

Multiple noncoding exons 1 of nuclear receptors NR4A family (nerve growth factor-induced clone B, Nur-related factor 1 and neuron-derived orphan receptor 1) and NR5A1 (steroidogenic factor 1) in human cardiovascular and adrenal tissues

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**Multiple noncoding exons 1 of nuclear receptors NR4A family (NGFIB,NURR1,NOR1) and NR5A1 (SF1) in human cardiovascular and adrenal tissues**

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## **Abstract**

**Objective:** Nuclear receptors are involved in a wide variety of functions, including aldosteronogenesis. Nuclear receptor families NR4A (NGFIB,NURR1,NOR1) and NR2F (COUP-TFI,COUP-TFII,NR2F6) activate, whereas NR5A1 (SF1) represses CYP11B2 (aldosterone synthase) gene transcription. The present study was undertaken to elucidate the mechanism of differential regulation of nuclear receptors between cardiovascular and adrenal tissues.

**Methods:** We collected tissues of artery (n=9), cardiomyopathy (CM) muscle (n=9), heart muscle (non-CM) (n=6),adrenal gland (n=9) and aldosterone-producing adenoma (APA) (n=9). 5'-rapid amplification of cDNA ends (5'-RACE) identified transcription start sites. Multiplex RT-PCR determined use of alternative noncoding exons 1 (ANEs).

**Results:** In adrenocortical H295R cells, angiotensin II, KCl or cAMP all stimulated CYP11B2 transcription and NR4A was up-regulated, whereas NR2F and NR5A1 were down-regulated. 5'-RACE and RT-PCR revealed 4 ANEs of NGFIB (NR4A1), 3 of NURR1 (NR4A2), 2 of NOR1 (NR4A3), and 2 of SF1 (NR5A1) in cardiovascular and adrenal tissues. Quantitative multiplex RT-PCR showed NR4A and NR5A1 differentially employed multiple ANEs in a tissue-specific manner. The use of ANEs of NGFIB and NURR1 was significantly different between APA and artery. Changes in use of ANEs of NGFIB and NOR1 were observed between CM and non-CM. The NR4A mRNA levels in artery were high compared to cardiac and adrenal tissues, whereas the NR5A1 mRNA level in adrenal tissues was extremely high compared to cardiovascular tissues.

**Conclusions:** NR4A and NR5A1 genes are complex in terms of alternative promoter use. The use of ANEs may be associated with the pathophysiology of the heart and adrenal gland.

## Introduction

Aldosterone classically plays an important role controlling fluid, electrolyte homeostasis and blood pressure. Aldosterone synthase, encoded by the CYP11B2 gene, catalyzes the final step from corticosterone to aldosterone. Although a major source of aldosterone production is the adrenal cortex, aldosterone production occurs in the extra-adrenal sites (heart, blood vessels, brain) via the renin-angiotensin-aldosterone system (RAAS) [1, 2, 3] and aldosterone is now considered a major cardiovascular risk hormone.

Aldosterone contributes to vascular smooth muscle cells hypertrophy [4]. Exogenous aldosterone administration to mice increases macrophage oxidative stress and atherosclerotic lesion development [5] and an aldosterone synthase inhibitor reduces atherosclerosis and inflammation in atherosclerotic apolipoprotein E-deficient mice [6]. Furthermore, mineralocorticoid blockade improves resistance artery remodeling in hypertensive patients [7]. These observations suggest that aldosterone is associated with atherosclerosis development.

A number of nuclear receptors families, including NR4A (NGFIB, NURR1, NOR1), NR2F (COUP-TFI, II, 2F6), and NR5A1 (SF1) have been reported to play a pivotal role in CYP11B2 gene transcription. NR4A and NR2F families function as activators [8, 9, 10, 11] whereas NR5A1 acts as a repressor [11, 12, 13, 14]. A recent study showed that differentially regulated, alternative promoters were common, and that about 60% of protein-coding genes employ two or more alternative promoters [15]. As a typical example, the CYP19 (aromatase) gene has 11 alternative promoters used preferentially by different tissues [16]. We previously cloned promoter I.8 of the CYP19 gene [17]. The ATG translation initiation site is located 38 bp downstream of a common splice acceptor site in coding exon II. The 93 kilobase (kb) 5' flanking region of the gene contains a number of „physiological“ promoters with alternative noncoding exons 1 (ANEs) that are controlled in a tissue-specific manner. These ANEs are spliced alternatively onto a common splice junction, leading to promoter-specific mRNA species that encode the identical aromatase protein.

We investigated nuclear receptor genes involved in aldosterone synthesis, focusing on differentially used alternative promoters with ANEs. In the present study, we identified a number of ANEs in nuclear receptor genes including NR4A family members and NR5A1. All these genes preferentially employed distinct ANEs between human cardiovascular and adrenal tissues.

## **Materials and Methods**

### **Cell culture and human tissues**

Adrenocortical H295R cells were purchased from ATCC (Manassas, VA) and maintained in DMEM/F12 medium (Invitrogen, Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 2.5% Ultrosor G (Life Sciences, Cergy, France), penicillin, streptomycin (Life Technologies), and 1% ITS premix (BD Biosciences, Bedford, MA). Human renal artery and adrenal gland were obtained directly after total nephrectomy for renal cell carcinoma (renal artery, n=4; adrenal gland, n=4). In addition, human aorta and adrenal gland were obtained from autopsy specimens (aorta, n=5 ; adrenal gland, n=5). Finally, human artery and adrenal gland with 9 samples each were obtained. Ventricular tissue was 9 patients with cardiomyopathy (CM) (hypertrophic cardiomyopathy, n=8; dilated cardiomyopathy, n=1) and obtained from 6 autopsy cases without heart muscle disease (non-CM). Tumor tissue was also obtained from 9 patients with aldosterone-producing adenoma (APA). Experimental protocols were approved by the Institutional Review Board of Kanazawa University and Tohoku University. The purpose of the study was explained to all patients, and written informed consent was obtained from each study participant.

