MicroRNA-24 Is a Novel Regulator of Aldosterone and Cortisol Production in the Human Adrenal Cortex

Stacy Robertson, Scott M. MacKenzie, Samantha Alvarez-Madrazo, Louise A. Diver, Junjun Lin, Paul M. Stewart, Robert Fraser, John M. Connell, Eleanor Davies

Abstract—Dysregulation of aldosterone or cortisol production can predispose to hypertension, as seen in aldosterone-producing adenoma, a form of primary aldosteronism. We investigated the role of microRNA (miRNA) in their production, with particular emphasis on the CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) genes, which produce the enzymes responsible for the final stages of cortisol and aldosterone biosynthesis, respectively. Knockdown of Dicer1, a key enzyme in miRNA maturation, significantly altered CYP11B1 and CYP11B2 expression in a human adrenocortical cell line. Screening of nondiseased human adrenal and aldosterone-producing adenoma samples yielded reproducible but distinctive miRNA expression signatures for each tissue type, with levels of certain miRNA, including microRNA-24 (miR-24), differing significantly between the 2. Bioinformatic analysis identified putative binding sites for several miRNA, including miR-24, in the 3′ untranslated region of CYP11B1 and CYP11B2 mRNAs. In vitro manipulation of miR-24 confirmed its ability to modulate CYP11B1 and CYP11B2 expression, as well as cortisol and aldosterone production. This study demonstrates that Dicer-dependent miRNA, including miR-24, can post-transcriptionally regulate expression of the CYP11B1 and CYP11B2 genes. Normal adrenal tissue and aldosterone-producing adenoma differ significantly and reproducibly in their miRNA expression profiles, with miR-24 significantly downregulated in the latter. Adrenal miRNA may, therefore, be a novel and valid target for the therapeutic manipulation of corticosteroid biosynthesis. (Hypertension. 2013;62:00-00.) ● Online Data Supplement

Key Words: aldosterone ■ aldosterone synthase ■ cortisol ■ hypertension ■ microRNAs ■ steroid 11-beta-hydroxylase

The corticosteroid hormones cortisol and aldosterone are important determinants of blood pressure and cardiovascular risk. Excess cortisol results in Cushing syndrome, associated with hypertension and accelerated atherogenesis, whereas excess aldosterone, in primary aldosteronism, leads to severe hypertension with markedly increased risk of myocardial infarction, stroke, and left ventricular hypertrophy. The frequency of primary aldosteronism in hypertensive patients is now accepted to be ≈5% to 10%; recent studies show that aldosterone is an important predictor of cardiovascular risk and outcome, with levels toward the high end of the normal range predicting blood pressure and development of hypertension. Controlled release of corticosteroids from the adrenal cortex is achieved, in part, by strictly regulated expression of genes encoding the steroidogenic enzymes that catalyze their biosynthetic pathways. The final enzymes in this process are particularly important: 11β-hydroxylase (CYP11B1) is responsible for the terminal conversion that produces cortisol, whereas aldosterone synthase (CYP11B2) fulfills the equivalent role in aldosterone production. The CYP11B1 and CYP11B2 genes lie in tandem on human chromosome 8 and have ≈93% sequence similarity within their coding regions. Changes in the expression of these genes have been observed in cases of aldosterone-producing adenoma (APA). In addition, the rate of aldosterone production is known to be heritable, and several polymorphisms in the CYP11B1 and CYP11B2 genes associate with altered 11β-hydroxylation, aldosterone production, or hypertension. Although it is relatively straightforward to propose mechanisms by which polymorphisms in the 5′ regulatory regions of these genes, such as rs1799998, could affect transcription, it is not immediately apparent how polymorphisms located in introns or the 3′ untranslated region (3′ UTR) of these genes could alter expression. However, in recent years, microRNAs (miRNAs) have emerged as key regulatory molecules that regulate ≈30% of human genes. They are endogenous, single-stranded noncoding RNA molecules of ≈22 nucleotides, produced through a series of maturation reactions mediated by the RNase III enzymes, Drosha and Dicer. These post-transcriptional regulatory molecules exert their effects by targeting the 3′ UTR of specific miRNAs. Through mRNA destabilization...
or transcriptional repression, miRNAs act not as simple on–off switches for expression but rather as fine-tuning modulators of mRNA levels. Differential binding of miRNAs, based on abundance or target sequence variation, might account for altered gene expression and, therefore, protein synthesis.

