## RESEARCH

# Early transcriptomic response of mouse adrenal gland and Y-1 cells to dexamethasone

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## Abstract

Glucocorticoids have short- and long-term effects on adrenal gland function and development. RNA sequencing (RNA-seq) was performed to identify early transcriptomic responses to the synthetic glucocorticoid, dexamethasone (Dex), *in vitro* and *in vivo*. In total, 1711 genes were differentially expressed in the adrenal glands of the 1-h Dex-treated mice. Among them, only 113 were also considered differentially expressed genes (DEGs) in murine adrenocortical Y-1 cells treated with Dex for 1 h. Gene ontology analysis showed that the upregulated DEGs in the adrenal gland of the 1-h Dex-treated mice were highly associated with the development of neuronal cells, suggesting the adrenal medulla had a rapid response to Dex. Interestingly, only 4.3% of Dex-responsive genes in the Y-1 cell line under Dex treatment for 1 h were differentially expressed under Dex treatment for 24 h. The heatmaps revealed that most early responsive DEGs in Y-1 cells during 1 h of treatment exhibited a transient response. The expression of these genes under treatment for 24 h returned to basal levels similar to that during control treatment. In summary, this research compared the rapid transcriptomic effects of Dex stimulation *in vivo* and *in vitro*. Notably, adrenocortical Y-1 cells had a transient early response to Dex treatment. Furthermore, the DEGs had a minimal overlap in the 1-h Dex-treated group *in vivo* and *in vitro*.

## Introduction

Glucocorticoids (GCs) are primary stress hormones and critical regulators of several physiological mechanisms. GCs commonly elicit their function through glucocorticoid receptor (GR)-mediated genomic effects. However, these hormones can also stimulate several non-genomic mechanisms via the membrane-bound GR (1). Non-transcriptional effects have short-term outcomes that control several critical physiological processes, including inflammation, behavioral changes, and even negative feedback from the hypothalamic–pituitary–adrenal (HPA) axis (2). Although GCs have non-genomic actions, they may still rapidly affect gene expression via different mechanisms such as mRNA destabilization and coactivator competition (3, 4). Transcriptional responses can occur within 1 h after the initial stimulus (5, 6). Therefore, the rapid response of gene expression is observed immediately after GC administration via both genomic and non-genomic actions.

Synthetic GC dexamethasone (Dex) is widely used for therapeutic and diagnostic purposes in clinical settings due to its potent GR activity and predominant effects. Treatment with Dex suppresses the HPA axis activity leading to decreased steroidogenesis in the adrenal cortex, eventually reducing endogenous GC secretion. The mechanisms of the abovementioned Dex-mediated negative feedback have been widely assessed (7). However, the immediate transcriptomic response (i.e. within approximately 1 h) of the adrenal gland to Dex administration is not completely elucidated at the genome-wide level. In this study, RNA sequencing (RNA-seq) and downstream bioinformatics

analysis were performed to explore the early transcriptional response to Dex exposure *in vivo* and *in vitro*. This study provided data about the transcriptomic response of Y-1 cell, a widely used murine adrenocortical cell line, to Dex treatment for 1 and 24 h. The rapid response (within 1 h) *in vitro* vs *in vivo* was also compared to highlight the difference between Y-1 adrenocortical cells and the C57BL/6 mice.

## Materials and methods

### Animals and dexamethasone treatment

Male C57BL/6 mice aged 35–60 days were housed in a 14 h light:10 h darkness cycle, were given free access to water and rodent chow, and were treated with Dex as previously described (8). In short, mice were familiarized with general handling, weighing procedures, and intraperitoneal injections for 4 days (once a day with 0.25 mL of sterile 1× PBS). On the fifth day, in the morning, animals were weighed, injected with 0.25 mL of either vehicle (sterile 1× PBS) or 10 μg dexamethasone (1DEX027, Covetrus, Portland, ME, USA), and returned to their cages. Mice were euthanized by $CO_2$ inhalation 1 h after injection, and all samples were collected immediately after sacrification. Adrenal glands were quickly removed from mice, and the surrounding adipose tissue was trimmed away. The right adrenal gland from each mouse was snap-frozen in liquid nitrogen and stored at –80°C until use. All procedures followed the protocols approved by the Institutional Animal Care and Use Committees at Auburn University.