### **Real-time RT-PCR**

Messenger RNA (1 µg) was reverse-transcribed from total RNA using Superscript III (Invitrogen, Life Technologies Japan Ltd., Tokyo, Japan) and random hexamer primers, and was quantified using real-time PCR. Real-time PCR using the SYBR Green method was carried out with gene-specific primer pairs (Table 1). Real-time PCR for CYP11B2 was carried out using the TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies Japan Ltd., Tokyo, Japan). PCR amplification was performed in triplicate employing an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The cDNA (2 µl) was added to the PCR mixture in a final volume of 20 µl. Thermal conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec, 60 °C for 1 min.

To estimate the copy number of each RNA investigated, 10-fold serial dilutions of appropriate cloned DNA were used as templates to generate standard curves, as we previously reported [17, 18, 19]. Briefly, a standard PCR was performed to obtain PCR products of target genes. Subsequently, PCR products were cloned using TA cloning. Clones containing PCR products were cultured overnight in 1 ml of LB medium with ampicillin. Plasmid DNA was extracted using Qiagen Miniprep Kit (Qiagen, Tokyo, Japan). A 10-fold serial dilution of these appropriate cloned DNA samples ranging from  $10^{-18}$  to  $10^{-23}$  mol/L was produced using DNase-free and RNase-free water (Qiagen) for the DNA standards for each target. Triplicates of each cDNA sample, serially diluted standards and no template controls were added to each real-time PCR run.

### **Western blot**

Proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Yokohama, Japan). Proteins in SDS-PAGE gels were electrophoretically transferred to PVDF membranes and visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Samples were incubated with anti-NGFIB (NR4A1) (CBX00289; Cosmo Biolabs Inc., Tokyo, Japan), anti-NURR1 (NR4A2) (sc-991; Santa Cruz Biotechnology), anti-NOR1 (NR4A3) (PP-H7833-00; Perseus Proteomics Inc., Tokyo, Japan), or anti-SF1 (NR5A1) (a generous gift from Dr. Ken-ichiro Morohashi).

### **Rapid amplification of 5' cDNA ends (5'-RACE)**



To identify transcription start sites (TSSs), 5'-RACE was performed using the CapFishing™ Full-length cDNA Premix Kit (Seegene, Inc., Seoul, Korea). The 5'-RACE was individually done using total RNA from H295R cells, artery, cardiac muscle, adrenal cortex and APA tissues. Briefly, CapFishing™ adaptor-ligated and double stranded cDNA were synthesized using SuperScript™ II (Life Technologies Japan Ltd., Tokyo, Japan). The PCR was performed using the 5'-RACE primer (5'-gtc tac cag gca ttc get tea t-3') and a 3' target-specific antisense primer (Table 1). The resulting PCR products were fractionated on a 1% agarose gel. All bands within the range 150 to 300 bp were subcloned using pGEM-T Easy vector system (Promega Corporation, Madison, WI, USA) and sequenced. The sequencing analysis was performed using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems). The sequencing reactions were performed according to the manufacturer's instructions and analyzed on an ABI310 DNA Sequencer (Applied Biosystems).

### **Multiplex RT-PCR for measuring noncoding exon 1 usage**

To characterize alternative noncoding exon usage, we performed multiplex RT-PCR as we previously reported [17]. Briefly, we designed several different amplicons (Table 1). The reverse primers were located at common coding exons and labeled with 6-FAM dye for NGFIB (NR4A1), VIC dye for NURR1 (NR4A2), NED dye for NOR1 (NR4A3), and PET dye for NR5A1 (SF1). Forward primers were located at different untranslated exons identified by 5'-RACE or in DBTSS (DataBase of Transcriptional Start Sites) (<http://dbtss.hgc.jp/>) (Table 1). Total RNA was treated with TURBO DNase (Applied Biosystems) before synthesizing cDNA. The PCR reaction for each gene was carried out as follows: denaturation at 96°C for 5 min, 45 cycles of 96°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. After PCR amplification, RT-PCR products were separated on an ABI 3100 capillary sequencer with an internal size standard GeneScan-600 LIZ (Applied Biosystems) and quantified by GeneScan software (Applied Biosystems).

## **Results**

### **Effect of angiotensin II, KCl, and cAMP on nuclear receptor expression in adrenocortical H295R cells**

Adrenocortical H295R cells were treated with 100 nM angiotensin II (Ang. II), 16 mM KCl, or 1mM cAMP for 6 hours. All three members of the NR4A family, NGFIB (NR4A1), NURR1 (NR4A2) and NOR1 (NR4A3), were upregulated in the presence of Ang. II, KCl or cAMP (Fig. 1), whereas NR2F family genes (COUP-TFI, COUP-TFII, NR2F6), and NR5A1 were downregulated in the presence of Ang. II, KCl or cAMP. Each treatment also upregulated transcription of the nuclear receptor target gene CYP11B2 in adrenocortical H295R cells (Fig. 1). NR4A and 2F families upregulate [8], whereas NR5A1 downregulates the CYP11B2 gene transcription [20], suggesting that NR4A family and NR5A1 were considered important for the CYP11B2 induction in H295R cells. These results led us to select NR4A family and NR5A1 for further analyses.

### **Identification of transcription start sites (TSSs) and noncoding exons 1 by means of 5'-RACE and RT-PCR**

According to a default setting in DBTSS (DataBase of Transcriptional Start Sites; <http://dbtss.hgc.jp/>), we separated alternative promoters using a 500-bp interval between two transcription start sites. The 5'-RACE for NGFIB cloned canonical ANE and novel ANE directly upstream of the coding exon in H295R cells and artery (Fig. 2a). The novel ANE-specific RT-PCR product was successfully amplified, confirming that the newly-identified NGFIB ANE was transcribed in H295R cells. We also identified a number of candidates for further NGFIB ANE in DBTSS. Using RT-PCR, we confirmed 4 different ANEs within 17 kb upstream of the first NGFIB coding exon. We designated these ANEs as ANE 1, 2, 3, and 4 serially from 5' to 3'. The ATG translation initiation site was located 3 bp downstream of a common splice acceptor site in

coding exon 1. Additionally, we observed two different splicing events of ANE1-derived transcripts (Fig. 3a).