The influence of miRNAs has been seen in cancer, cardiovascular disease, developmental processes, and tumorigenesis. However, few studies have investigated their impact on the regulation of corticosteroid production, and their role in adrenal gland pathophysiology remains poorly understood. Targeted deletion of Dicer elicits effects on adrenal cell development and survival, and miRNAs are also known to be dysregulated in adrenal carcinomas. Although the regulation of aldosterone production by miR-21 has been demonstrated, no direct effect on specific target mRNAs was shown.

Here, we investigate the post-transcriptional effects of miRNAs on CYP11B2 and CYP11B1 expression and on corticosteroid production in the human adrenal cortex. Through short inhibitory RNA (siRNA) knockdown of Dicer expression in a human adrenocortical cell line, we demonstrate that CYP11B1 and CYP11B2 mRNAs are subject to Dicer-mediated miRNA regulation. We also use bioinformatic analysis to identify putative miRNA target sites located in the 3′ UTR of CYP11B1 and CYP11B2 mRNAs and determine whether miRNAs predicted to bind to these sites are expressed in nondiseased adrenal tissue. We then identify miRNAs that are differentially expressed in adrenal tissue from patients with constitutive production of aldosterone as a result of APA, demonstrating reproducible differential expression of specific miRNAs between these tissue types. Finally, we show that one such miRNA, microRNA-24 (miR-24), is able to target both CYP11B1 and CYP11B2 mRNAs directly, significantly decreasing aldosterone and cortisol production in adrenocortical cells.

Methods

For full Materials and Methods, see the online-only Data Supplement.

H295R Cells

These were a gift from Prof William Rainey (Medical College of Georgia, Augusta, GA).

Human Tissue Samples

Four frozen nondiseased adrenal tissue samples were obtained from white adult patients undergoing nephrectomy, with full local ethical approval from the University of Birmingham, United Kingdom. The analyzed tissue was predominantly cortical. Four samples of formalin-fixed paraffin-embedded APA tissue were obtained from the University of Georgia Biobank. Use of tissue in this study was conducted in accordance with the requirements of the Human Tissue Act and with appropriate permission from the local ethical review board. Participants provided informed consent.

Data Analysis

Real-time quantitative reverse transcriptase polymerase chain reaction results were analyzed using the relative quantification method of comparative C\text{ΔΔ}C. In vitro results were analyzed using either an unpaired Student t test or 1-sample t test, as stated. Analysis of results for multiple groups was performed by 1-way ANOVA and Bonferroni post hoc test, comparing all results to a designated control group or to all sets of data, as required. Microarray signal intensities were compared between tissue groups using a 2-way ANOVA and Bonferroni post hoc test. Statistical analysis was performed using GraphPad Prism 4.0 software. All results are expressed as mean±SEM. Unless stated otherwise, in vitro experiments were performed in ≥3 technical replicates on 3 biologically independent occasions.

Results

siRNA Knockdown of Dicer

Two prevalidated Dicer1-targeted siRNA molecules, Dicer1A and Dicer1B (Applied Biosystems), were separately transfected into the H295R adrenocortical cell line. Real-time reverse transcriptase polymerase chain reaction analysis of RNA isolated 48 hours post-transfection showed that each siRNA significantly reduced the level of Dicer1 mRNA relative to a scrambled negative control siRNA (Figure 1A). Dicer1A inhibition of Dicer1 significantly increased CYP11B1 and CYP11B2 mRNAs relative to scrambled negative control RNA, although a statistically significant effect was not observed with Dicer1B siRNA (Figure 1B and 1C).

Bioinformatic Prediction of miRNA-Binding Sites in CYP11B1 and CYP11B2 3′ UTRs

Site predictions generated by each of the 4 algorithms (MicroRNA.org [v. Aug 2010], miR-Viewer [v. June 2005], TargetScan [v.5.1], and MicroCosm Targets [v.5]) were recorded and pooled to create a full list of miRNAs predicted to bind one or both target mRNAs. The number of miRNAs predicted to have binding sites within the 3′ UTR of each gene varied considerably depending on the algorithm used (Table S1 in the online-only Data Supplement). The number of unique miRNAs predicted by ≥1 algorithm to have a target site within the 3′ UTR totaled 202 for CYP11B1 and 172 for CYP11B2. Bioinformatic analysis of the entire human CYP11B1 and CYP11B2 genes, including introns and the intragenic region, did not identify any sequence known to produce existing miRNAs (miRBase v.18).