### Cell culture

Y-1 mouse adrenocortical cells (ATCC #CCL-79) were purchased from American Type Culture Collection. Y-1 cells were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum and 1% streptomycin/ penicillin (E490-20ML, VWR, Radnor, PA, USA). Cells were subcultured separately into four groups (control, 1-h Dex-treated, 24-h Dex-treated, and 48-h Dex-treated) using six-well plates with 400,000 cells per well. After reaching 70% confluency, the growth media was aspirated and replaced by either vehicle- (growth media with 0.05% ethanol) or Dex- (growth media with 0.05% ethanol and 100 nM Dex) containing media. The 100 nM Dex is commonly used in the cell culture system for studying the Dex effect and is considered a high-dose treatment (9, 10, 11, 12). At the end of the treatment, the culture medium was aspirated, immediately followed by RNA extraction. Vehicle-treated

control groups were harvested along with the 1-h Dex-treated group and the 24-h Dex-treated group. For the RNA-seq study, the 24-h vehicle-treated samples were used as the control group. Three experimental replicates were performed independently on different days starting from different cell passages. For the qPCR study, the control group was the 1-h vehicle-treated samples.

### RNA isolation and sequencing

Total RNA from cell cultures was extracted using 400 μL TRIzol (Invitrogen). Total RNA from tissues was extracted from frozen adrenal glands using Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Adrenal glands from two to three animals were pooled as one biological replicate, with two biological replicates per condition (a total of five mice were included in each condition). Total RNA was sent to Novogene Life Sciences Co., Ltd., for the PE150 RNA-seq service. All samples that passed the quality control (concentration > 25 ng/μL, RIN ≥ 7.5) were used for library preparation and subsequent RNA-seq.

### Bioinformatics data analysis

Unstranded paired-end read files were first analyzed using the FASTQ error check feature of Chipster (13). Quality control was then run on the raw read files using FastQC. The trimmed reads were then aligned to the mouse genome (mm10, GRCm38.95) using STAR (v. 2.5) (14). Reads were mapped to each gene and counted using HTSeq (v.0.6.1) (15). The maximal mappable prefix (MMP) method ensures precise mapping results for exon–exon junction reads. Differential expression analysis between control and Dex-treated groups was performed using the DESeq2 (16) in R version 3.6.2 with FDR cutoff $\alpha = 0.05$. Differential expression analysis between the 1-h and 24-h Dex treatment groups was also performed using the same parameter (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). Three replicates per group were included in the *in vitro* study and two replicates per group were used for the *in vivo* study. Statistical significance of differential expression was assigned to genes with adjusted *P*-values < 0.05, irrespective of fold change. Genes must have at least one fragment per kilobase per million mapped reads (FPKM) in at least one sample to be considered differentially expressed genes (DEGs). Heatmaps for DEGs were generated using heatmap.2 in R, with Ward's

minimum variance method for clustering (ward.D2) and Euclidean distance measurement method. The Panther Gene Ontology website (geneontology.org) was used for GO analysis. GO-Figure! was used to generate Fig. 2D. GO enrichment analysis in Fig. 3 was analyzed using g:Profiler and the enrichGO function in the clusterProfiler package (3.16.0) in R with $P < 0.05$ as a cutoff value (17, 18).

### Quantitative RT-PCR

The relative expression of some key DEGs was verified using quantitative RT-PCR (qPCR). Total RNA was used to generate cDNA using SuperScript IV transcriptase and dT primers. qPCR was performed using the off-the-shelf TaqMan Gene Expression Assays (Supplementary Table 1). *Gapdh* and *Rplp0* were used as the internal control. The relative gene expressions were calculated using the relative standard curve method. Each data point was an average of three independent cell culture experiments with three technical replicates in each experiment.

### Statistical analysis

Student's *t*-test was used to compare the expressions of DEGs between the control and Dex-treated groups in Y-1 cells (Fig. 5).