We also cloned novel ANE for NURR1 (Fig. 2b) directly upstream of the first coding exon. As with the novel NGFIB ANE, we confirmed that the newly-identified ANE was truly transcribed. NURR1 differentially employed 3 promoters with distinct ANEs within 2.5 kb of the coding region (Fig. 2b). A common splice site was located in the 124 bp noncoding exon 2 (Fig. 3b). The ATG translation initiation site was 127 bp downstream of a common splice acceptor site.

We were not able to clone any full-length mRNA species for NOR1 from the 5'-RACE cDNA library. According to bioinformatics from NCBI, the 6.2 kb 5' flanking region of the NOR1 gene contains 2 different promoters with distinct ANEs. Those canonical promoters produce 4 different transcript variants (TVs), encoding different isoforms, from the single NOR1 gene. TV1 (NM\_00698.2) and 2 (NM\_173198.1) produce the same isoform a. TV3 (NM\_173200.1) and 4 (NM\_173199.1) make isoform b and c, respectively. In terms of promoter use, TV1, 2, and 4 are derived from an alternative promoter using ANE1, whereas TV3 uses ANE2. Using RT-PCR, we confirmed the expression of both ANE1 and 2-derived transcripts in H295R cells. The ATG translation initiation site is located 3 bp downstream of a common splice acceptor site in coding exon 1 (Fig. 3c).

Using the same strategy we determined the ANEs of NR5A1 (Fig. 3d). 5'-RACE identified ANE1a in H295R cells. Additionally, RT-PCR confirmed ANE1b-derived transcripts in H295R cells among a number of candidate ANEs from DBTSS.

All ANEs of the 4 nuclear receptor genes identified in this study were spliced alternatively onto a common splice junction, leading to promoter-specific mRNA species that encode an identical protein.

### **Analysis of alternative noncoding exon 1 usage in adrenocortical H295R cells**

ANE3 was the dominant NGFIB noncoding exon 1 in H295R cells as determined by quantitative multiplex RT-PCR (Fig. 4). Compared to basal levels, NGFIB-ANE3 transcription was significantly increased in the presence of Ang. II ( $56.9 \pm 0.3\%$  vs  $50.0 \pm 0.2\%$ , mean  $\pm$  (standard deviation (SD))). NURR1 ANE1a was primarily utilized in H295R cells whereas ANE1b usage was extremely low (Fig. 4). Compared to basal levels, NURR1 ANE1a usage was significantly increased in the presence of Ang. II ( $62.5 \pm 2.7\%$  vs  $58.0 \pm 3.4\%$ , mean  $\pm$  SD). ANE2 was the dominant ANE for NOR1 (NR4A3) under basal, Ang. II and KCl-stimulated conditions in H295R cells. cAMP stimulation appeared to switch the NOR1 dominant ANE from ANE2 to ANE1 (Fig. 4). NR5A1 ANE usage appeared to show no significant change (Fig. 4). These observations indicate that different stimuli stimulate ANEs of NR4A family in a different manner in H295R cells (Fig. 4), and that cAMP may be one pathway used to differentially regulate NOR1.

#### **Analysis of gene expression in human cardiovascular and adrenal tissues**

Arterial gene expression of the NR4A family genes was higher than in other tissues from non-CM, CM, adrenal cortex and APA. In contrast, the mRNA levels of NR5A1 and the target gene CYP11B2 in adrenal cortex and APA were significantly higher than those in artery, non-CM and CM (Fig. 5a).

#### **Analysis of alternative noncoding exon 1 usage in human cardiovascular and adrenal tissues**

NGFIB ANE3 was preferentially used in human artery, adrenal cortex and APA. ANE1 was used only at low levels in all tissues analyzed in this study. NGFIB employed ANE2 at distinct levels between cardiovascular and adrenal tissues: ANE2 was the dominant noncoding exon 1 used in cardiovascular tissues. CM tissue showed up-regulation of ANE4 and down-regulation of ANE3 compared to cardiac muscle tissue without disease (Fig. 5b).

NURR1 ANE1a usage was high in artery compared to other tissues. NOR1 ANE1 usage was high in CM compared to other tissues. SF1 used ANEs differentially between cardiovascular and adrenal tissues (Fig. 5b).

### **NGFIB and NURR1 differentially use ANEs between APA and artery**

NGFIB and NURR1 were regulated between APA and artery in a tissue-specific manner. The use of NGFIB ANE 4 in APA was twice that seen in artery (42.3% vs. 21.5%;  $P < 0.0001$ ) (Fig. 6). NGFIB serves a protective function for atherosclerosis [21, 22] whereas the role of NURR1 in atherosclerosis remains unknown. Targeting ANE4-specific transcripts may reduce NGFIB function specifically in APA without loss of its function in artery. Likewise, we could target either ANE 1a or 2 of NURR1 to decrease NURR1 activity only in APA, avoiding anticipated adverse effects on blood vessels (Fig. 6). Therapeutic strategies that target differentially-regulated ANEs of NGFIB and NURR1 may show clinical utility against primary aldosteronism.

## Discussion

We report the genomic organization and structure of NR4A family and NR5A1 genes. We found a number of alternative promoters with noncoding first exons in the genes encoding the NR4A and NR5A families of nuclear receptors that regulate aldosterone synthase expression and thus aldosterone production. NGFIB and NURR1 showed significant differences in use of ANEs between APA and artery.

Primary aldosteronism is the most frequent form of secondary hypertension, with prevalence rates of 5% to 13% [23]. APA and bilateral idiopathic hyperaldosteronism are the most common subtypes of primary aldosteronism. Patients with primary aldosteronism exhibit a higher rate of cardiovascular complications, target organ damage and metabolic syndrome. Unilateral adrenalectomy is a treatment option for patients with APA. Patients with bilateral idiopathic hyperaldosteronism are treated medically. In addition, APA patients may be treated medically. Mineralocorticoid receptor (MR) antagonists are currently the most commonly used treatment of primary aldosteronism. Although the use of MR antagonists shows clinical benefit in the treatment of primary aldosteronism, it also leads to severe side effects such as gynecomastia and endocrinal dysregulation. New therapeutic strategies that directly target the synthesis of aldosterone are preferred.