miRNA Profiling of APA and Nontumorous Adrenal Tissue

miRNA expression was assessed by µParflo technology microarray analysis (v.10.1, LC Sciences, Houston, TX) in total RNA isolated from 4 nondiseased adrenal tissue samples and 4 APA tissue samples. One hundred and three miRNAs were present in nontumorous tissue above the stated threshold level (500 arbitrary units) and 62 in APA samples. Microarray results for individual miRNAs within each tissue group were highly consistent across each of the 4 samples, whereas signals for individual miRNAs were also highly correlated between nontumorous tissue and APA (P<0.0001; r², 0.821; Figure 2A). We combined these microarray data, indicating the presence of miRNAs, with the previous bioinformatic analysis predicting possible miRNA-binding sites in the CYP11B1 and CYP11B2 3′ UTRs, thereby enabling us to identify those miRNAs that could plausibly have a direct post-transcriptional role in CYP11B1 and CYP11B2 regulation. Seven such miRNAs were each predicted to bind both the CYP11B1 and the CYP11B2 3′ UTRs, of which 3 (miR-10b, miR-24, and miR-34c-3p) were present at significantly different levels in nontumorous adrenal and APA tissue (Figure 2B). Of these, only miR-10b and miR-24 were expressed above the
miR-24 Effects on CYP11B1, CYP11B2, and Steroid Production

The effects of miR-10b and miR-24 on the regulation of CYP11B1 and CYP11B2 were assessed by transfecting H295R cells with pre-miR molecules, which artificially supplement the endogenous levels of specific miRNAs. Real-time reverse transcriptase polymerase chain reaction analysis conducted using RNA isolated 48 hours post-transfection showed that miR-10b pre-miRs caused no significant change in CYP11B1 or CYP11B2 mRNA levels relative to a scrambled negative control miRNA (data not shown); therefore, miR-10b was not subjected to any further investigation. However, relative to scrambled negative control RNAs, transfection with miR-24 pre-miR significantly reduced CYP11B1 mRNA 0.64±0.06-fold (P=0.037; Figure 3A) and CYP11B2 mRNA 0.58±0.07-fold (P=0.026; Figure 3B). Cells were then transfected with the miR-24 anti-miR, which, by competitive binding of endogenous miR-24, specifically reduces its availability. Anti-miR had the opposite effect to pre-miR, increasing the abundance of CYP11B1 mRNA 1.38±0.05-fold (P=0.02; Figure 3A) and CYP11B2 mRNA 1.32±0.06-fold (P=0.035; Figure 3B) relative to control. These results are consistent with canonical regulation of CYP11B1 and CYP11B2 by miR-24 in H295R cells. Real-time reverse transcriptase polymerase chain reaction measurement of miR-24 confirmed the respective increase and decrease in its levels as a result of pre-miR and anti-miR transfection (both P<0.001; Figure 3C and 3D).

Furthermore, we observed significant alterations in steroid production rates (as measured by liquid chromatography tandem mass spectrometry), which correlated with changes in CYP11B1 and CYP11B2 mRNA abundance. As CYP11B2 mRNA level fell, so too did aldosterone levels within the cell media, declining significantly 0.78±0.02-fold...
relative to controls ($P=0.044$) in response to miR-24 pre-miR and rising 1.22±0.03-fold in response to miR-24 anti-miR ($P=0.014$; Figure 3E). Similarly, cortisol production fell in response to the pre-miR (0.71±0.05-fold; $P=0.033$), although a corresponding significant increase in response to anti-miR was not observed (0.93±0.03-fold; $P=0.144$; Figure 3F).