### Results

#### Differentially expressed genes had a minimal overlap in the 1-h and 24-h Dex-treated Y-1 cells

RNA-seq analysis identified 313 and 319 DEGs in Y-1 cells in the 1-h and 24-h Dex-treated groups, respectively (Fig. 1). There were 203 upregulated genes and 110 downregulated genes in the 1-h Dex-treated group. Meanwhile, 200 and 119 genes were upregulated and downregulated, respectively, in the 24-h Dex-treated group (Supplementary Table 2). The clustered heatmap showed that these DEGs belong to four major groups, which were as follows: (a) upregulated mainly in the 1-h Dex-treated group, (b) upregulated mainly in the 24-h Dex-treated group, (c) downregulated mainly in the 24-h Dex-treated group, and (d) downregulated mainly in the 1-h Dex-treated group (Fig. 1A). Interestingly, most DEGs in the 1-h Dex-treated group were not considered DEGs in the 24-h Dex-treated group. The expressions of these genes returned to basal levels similar to those of the non-treated control group. Among 606 Dex-responsive genes found in Y-1 cells, only 26 (4.3%) were differentially

expressed in the 1-h and 24-h Dex-treated groups, which is equivalent to 8.3 and 8.2% of DEGs in each group, respectively (Fig. 1B). Interestingly, the heatmap showed that almost all of the 26 genes had a similar response to Dex treatment for 1 and 24 h. *Sdf2l1* and *Plk2* might have an opposite direction of response on the heatmap. However, their expression did not significantly differ, as revealed on qPCR. *Sdf2l1* was differentially expressed on qPCR only in the 24-h Dex-treated group but not in the 1-h Dex-treated group ($P = 0.003$ vs $P = 0.94$). In contrast, *Plk2* remained stable in the 1-h and 24-h Dex-treated groups ($P = 0.57$ vs
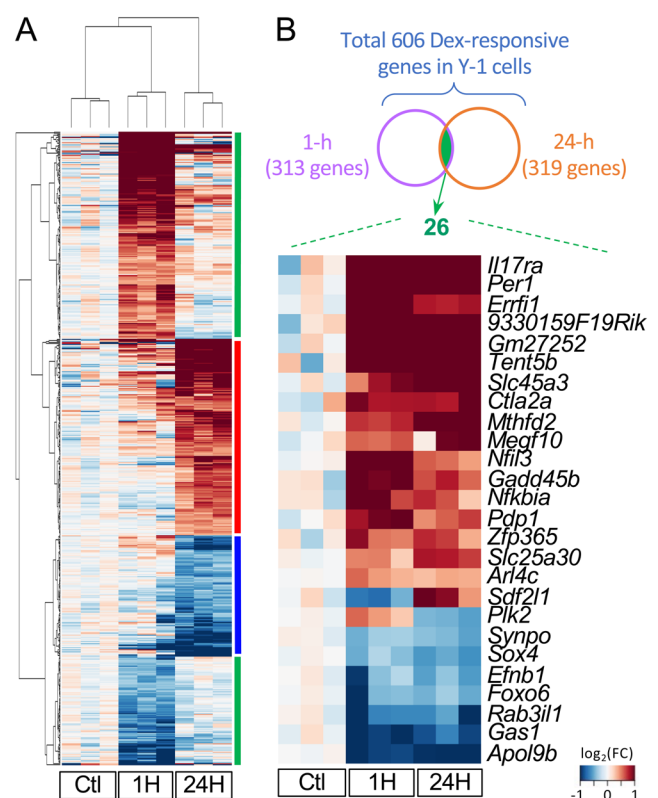