The NGFIB response element (NBRE) within the CYP11B2 promoter has been shown to control transcription by its binding of members of the NR4A family (NGFIB, NURR1, NOR1) [8, 9]. NGFIB and NURR1 are thought to up-regulate CYP11B2 expression in human adrenal cortex and APA [8, 10]. Thus, NR4A nuclear receptors are emerging as a candidate therapeutic target in primary aldosteronism. All these nuclear receptors are also expressed in atherosclerotic lesions [24], and appear to exert a direct influence on vascular remodeling, including cell viability and proliferation, inflammation [25]. NGFIB exerts a favorable effect on artery in terms of atherosclerosis [21, 22] whereas NOR1 appears atherogenic [26]. The pathophysiological role of NURR1 remains to be elucidated in atherogenic responses. Thus, NGFIB should be differentially

regulated between APA and artery to inhibit aldosterone synthase expression and prevent atherosclerosis: NGFIB should be down-regulated in APA, but not in artery.

Although NGFIB and NURR1 transcripts from different tissues contain the same coding exons, they differ in the noncoding exon 1, which is transcribed from individual alternative promoters and subsequently spliced onto the common coding exons (Fig. 3a). The use of NGFIB ANE 4 in APA was twice that seen in artery (42.3% vs. 21.5%;  $P < 0.0001$ ) (Fig. 5b). Targeting ANE4-specific transcripts may lead to efficient reduction of NGFIB function specifically in APA, maintaining its function in artery. Since we observed a significant difference in use of ANEs (Fig. 5b), NURR1 function could also be differentially controlled between APA and artery. Modulating alternative promoter usage of NGFIB and NURR1 may have clinical utility in the treatment of APA or cardiovascular disease.

A number of approaches could be employed to target NGFIB and NURR1. Oligo-DNA molecules may be introduced as „decoy“ *cis*-elements to block the activation of tissue specific promoters [27]. Alternatively, siRNA-directed transcriptional silencing may also be applicable if they were designed to target the tissue-specific ANE [28, 29]. Therefore, a difference in promoter usage among tissues is a potentially important target for effective treatments. We believe that our attempt to characterize this difference in aldosterone regulation between artery and primary aldosteronism will lead to a novel therapeutic option that efficiently suppresses aldosterone.

In contrast to NR5A1 (SF1), NR4A family members are differentially expressed in a number of tissues of metabolic (heart, liver and adipose) and endocrine (hypothalamus, pituitary and adrenal glands) origin. A growing body of evidence is accumulating to demonstrate a critical role of NR4A receptors in regulating glucose and lipid homeostasis, adipogenesis, inflammation and vascular remodeling [25, 30]. Although NR4A function remains unclear in the heart, the acute induction of NR4A expression following  $\beta$ -adrenergic stimulation in the heart implicates the NR4A family as potential mediators of cardiac muscle metabolism associated with  $\beta$ -adrenergic signaling [31]. We observed changes in use of ANEs of NGFIB and NOR1 in CM compared to

cardiac muscle without disease (Fig. 5b). These observations may be associated with myocardial energetic impairment in CM.

The tissue-restricted pattern of gene expression is predominantly determined by the action of alternative promoters. However, alternative noncoding exons can also modulate gene expression in a posttranscriptional manner [32]. Further experiments are required to elucidate the underlying mechanisms by which NR4A family and NR5A1 are differentially regulated between cardiovascular and adrenal tissues, for example by identifying *cis*-acting elements in the alternative promoter regions and determining the effect of alternative exons 1 on mRNA stability and protein translation.

In summary, we reveal that nuclear receptors including NR4A family and NR5A1 differentially used multiple promoters with ANEs in human cardiovascular and adrenal tissues. All these genes showed significant differences in use of ANEs between those tissues. These findings may provide opportunities for further investigation into the regulatory mechanisms of those genes.



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## Tables

Table 1. Primers used in this study

Real time RT-PCR

Name	Sequence (5'-3')	Locus version	*Position	AT	Product size (bp)
NGFIB (NR4A1) F	gcatggtgaaggaagttgctc	NM_173157.1	1156-1268	60	113
NGFIB (NR4A1) R	cagggaagtgaggagattgg				
NURR1 (NR4A2) F	agtctgatcagtcacctgt	NM_006186.2	1422-1503	60	82
NURR1 (NR4A2) R	tcgacctggaacctggaat				
NOR1 (NR4A3) F	tggaatggtaaaagaagttgctc	NM_006981.2	1794-1875	60	82
NOR1 (NR4A3) R	taatgggctctttggtttgg				
SF1 (NR5A1) F	atggtcttcaaggagctgga	NM_004959.3	1037-1131	60	95
SF1 (NR5A1) R	acctggcggtagatgtggt				
GAPDH F	tcattgacctcaactacatggttt	NM_002046.3	212-359	60	148
GAPDH R	ttgattttggagggatctcg				
COUP-TFI (NR2F1) F	atcgtgctgttcacgtcaga	NM_005654.4	2660-2769	60	110
COUP-TFII (NR2F1) R	gggtactggctcctcacgta				
COUP-TFII (NR2F2) F	gcaagtggagaagctcaagg	NM_021005.2	1472-1570	60	99
COUP-TFII (NR2F2) R	tcagagagaccacagcatct				
NR2F6 F	ctacacctgccggtccaac	NM_005234.3	389-476	60	88

NR2F6 R	ccggaagcacttcttgagac				
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5'-rapid amplification of cDNA ends (5'-RACE)