**Target Validation of miR-24 by Reporter Constructs**

Reporter construct studies investigated whether miR-24 effects could be attributed to direct targeting of the *CYP11B1* and *CYP11B2* mRNA 3′ UTRs, as predicted by bioinformatic analysis (Figure 4A). Expression vectors fusing the coding region of the firefly luciferase gene to the 3′ UTR of *CYP11B1* (pEZX-B1) or *CYP11B2* (pEZX-B2) were constructed. Each construct also contained a renilla luciferase gene fused to a separate constitutive promoter, which provided an index of transfection efficiency. A single nucleotide in each of the bioinformatically predicted miR-24 seed sites within these constructs was then mutated, as illustrated in Figure 4A; for pEZX-B1 constructs, where 2 miR-24 sites were predicted, singly mutated constructs were prepared, as well as a construct containing mutations at both sites (combined). A no-insert vector (pEZX-C) acted as control. Luciferase activity was found to be significantly increased in cells transfected with pEZX-B1 mutated at either of the predicted miR-24 sites, relative to the original nonmutated pEZX-B1 construct.
sumably because of a greater silencing effect by Dicer1A.

and

CYP11B1

Dicer1A siRNA resulted in significantly increased

0.5-fold after 48 hours. This fairly modest reduction in transcript levels possi-

ble anti-miR. Mutation of either of the predicted miR-24 target sites located in the 3′ UTR of CYP11B2 and CYP11B1 attenuated this anti-

Nevertheless, the derepression of CYP11B1 and CYP11B2 on miRNA depletion confirms that these genes are subject to endogenous negative regulation by miRNAs.

As our bioinformatic analysis indicates that no miRNAs originate from the CYP11B1/CYP11B2 locus, we focused on identifying sites at which miRNAs might act. Putative miRNA target sites located in the 3′ UTR of CYP11B1 and CYP11B2 miRNAs were predicted using 4 different bioinformatic algorithms and resulted in highly variable results from each (Table S1). We pooled these data to improve accuracy and minimize the risk of falsely excluding certain sites. A large number of predicted binding sites were common to both miR-24 and the 3′ UTR sequence, presumably because of the 3′ UTR similarity of the 2 genes (≈80%), which is comparable with that of transcription factor–binding sites in their 5′ regulatory regions.20,21 Nevertheless, numerous miRNAs are predicted to bind only 1 of the 2 transcripts, so the potential exists for independent, as well as common, miRNA-mediated regulation of these genes.

The number and quantity of miRNAs detected in adrenal tissue were remarkably consistent. Other studies analyzing adrenal miRNAs have been published,15,22–25 but comparison is difficult as a result of the various profiling platforms used and the incomplete presentation of data; only Soon et al15 used a microarray system with a level of miRNA coverage equal to that used here (ie, 100% of the miRNAs in miRBase v.10). Our results from APA tissue were also highly consistent, and levels of many miRNAs correlated with those in nondiseased adrenal tissue. However, several miRNAs present in APA were either absent or present at significantly different levels from those in normal tissue. Therefore, APAs have an miRNA profile distinct from that of nontumorous adrenal tissue.

Discussion

Our disruption of miRNA production in an adrenocortical cell line by silencing of Dicer1 used 2 different siRNAs, each of which reduced Dicer1 mRNA levels by ≈0.5-fold after 48 hours. This fairly modest reduction in transcript levels possibly reflects the relative difficulty of transfecting H295R cells. Dicer1A siRNA resulted in significantly increased CYP11B1 and CYP11B2 mRNA levels, whereas Dicer1B did not, presumably because of a greater silencing effect by Dicer1A.
Seven miRNAs predicted to bind both CYP11B1 and CYP11B2 mRNAs were present in both normal and APA tissue: miR-10b, miR-140-3p, miR-143, miR-24, miR-22, miR-34a, and miR-34c-3p. Three were present at significantly different levels in APA and normal tissue, and, of these, miR-10b and miR-24 were expressed above threshold levels in both APA and nontumorous tissue. Although these were selected for target validation studies, there are likely to be further miRNAs that regulate corticosteroidogenesis.

Target validation confirmed that miR-24, but not miR-10b, regulates CYP11B1 and CYP11B2 mRNA abundance and steroid production. Although reporter construct studies found that reduction in miR-24 levels increases the activity of luciferase genes fused to CYP11B1 and CYP11B2 3′ UTRs, a corresponding decrease after overexpression of miR-24 was not observed. One possible explanation for this is that high endogenous levels of miR-24 in HeLa cells may already saturate available miR-24 target sites and repress the construct at a maximal level.30 Nevertheless, experiments with anti-miRs provided further confirmation that miR-24 regulation of CYP11B1 and CYP11B2 occurs via mRNA 3′ UTR sequence. Results from H295R cells are consistent with miR-24 acting to repress both of these genes in a canonical miRNA manner. Furthermore, our mutagenesis studies confirm that miR-24 represses CYP11B1 and CYP11B2 via the bioinformatically predicted sites. The complete abolition of miR-24 effects relative to controls suggests that no further miR-24 sites are present in the CYP11B1 and CYP11B2 transcripts and that all functional miR-24 sites at these loci were predicted by our in silico analysis. Our results also show that both sites in the CYP11B1 3′ UTR are functional and seem to mediate repression by miR-24 to a similar degree.