**Figure 1**
Y-1 cells responded differently to the 1-h and the 24-h dexamethasone treatment. (A) The clustered heatmap showed the fold change of all DEGs identified either in the 1-h or the 24-hour Dex treatment in Y-1 cells. Genes were clustered into four major groups. Genes in two clusters (marked by the red bar and the blue bar on the right) were either upregulated or downregulated mainly in the 24-h group with no or minor changes in the 1-h group. Genes in another two clusters (marked by green bars) had a transient response to Dex. In these two clusters, expressions of genes in the 24-h group moved back toward the non-treated control group. (B) There were 313 and 319 genes differentially expressed in the 1-h treatment group and the 24-h treatment group, respectively. A total of 26 genes were differentially expressed in both groups. *Sdf2l1* and *Plk2* are the only two genes that showed an opposite change between the 1-h and 24-h treatment groups. Ctl, vehicle control. Genes were excluded if the average FPKM of each group was less than 1 across all three groups. The fold change used for heatmaps was calculated based on FPKM +0.001.
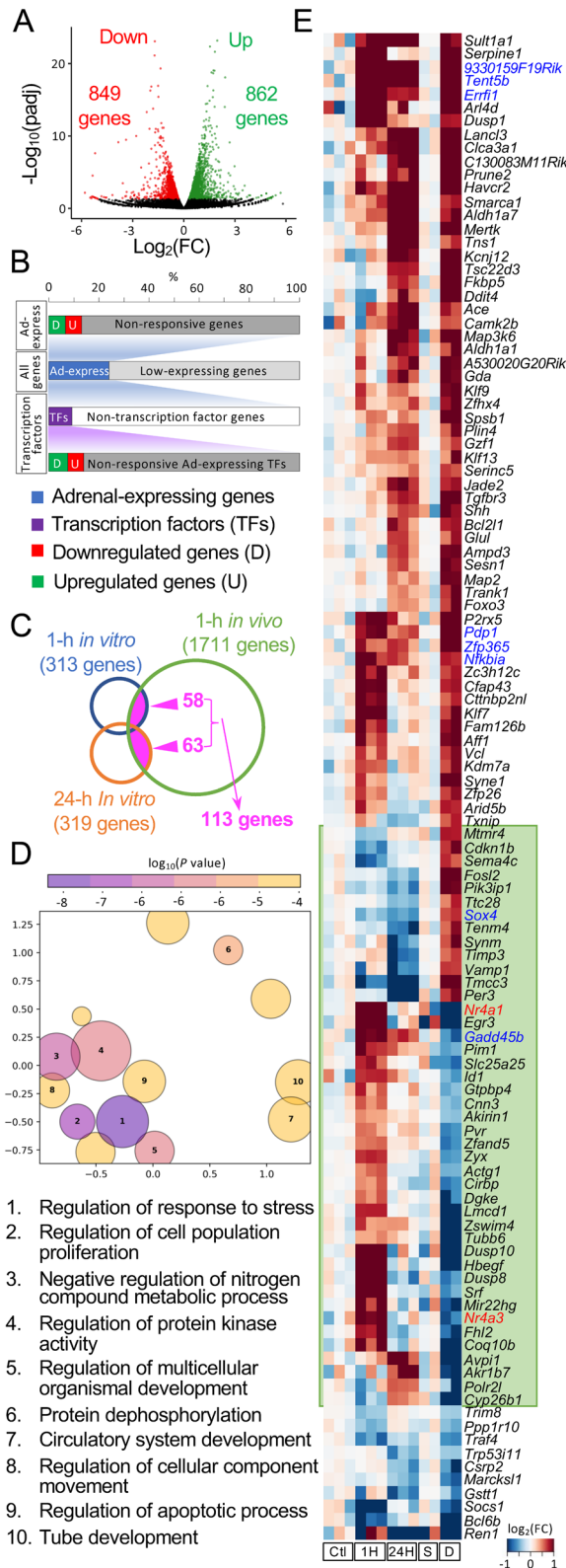
**Figure 2**

Transcriptomic response of adult mouse adrenal gland to 1-h dexamethasone treatment. (A) The volcano plot showed the DEGs in the whole adrenal gland transcriptome in male mice under a 1-h Dex

$P = 0.42$). Notably, Y-1 cells had low *Plk2* expression levels (FPKM of <20 in all samples, Supplementary Table 2).

## Genes responsive to Dex treatment *in vivo* and *in vitro*

To compare the early response to Dex treatment *in vitro* vs *in vivo*, adult C57BL/6 mice were also treated with high-dose Dex, which can have a sufficient effect on the HPA axis at the pituitary level within 1 h (8). Approximately 26.2% of 54,533 genes in the mouse genome had at least 1 FPKM in 1 sample. These genes were classified as adrenal-expressed genes (14,300 genes). The number of adrenal-expressed genes in the saline-treated group was similar to that in another genome-wide study of adult C57BL/6 mice (19). The *in vivo* 1-h Dex-treated group had 862 upregulated genes and 849 downregulated genes (Fig. 2A, Supplementary Table 3). These 1711 Dex early responsive genes comprise 12.0% of all adrenal-expressed genes (Fig. 2B). Approximately 13.3% of adrenal-expressed transcription factors (TFs) were differentially expressed in the 1-h Dex-treated group. The percentage of Dex early responsive adrenal-expressed genes and Dex-responsive adrenal-expressed TFs was similar. Hence, the response rate to Dex treatment was similar between the TF and non-TF genes (Fig. 2B). Interestingly, only a small percentage of DEGs *in vitro* was also identified  (Fig. 2C): 58 DEGs in the 1-h Dex-treated Y-1 cells were differentially expressed in the *in vivo* study; meanwhile, 63 genes in the 24-h *in vitro* Dex-treated group were also identified in the *in vivo* treatment group. The Venn diagram showed that only a small portion of DEGs overlapped among three groups (58 + 63 − 113 = 8 genes). Gene ontology (GO) analysis showed that 58 overlapping DEGs between the 1-h *in vitro* and *in vivo* groups were highly associated with the 'regulation of response to stress' (GO: 0080135, fold enrichment of >5.00, FDR $P = 0.0003$) (Fig. 2D).