Name	Sequence (5'-3')	Locus version	*Position	AT	
NGFIB 268R	atggtgggcttgatgaactc	NM_173157.1	c249-268	60	1st round PCR
NGFIB 206R	actcgggtgctggtgtcccatatt	NM_006186.2	c184-206	66	2nd round PCR (nested PCR)
NURR1 338R	catggttcagccgagtta		c320-338	60	
NR5A1 525R	atctgtgccttctctgctgttca	NM_004959.3	c501-525	66	

Quantitative multiplex RT-PCR

Name	Sequence (5'-3')	Locus version	*Position	AT	Product size (bp)	
NGFIB ANE1 F	aaaagcgggggtggagag	NT_029419. 12 (genomic DNA)	14574493- 14574510	60	147, 267	DBTSS
NGFIB ANE2 F	tgtctgggacctttccag		14580953- 14580972		155	DBTSS
NGFIB ANE3 F	cagggaccaggctgagac		14588600- 14588617		165	5'-RACE, NCBI (canonical)
NGFIB ANE4 F	attctgccaccttgetg		14591313- 14591330		210	5'-RACE, (novel)
NGFIB 268R-	6-FAM-	NT_029419.	c14591503-			

FAM	atggtgggcttgatgaactc	12 (genome DNA)	14591522			
		NM_173157.1 (mRNA)	c249-268			
NURR1 ANE1a F	aactgcacttcggcagagtt	NT_005403.17 (genome DNA)	c7398443-7398462	60	183	5'-RACE, NCBI (canonical)
NURR1 ANE1b F	tcagtttatgcgttgcttgg		c7397981-7398000		191	DBTSS
NURR1 ANE2 F	aacaggggaaggcatggaag		c7396803-7396821		229	5'-RACE, (novel)
NURR1 12.5kb F	ggaagggaggaggagacct		c7408567-7408585		(UD)	DBTSS (12.5 kb upstream of coding region)
NURR1 4kb F	gctgcagattaggggtgagc		c7400200-7400219		(UD)	DBTSS (4 kb upstream of coding region)
NURR1 1.8kb F	ggggttcagagtctcttct		c7397725-7397744		(UD)	DBTSS (1.8 kb upstream of coding region)
NURR1 338R-VIC	VIC-catggcttcagccgagtta		NT_005403.17 (genome)		7396114-7396118,	

		DNA)	7396598- 7396611			
		NM_006186. 2 (mRNA)	c320-338			
NOR1 ANE1 F	accgccacagcactcaaag	NM_006981. 2 (mRNA)	537-555	289	NCBI (canonical)	
NOR1 ANE2 F	aatcagatttgaaatgtgga tatg	NM_173200. 1 (mRNA)	56-80	118	NCBI (canonical)	60
NOR1 825R- NED	NED- gttcgatctccgtggtgat t	NM_006981. 2 (mRNA)	c803-825			
		NM_173200. 1 (mRNA)	c151-173			
NR5A1 ANE1a F	gtggtgtgagggggttc		c56434167- 56434184	254	5'-RACE, NCBI (canonical)	
NR5A1 ANE1b F	gggtctcccagactcacc	NT_008470. 19 (genome	c56433911- 56433928	289	DBTSS	60
NR5A1 17kb F	gctctctgatgaggcaagaa	DNA)	c56447311- 56447330	(UD)	DBTSS (17kb upstream of coding region	
NR5A1 2kb F	aaacgaatccaatgaag		c56430507-	(UD)	DBTSS	

			56430525			(2kb upstream of coding region)
NR5A1 301R- PET	PET- cttgaagaagcccttgag	NT_008470. 19 (genome DNA)	56430105- 56430111, 56430020- 56430031			
		NM_004959. 3 (mRNA)	c283-301			

F, forward primer; R, reverse primer; AT, annealing temperature; c, complementary; UD, undetectable; DBTSS, DataBase of Transcriptional Start Sites (<http://dbtss.hgc.jp/>).

\*Amplicon and primer positions refer to numbering of the original sequence submission from National Center for Biotechnology Information (NCBI).



## Figure legends

### Figure 1

Responses of the CYP11B2 gene to angiotensin II (Ang. II), KCl, and cAMP. (a) NR4A family. (b) NR2F family. Values are means  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , vs basal, two tailed Student's t-test.

### Figure 2

Identification of novel NGFIB and NURR1 noncoding exons 1 in H295R cells. (a) NGFIB ANE4. 2 transcription start sites were identified: the distal site was cloned in H295R cells whereas the proximal site in artery. Nucleotide numbers are relative to the distal transcription start site. The translation start site, "ATG" of NGFIB is in uppercase. (b) NURR1 ANE2. 3 transcription start sites were identified. Nucleotide numbers are relative to the most distal transcription start site. Bold and capital letters indicate transcription start sites identified by means of 5'-RACE.

### Figure 3

Schematic representation of alternative promoter regions and splicing events. Multiple alternative noncoding exons 1 and splicing events were observed in NR4A family (NGFIB, NURR1, NOR1) and NR5A1 (SF1). (a) NGFIB, (b) NURR1, (c) NOR1, (d) NR5A1.

### Figure 4

Alternative noncoding exon 1 (ANE) usage (%) of NR4A family (NGFIB, NURR1, NOR1) and NR5A1 (SF1) in H295R cells. Values are means  $\pm$  SD. \* $p < 0.001$ , vs basal, two tailed Student's t-test.

### Figure 5

Gene expression levels and promoter usage of NR4A family (NGFIB, NURR1, NOR1), NR5A1 and CYP11B2 in human cardiovascular and adrenal tissues. (a) mRNA expression levels (mmoles/GAPDH moles). (b) Promoter usage (%). Values are means  $\pm$  SEM. Statistically significant differences determined by two-tailed Student's or Welch's t-test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . CM, cardiomyopathy; APA, aldosterone-producing adenoma.

#### Figure 6

Summary of preferential regulation of the NGFIB and NURR1 genes. Values are means  $\pm$  SEM. Statistically significant differences determined by two-tailed Student's or Welch's t-test. AP; alternative promoter, ANE; alternative noncoding exon 1; APA, aldosterone-producing adenoma.