This study indicates that miR-24 targets a single sequence common to the CYP11B1 and CYP11B2 genes: an offset 6-mer site with an additional G-U wobble pairing at base 2, which can be tolerated in miRNA seed sites (Figure 4A).27 There is a further miR-24 target site unique to the CYP11B1 3′ UTR; this 7-mer-m8 site does have a mismatch in the seed region, but the large degree of base-pairing complementarity within the center region and 3′ end of the miRNA most likely compensate for this.28 miR-24 is transcribed from 2 different genomic locations, each as part of a triplet cluster: miR-24-2 is grouped with miR-23a and miR-27a in an intergenic region on chromosome 19, whereas miR-24-1, miR-23b, and miR-27b cluster in an intron on chromosome 9 (Figure S1). As the 2 miR-24s have identical mature sequences but different primary transcripts, they will share biological function but may have different expression patterns.29 Our data show that miR-23b and miR-27b are also downregulated in APA, in agreement with the observed reduction in miR-24 (Figure S1B). We tested other members of the miR-24-1 cluster for effects on CYP11B1 and CYP11B2 (miR-23b has a putative binding site on CYP11B1), but neither yielded results consistent with canonical miRNA action (data not shown).

The genomic locus spanning the miR-24-1 cluster, C9orf3, encodes a novel metalloprotease called aminopeptidase-O.30 A closely related enzyme, aminopeptidase-A, plays a crucial role in blood pressure regulation and is integral to the renin–angiotensin system, cleaving angiotensin II to angiotensin III.31,32 Although the role of aminopeptidase-O is less well defined, it can cleave angiotensin III to angiotensin IV.30 Angiotensin III, but not angiotensin IV, stimulates aldosterone production in the adrenal gland by a mechanism thought to involve type 2 angiotensin II receptors.33 Reduced transcription at this locus in APA could, therefore, stimulate aldosterone production via post-transcriptional CYP11B2 regulation while simultaneously reducing angiotensin III cleavage. Further investigation of aminopeptidase-O and of the miRNAs transcribed within its introns will be of interest.

A regulatory role for miR-24 has been identified in several other cellular pathways, and its action often seems to be cell-type specific, contributing to opposing cell mechanisms, such as cell proliferation and cell apoptosis, depending on context.34,35 The majority of validated targets for miR-24 are related to regulation of the cell cycle,29 which may explain its observed differential expression in tumor tissue; whether miR-24 contributes to adrenal plasticity remains to be determined.

 Perspectives

We have shown that Dicer-mediated miRNAs in adrenocortical tissue regulate corticosteroid production. Generation of miRNA profiles from nontumorous adrenal tissue, in combination with bioinformatic analysis, enabled us to identify those miRNAs most likely to regulate the terminal stages of aldosterone and cortisol biosynthesis through repression of CYP11B1 and CYP11B2 mRNA function. Comparison of these with APA profiles highlighted miRNAs likely to be involved in the dysregulation of corticosteroidogenesis and to contribute to tumorigenesis. In this way, we identified miR-24 as a probable repressor of these transcripts and confirmed this through in vitro experiments, which also demonstrated significant changes in steroid production. Future research will focus on the identification and validation of further miRNA species likely to act on CYP11B1, CYP11B2, and the various other components of this pathway. We expect such work to reveal a complex regulatory network capable of modulating steroid production with a fine degree of control, which may also provide insights into the development of functional adrenal tumors.

 Acknowledgments

We are grateful to Professor William E. Rainey (Medical College of Georgia, Augusta, GA) for the kind gift of H295R cells, to Tim Harvey (University of Glasgow) for his technical assistance and to Dr John D. McClure (University of Glasgow) for his statistical advice.

 Sources of Funding

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 Disclosures

None.

 References

MicroRNA-24 Regulates Corticosteroid Production

What Is New?

• Comparison of microRNA profiles reveals consistent differences between aldosterone-producing adenoma and nondiseased adrenal tissue. We demonstrate that 1 differentially expressed microRNA, miR-24, represses expression of the steroidalogenic CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) genes through direct binding of their respective mRNAs.