treatment. (B) Around 26.2% of genes were identified as adrenal-expressed genes. 12.0% of adrenal-expressed genes were differentially expressed under a 1-h Dex treatment. Transcription factors (TFs) had a similar response rate compared to non-TF genes. (C) Minimal overlap of DEGs among three groups. (D) GO analysis of the 58 overlapping DEGs in the 1-h treated *in vivo* and *in vitro* studies. GO terms that are most similar in semantic spaces (indicated on the X and Y axis) are placed nearest to each other. The ten most significant terms are labeled with numbers and their descriptions are provided below the plot. (E) The clustered heatmap of the 113 genes that were differentially expressed both *in vivo* and *in vitro*. Genes in the green box had opposing responses to Dex treatment *in vivo* vs *in vitro*. DEGs identified in all three conditions are highlighted in blue. The fold change used for heatmaps was calculated based on FPKM +0.001. Ctl, untreated control (*in vitro*); 1H, 1-h Dex-treated group (*in vitro*); 24H, 24-h Dex-treated group (*in vitro*); S, saline-treated group (*in vivo*); D, Dex-treated group (*in vivo*).
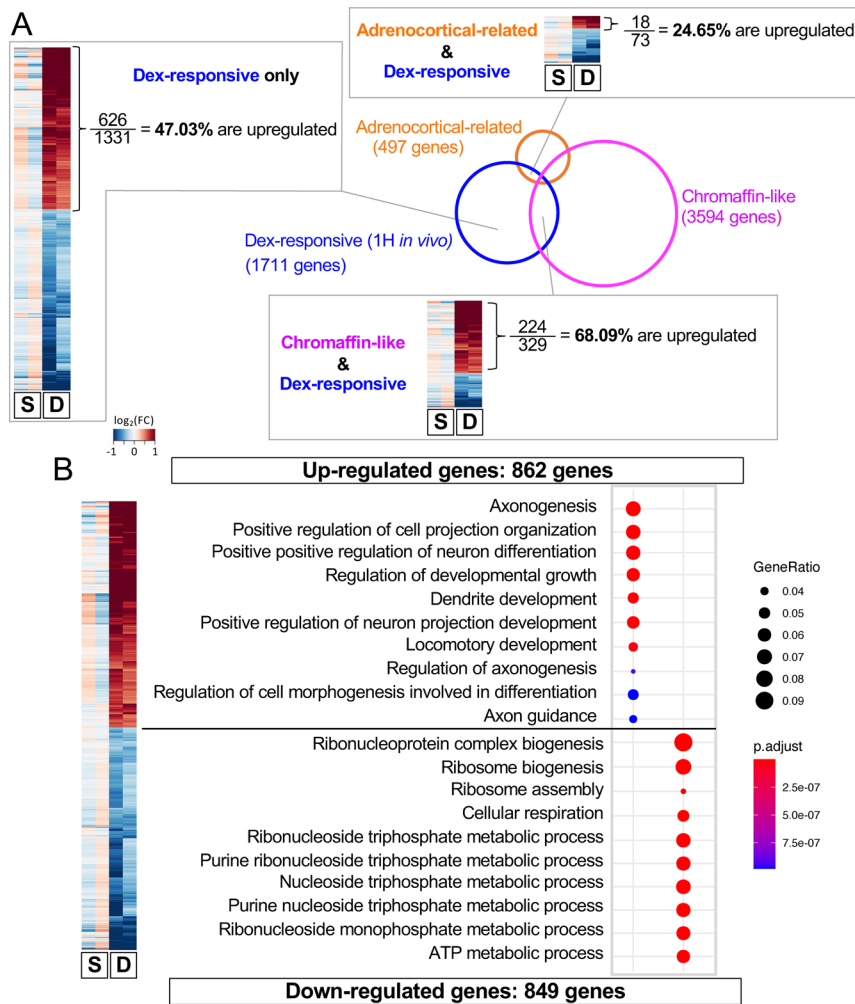
**Figure 3**