# Figures

Figure 1a

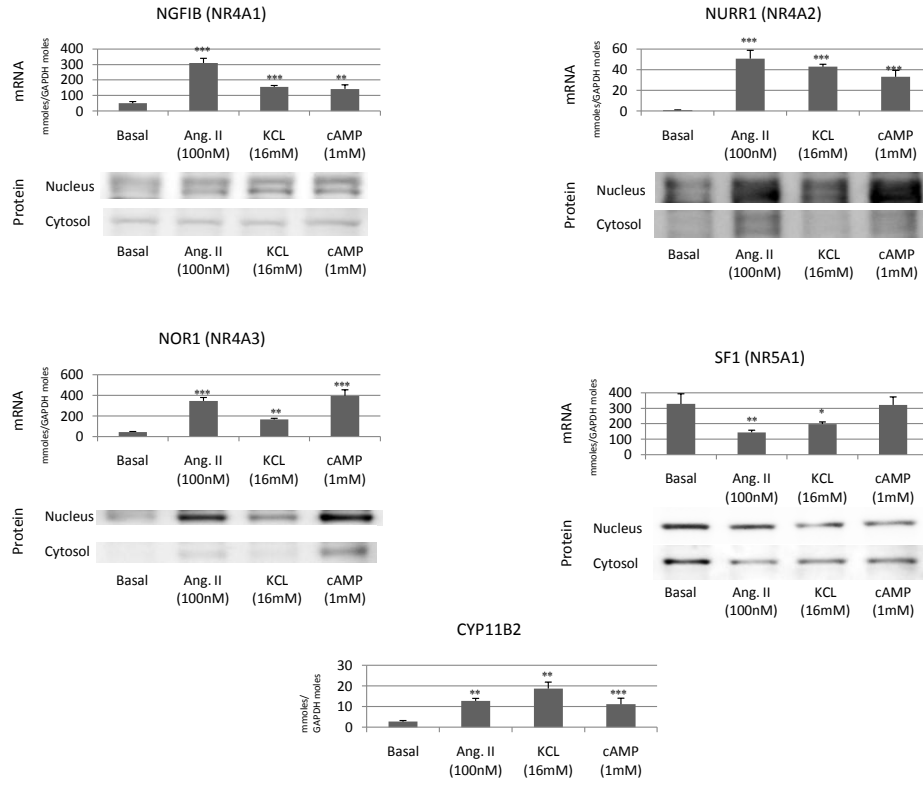


Figure 1b

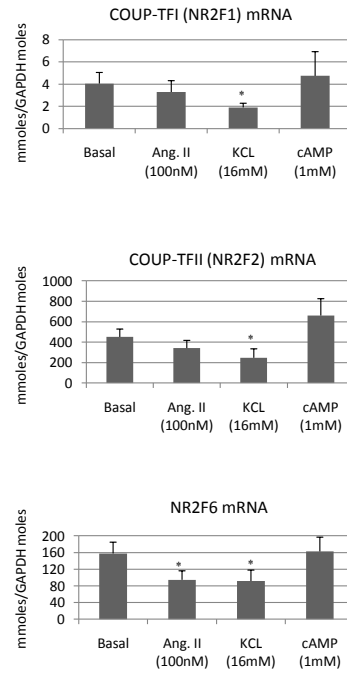


Figure 2a

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+1  Ggtcccggg cctctgtctc atctttaggc tgggattcct gccaccttgc tgctctgggg
+61 cccaaatact ttgagacaag gctatagget tgtcccactg actctccttt ccctccCtgg
+121 ggtctcctct ctctccagag ATGccctgta tccaagccca atatgggaca ccagcaccga
    
```

Figure 2b

```

-60 ttttcatact caggtcctga attaacctaa gatggaatg acctctccac ctacactgta
+1  Gcaaaggggc cagttcatta catcataaat gttaaatgag ttcattggact agctttcctc
+61 ttgcaggatc ttctctctgc aaggatttac acagtgaat ggggtggatt ttctGttggt
+121 tcaagtcatt tcttttatac attcatttta agtgctatgt ttggtaaag cttcccactc
+181 atttccaatg agacaaaacG ggaaggcatg gaagggcctg cctggtgagt ctacatatgc
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Figure 3a