What Is Relevant?

• MicroRNAs are shown to play a direct role in adrenal steroid production. This novel regulatory mechanism may be relevant to common forms of hypertension, such as primary aldosteronism, and provide new targets for intervention.

Novelty and Significance

What Is New?

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microRNA-24 is a novel regulator of aldosterone and cortisol production in the human adrenal cortex

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Short title: microRNA-24 regulates corticosteroid production

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Supplemental Methods

Cell Culture – The H295R human adrenocortical tumour cell line (a gift from Prof. William Rainey, Medical College of Georgia, U.S.A.) was maintained in Dulbecco’s modified Eagles medium/F12 (1:1) with HEPES buffer (Invitrogen). This was supplemented with 2.5% Ultroser G (Pall Bioscience), 1% Insulin-transferrin-selenium (ITS) (BD Biosciences), 1IU penicillin, 100µg/mL streptomycin (Invitrogen). HeLa cells (European Collection of Animal Cell Cultures) were maintained in Dulbecco’s modified Eagles medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 1IU penicillin and 100µg/ml streptomycin (Invitrogen).

Human Tissue Samples – Four frozen non-diseased adrenal tissue samples were obtained from white adult patients undergoing nephrectomy, with full local ethical approval from the University of Birmingham, UK. The analysed tissue was predominantly cortical. Four samples of formalin-fixed paraffin-embedded (FFPE) APA tissue was obtained from the University of Glasgow Biobank. Use of tissue in this study was conducted in accordance with the requirements of the Human Tissue Act and with appropriate permission from the local ethical review board. Participants gave informed consent.

RNA Isolation – Total RNA, including the miRNA fraction, was isolated from H295R cells grown in 6-well plates and from frozen tissue samples using the miRNeasy mini kit (QIAGEN), according to the manufacturer’s instructions. Cells were directly lysed using 700µl of QIAzol per well, or else 40mg of frozen tissue was lysed in a FastPrep Lysing Matrix D Tube with 700µl of QIAzol. Total RNA was isolated from four 20µm FFPE tissue sections using the RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems) according to the manufacturer’s instructions. RNA concentration was determined by NanoDrop® and its quality assessed using the Bioanalyser 2100 apparatus (Agilent).

miRNA Microarray Analysis – miRNA microarray analyses were performed by LC Sciences (Houston, Texas, U.S.A.), using 5µg of the total RNA. Samples were quality checked prior to proceeding with the miRNA µParaflo® technology microarray (version 10.1), then labelled with a fluorescent dye and hybridised overnight to a microfluidic chip containing complementary probes for 723 human miRNAs and other RNA controls. Post-array analysis utilised internal spike-in controls to cross-normalise the array data output, allowing signals to be compared between chips.

Cell Transfection – H295R Cells were transfected using siPORT NeoFX Transfection Agent (Ambion), according to manufacturer’s instructions. They were transfected with Pre-miR™ or Anti-miR™ molecules (Applied Biosystems) to a final concentration of 50nM, or with pre-validated siRNA molecules (Dicer 1A: Product no. s23755; Dicer 1B: s23756, Applied Biosystems) to a final concentration of 30nM, in 6-well plates at a final cell density of 4.8×10^5 cells/well. HeLa cells were co-transfected with pEZX-construct (500ng) and either Pre-miR™ or Anti-miR™ in 24-well plates at a final cell density of 8×10^4 cells/well.

Real-Time Quantitative Reverse Transcriptase PCR (qRT-PCR) – First-strand cDNA was generated in a 20µl volume using the miScript RT Kit (QIAGEN) and 200ng of total RNA. The resulting cDNA was diluted to a final volume of 100µL and 2µl of this
was amplified by PCR (ABI PRISM 7900HT, Applied Biosystems). Reactions used the miScript SYBR Green PCR Kit (QIAGEN) to measure mature miRNA-derived cDNA sequences and the Universal ProbeLibrary (UPL) System (Roche Applied Science) and ABsolute™ QPCR ROX Mix (Abgene) to measure CYP11B1 and CYP11B2 cDNA.

