C, Mosso L, Marafetti L, Veglio F, Maccario M. Comparison of confirmatory tests for the diagnosis of primary aldosteronism. *J Clin Endocrinol Metab.* 2006;91:2618-2623.
- 3- Mulatero P, Curnow KM, Aupetit-Faisant B, Foeking M, Gomez-Sanchez C, Veglio F, Jeunemaitre X, Corvol P, Pascoe L. Recombinant CYP11B genes encode enzymes that can catalyse conversion of 11-deoxycortisol to cortisol, 18-hydroxycortisol and 18-oxocortisol. *J Clin Endocrinol Metab.* 1998;83:3996-4001.
- 4- Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA.* 2001;98:31-36.
- 5- Fallo F, Pezzi V, Barzon L, Mulatero P, Veglio F, Sonino N, Mathis JM. Quantitative assessment of CYP11B1 and CYP11B2 expression in aldosterone-producing adenomas. *Eur J Endocrinol.* 2002;147:795-802.
- 6- Enberg U, Farnebo LO, Wedell A, Gröndal S, Thorén M, Grimelius L, Kjellman M, Bäckdahl M, Hamburger B. *In vitro* release of aldosterone and cortisol in human adrenal adenomas correlates to mRNA expression of steroidogenic enzymes for genes CYP11B2 and CYP17. *World J Surg.* 2001;25:957-966.
- 7- Friess H, Yamanaka Y, Büchler M, Kobrin MS, Tahara E, Korc M. Cripto, a member of the epidermal growth factor family, is over-expressed in human pancreatic cancer and chronic pancreatitis. *Int J Cancer* 1994;56:668-674.
- 8- Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. *Mol Cell Endocrinol.* 2004;228:23-38.
- 9- Bird IM, Hanley NA, Word RA, Mathis JM, McCarthy JL, Mason JI, Rainey WE. Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. *Endocrinology.* 1993;133:1555-1561.
- 10- Williams TA, Verhovez A, Milan A, Veglio F, Mulatero P. Protective effect of spironolactone on endothelial cell apoptosis. *Endocrinology.* 2006;147:2496-2505.

Gene	Name	Accession No.	N° probe sets	Fold change		
				Mean	lower limit	upper limit
TDGF1	teratocarcinoma-derived growth factor 1 (Cripto)	NM_003212	1	14.7	3.66	43
CYP11B2	aldosterone synthase	X54741	1	8.51	4.57	25.27
LRRC2	leucine rich repeat containing 2	NM_024512	1	7.69	2.8	25.7
ALDH1A2	aldehyde dehydrogenase, family 1, member A2	AB015228	1	7.13	3.5	14.84
SLC24A3	Na/K/Ca exchanger	NM_020689	2	6.81	4.1	11.08
SNAP25	synaptosomal-associated protein	NM_003081	1	6.41	2.72	13.48
HOP	homeodomain-only protein	AB059408	2	6.28	2.88	20.56
CLRN1	clarin 1	AF482697	1	5.1	2.38	17.46
LMOD1	leiomodoin 1	BC001755	1	5.05	2.92	12.65
TPD52	tumor protein D52	AA524023	3	4.34	2.98	6.79
HTR4	5-hydroxytryptamine receptor 4	AJ131724	1	4.33	2.05	7.79
HLA-DQA1	Major histocompatibility complex, IIDQ alpha1	BG397856	1	3.91	2.12	9.83
ATP2A3	ATPase, Ca ²⁺ transporter	NM_005173	1	3.86	2.05	7.08
CALN1	calneuron 1	AF282250	2	3.82	2.29	7.15
VSNL1	visinin-like 1	NM_003385	1	3.63	2.14	7.77
IGSF4	immunoglobulin superfamily, member 4	NM_014333	3	3.62	2.56	5.89
ENPP2	autotaxin	D45421	1	3.37	2.08	4.86
PRRX1	paired related homeobox 1	NM_006902	1	3.31	2.4	4.71
PCDHGC3	protocadherin gamma	AF152504	1	3.24	2.2	4.51
ABAT	4-aminobutyrate aminotransferase	AF237813	2	3.14	2.35	4.13
LRRC39	leucine rich repeat containing 39	BF111992	1	3.01	2.01	4.72
DUSP4	dual specificity phosphatase 4	NM_001394	1	2.84	2.03	3.84
SV2A	synaptic vesicle glycoprotein 2A	NM_014849	1	2.82	2.09	4.13
DKFZP586H2123	regeneration associated muscle protease	AI671186	1	2.8	2.03	3.9
TIMP3	tissue inhibitor of metalloproteinase 3	BF347089	1	2.61	2.3	3

Table S1. Microarray: genes with elevated expression levels in APAs compared to normal adrenals

Gene	Name	Accession No.	N° probe sets	Fold change		
				Mean	lower limit	upper limit
PCK1	phosphoenolpyruvate carboxykinase 1	NM_002591	1	-29.77	-7.4	-252.33
GLYAT	glycine-N-acyltransferase	AW024233	1	-17.96	-6.39	-1E+08
PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A	NM_006741	1	-15.89	-4.86	-97.96
ADIPOQ	Adiponectin, C1Q	NM_004797	1	-14.79	-4.83	-165.98
FABP4	fatty acid binding protein 4	NM_001442	2	-14.01	-3.57	-41.6
PLIN	perilipin	NM_002666	1	-13.41	-3.11	-39.69
CD36	CD36 antigen (collagen type I receptor)	NM_000072	3	-7.83	-4.98	-14.35
TSSK1B	testis-specific serine kinase 1	AF348076	1	-7.74	-2.63	-50.6
ATP1B3	ATPase, Na ⁺ /K ⁺ transporting, beta 3 polypeptide	AI928218	1	-4.96	-3.62	-7.38
FABP5	fatty acid binding protein 5	NM_001444	1	-4.39	-2.22	-7.29
ALS2	amyotrophic lateral sclerosis 2	AB046783	1	-4.16	-2.72	-7.51
SFRP2	secreted frizzled-related protein 2	AF311912	1	-3.94	-2.14	-6.69
GNB1L	G protein, beta polypeptide 1-like	NM_024627	1	-3.64	-2.24	-6.88
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	AI821404	1	-3.39	-2.47	-4.7
NUB1	negative regulator of ubiquitin-like proteins 1	AK026433	1	-2.91	-2.19	-3.93
IL1RL1	Interleukin 1 receptor-like 1	NM_003856	1	-2.62	-2.06	-3.3
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	U55764	1	-2.45	-2.03	-3.01

Table S2. Microarray: genes with decreased expression in APAs compared to normal adrenals