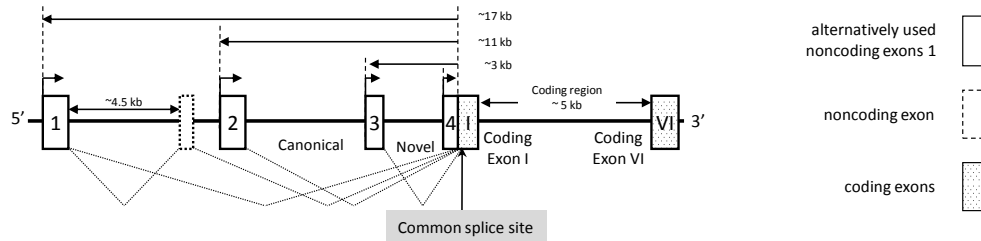


Figure 3b

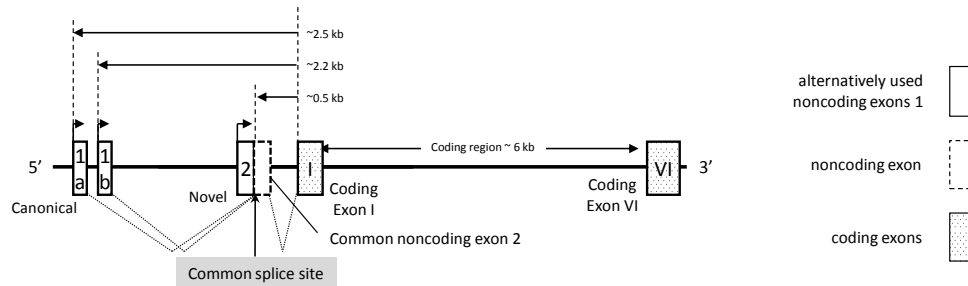


Figure 3c

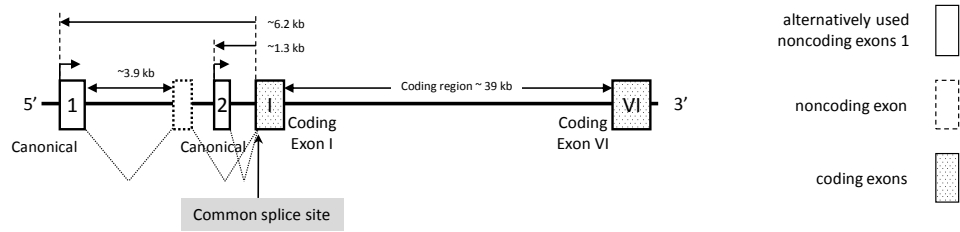


Figure 3d

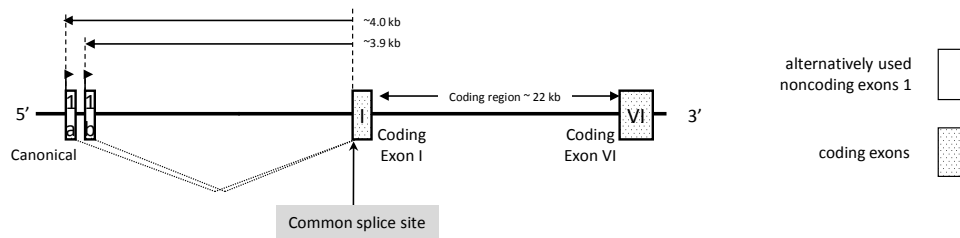