Steroid Measurement – Steroids were extracted from cell media using ChemElute cartridges (Varian), and eluted from the cartridge with dichloromethane. The samples were evaporated to dryness and reconstituted in 10% acetonitrile. Identification and quantification of steroid products was achieved by tandem mass spectrometry using a Varian 1200L mass spectrometer with a triple quadrupole detector.¹

Bioinformatic prediction of miRNA binding sites in CYP11B1 and CYP11B2 3’UTR – Four commonly-used target prediction algorithms (MicroRNA.org (v. Aug 2010), miR-Viewer (v. June 2005), TargetScan (v.5.1) and MicroCosm Targets (v.5)) were employed to identify putative miRNA binding sites within the 3’UTR sequences of CYP11B1 (2,022 bases) and CYP11B2 (1,428 bases). 3’UTR length and sequence was identified and confirmed using the UCSC Genome Browser Gateway and Ensembl Genome Browser (CYP11B1: ENST00000292427 and CYP11B2: ENST00000323110).

Reporter Construct Studies – Luciferase reporter constructs containing the full-length 3’UTR sequence of the CYP11B1 (pEZX-B1) or CYP11B2 (pEZX-B2) genes (LabOmins) were co-transfected together with Pre-miR or Anti-miR; a ‘no-insert’ vector (pEZX-C) was used as a control (LabOmins). The Dual Luciferase Reporter Assay system (Promega) and a Lumat LB 9507 tube luminometer (Berthold Technologies) were used to measure Firefly and Renilla luciferase activity in cell lysates containing 1x Passive Lysis Buffer, according to the manufacturer’s instructions.

Site-directed Mutagenesis - A single nucleotide in the seed region of the putative miR-24 binding site was mutated in each of the pEZX-B1 3’UTR and pEZX-B2 3’UTR plasmids (Figure 4a). There are two predicted binding sites in the 3’UTR of the CYP11B1 gene and these were mutated singly (Site 1 or Site 2) and in combination (Combined). Mutagenesis was carried out using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) and pairs of oligonucleotide primers at each site (Eurofins MWG Operon). The sequences of the primer pair for the common miR-24 site were 5’-GCCCTGCAGGACTCACCTGCCTGTACAGG-3’ (Forward) and 5’-CTGTACAGGGTGAGTCTGAGGGCG-3’ (Reverse); sequences for the CYP11B1-only miR-24 site were 5’-CTCGAGCATGCTGGACCTGAGCGCTGCTGTTGTA-3’ (Forward) and 5’-GACAATTTTCAGGAGGCTAGGAGGCACTGCTGAGG-3’ (Reverse; mutated nucleotides are underlined). Successful incorporation of mutations were confirmed by direct sequencing.

Data Analysis – qRT-PCR results were analysed using the relative quantification method of comparative Ct (ΔΔCt).¹⁸ In vitro results were analysed using either an unpaired Students’ t-test or one-sample t-test, as stated. Analysis of results for multiple groups was performed by one-way analysis of variance (ANOVA) and Bonferroni’s post-hoc test, comparing all results to a designated control group or to
all sets of data, as required. Microarray signal intensities were compared between tissue groups using a two-way ANOVA and Bonferroni’s post-hoc test. Statistical analysis was performed using Graph Pad Prism 4.0 software. All results are expressed as mean ± SEM (standard error of the mean). Unless stated otherwise, in vitro experiments were performed in at least three technical replicates, on three biologically-independent occasions.

Reference
**Supplemental Table**

Table S1: Bioinformatic analysis identifying putative miRNA-binding sites in the human CYP11B1 and CYP11B2 3'UTRs. The total number of miRNA binding sites identified by each database within the relevant 3'UTR is shown, with the number of different miRNAs predicted to bind that 3'UTR in parentheses. The right-hand column shows the total number of different miRNAs predicted, by at least one database, to bind that 3'UTR.

<table>
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<th>Gene</th>
<th>3'UTR Length (Base pairs)</th>
<th>Microrna.org</th>
<th>miR-Viewer</th>
<th>TargetScan</th>
<th>MicroCosm Targets</th>
<th>Unique miRNAs with Binding Sites</th>
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</table>
Supplemental Figure

Figure S1 A: Illustration of pre-miR-24-1 cluster located on human chromosome 9. B: Microarray expression levels in normal and aldosterone-producing adenoma tissue of the miRNAs from the two miR-24 clusters. Normalised microarray signals are shown, with each achieving the threshold value of >500AU (represented by the dotted line) in at least one tissue type; ** p<0.01, *** p<0.001.