Figure 4

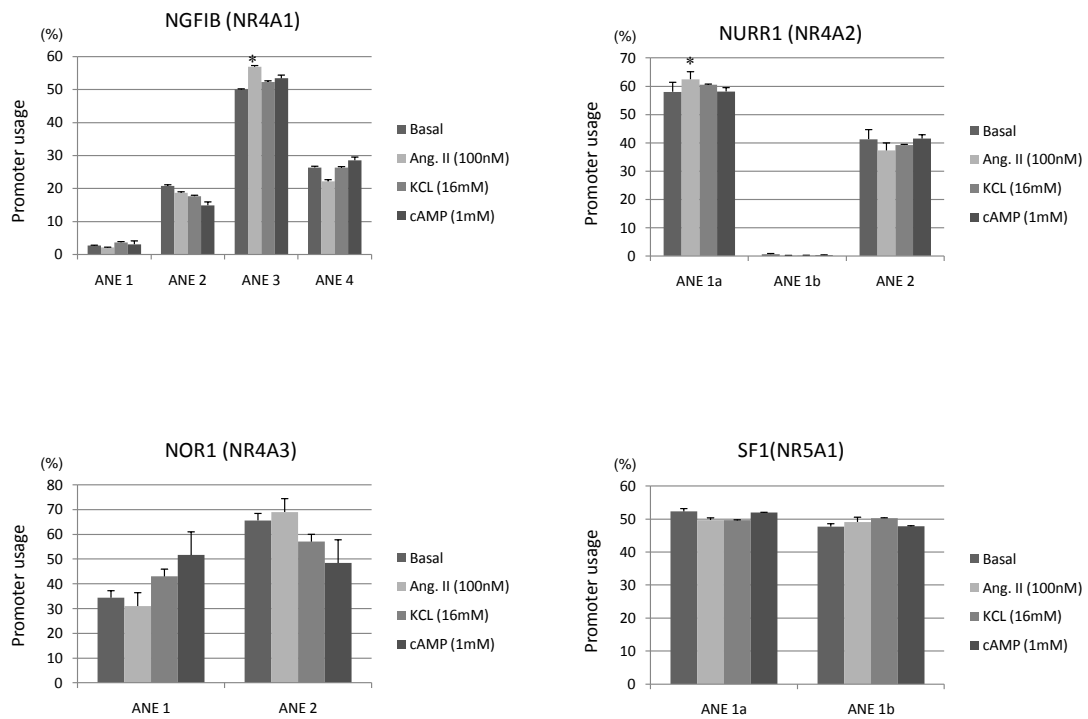


Figure 5a

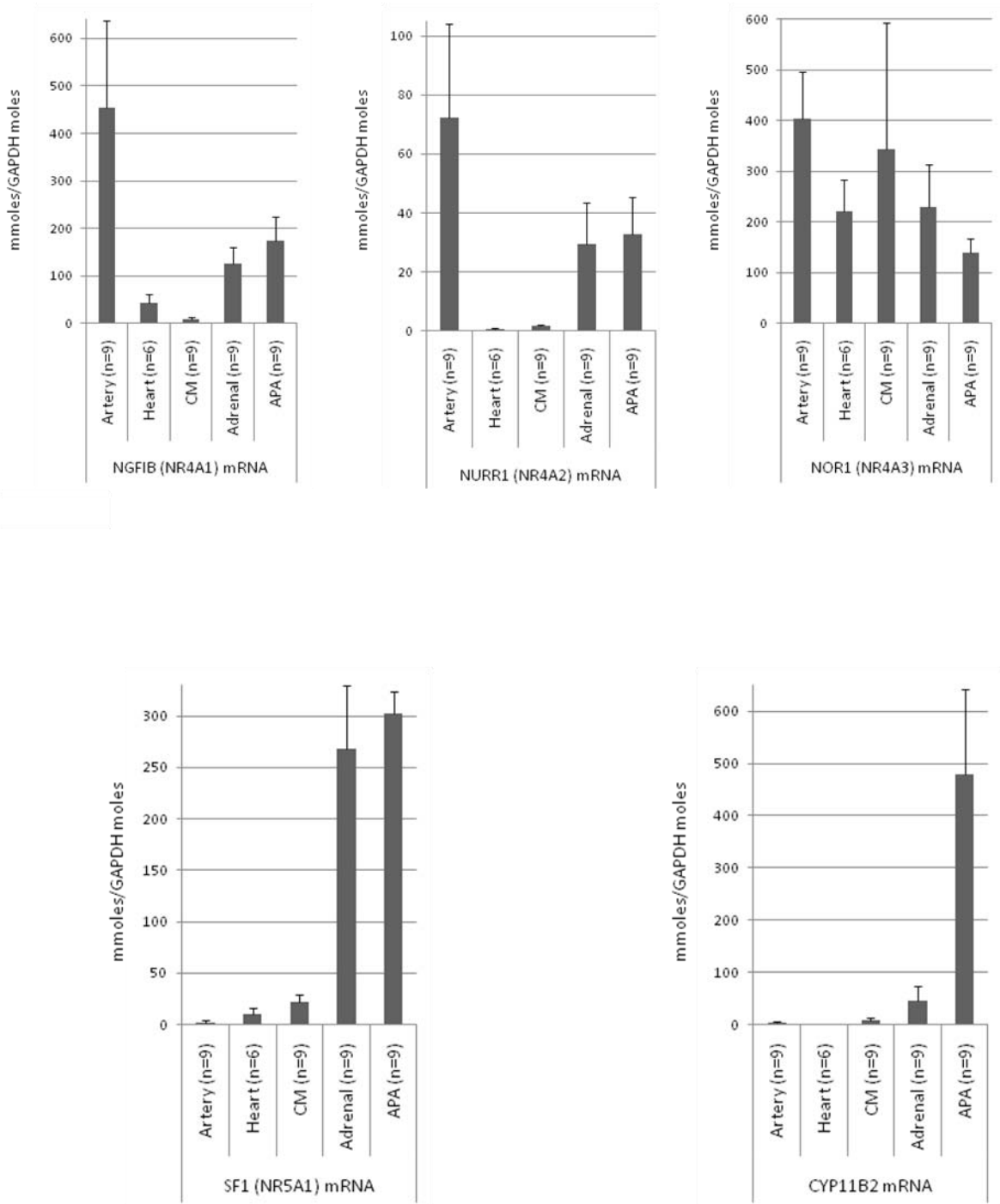


Figure 5b

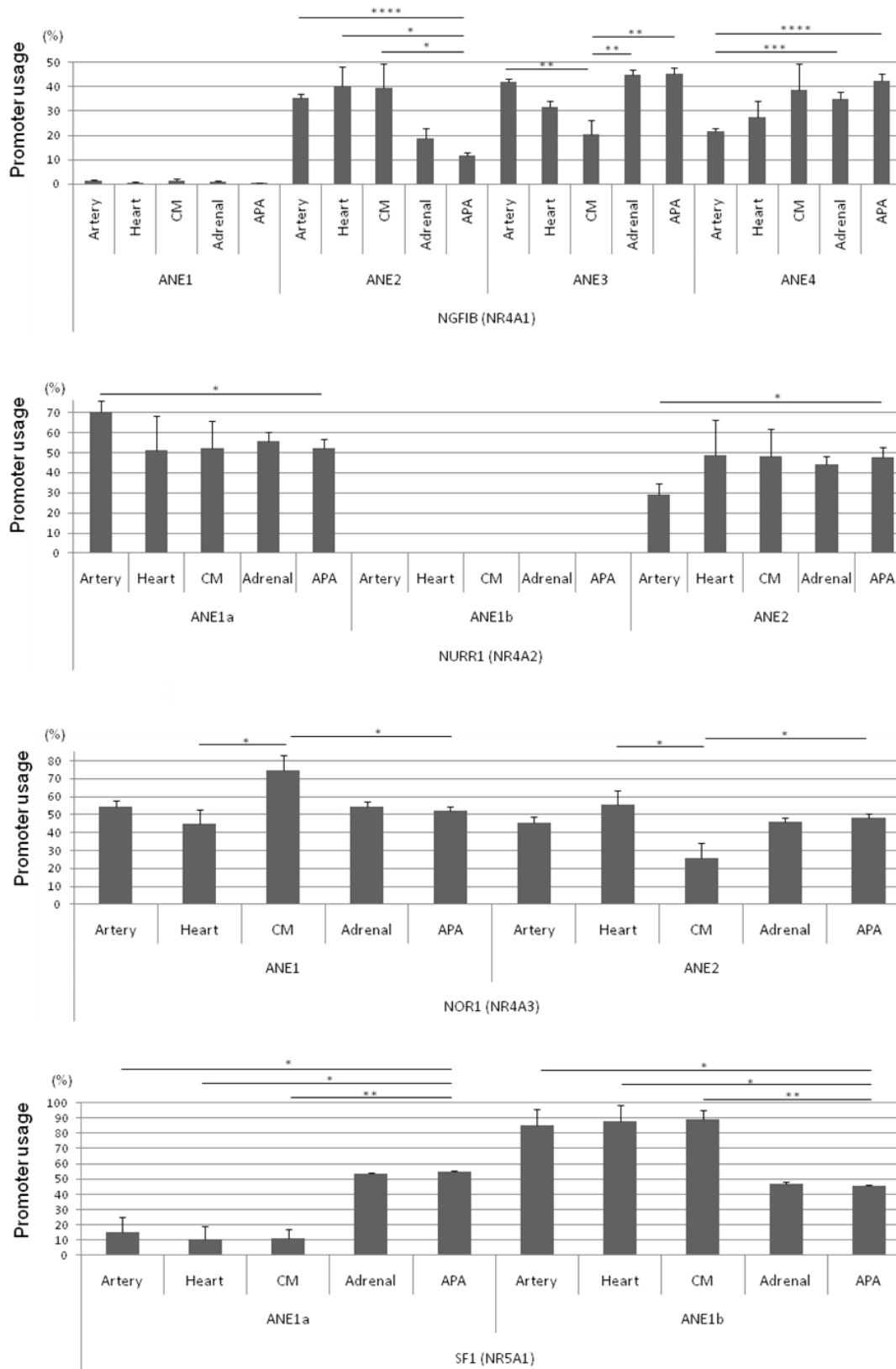


Figure 6