In the *in vivo* 1-h dexamethasone treatment, the upregulated genes and the downregulated genes were linked to different functions and different groups of cells. (A) Venn diagram comparing Dex-responsive genes with 'adrenocortical-related genes' and 'chromaffin-like' genes. Clustered heatmaps showed how genes were differentially expressed in each overlapping gene set. In Dex-responsive genes that overlap with the 'chromaffin-like' gene set, 68.09% were upregulated (significantly higher than the 'Dex-response only' group, $z = 6.84$, $P < 0.0001$). In genes that were both 'Dex-responsive' and 'adrenocortical-related', only 24.65% were upregulated (significantly lower than the 'Dex-response only' group, $z = -3.74$, $P < 0.0002$). (B) GO analysis showed that all upregulated genes in the *in vivo* 1-h Dex treatment group were highly associated with neural cell development and function. The fold change used for heatmaps was calculated based on FPKM +0.001. S, saline-treated group; D, Dex-treated group.

## Dynamic expression of differentially expressed genes *in vivo* and *in vitro*

A clustered heatmap was used to better visualize the response of 113 genes to Dex *in vivo* and *in vitro* (Fig. 2E). Interestingly, genes that exhibited an opposite direction of response during *in vivo* and *in vitro* treatments were clustered together (highlighted in the green box in Fig. 2E, containing 44 genes). For example, the expressions of nuclear receptor genes *Nr4a1* and *Nr4a3* and the zona fasciculata marker gene *Akr1b7* were upregulated either in the 1- or 24-h Dex-treated Y-1 cells. However, the expressions were downregulated *in vivo*. 'cellular response to corticotropin-releasing hormone stimulus' (GO: 0071376, fold enrichment of >100, FDR $P = 0.047$) was the leading GO term obtained from the 44 clustered genes. The other GO terms included 'cell migration involved in sprouting angiogenesis' (GO:0002042, fold enrichment of 84.7, FDR $P = 0.020$), 'blood vessel endothelial cell migration' (GO:0043534, fold enrichment of 53.6, FDR $P = 0.035$), and 'tissue development' (GO:0009888, fold enrichment of 4.47, FDR $P = 0.009$).

## Opposite response of genes associated with steroidogenesis and catecholamine synthesis

We then compared 1711 Dex-early responsive genes identified in the *in vivo* treatment, chromaffin-like genes, [20] and adrenocortical-related genes [21]. In the Dex-treated adrenal glands of mice, 24.65% of the adrenocortical-related Dex-responsive genes and 68.09% of the chromaffin-like Dex-responsive genes were upregulated (Fig. 3A, Supplementary Table 2). This difference was in accordance with the current understanding that the effect of GCs in the adrenal cortex could be opposite to that in the adrenal medulla. GCs can both suppress adrenocortical steroidogenesis directly or indirectly [22] and can induce medullary function by upregulating the expression of phenylethanolamine N-methyltransferase (PNMT) [23, 24], an epinephrine synthesis enzyme. To further understand

the functional meaning of this dynamic response at the transcriptome level, GO analysis was performed to evaluate the potential function of upregulated and downregulated genes. GO analysis revealed that several GO terms associated with the 862 Dex-upregulated genes were associated with the development or function of neuronal cells (Fig. 3B). To provide better visualization of the response of adrenal glands to Dex, the genes were classified based on their function, and clustered heatmaps were used to show the relative expression of genes both in the *in vivo* and *in vitro* treatment groups. Interestingly, results revealed that genes associated with catecholamine synthesis may be upregulated in general in the *in vivo* group (Fig. 4), which was in accordance with the result of GO analysis. Although none of these catecholamine synthesis-associated genes were considered DEG, several genes associated with the regulation of neurotransmitter secretion (GO:0046928, fold enrichment of 4.0, FDR *P* = 0.000407) were included in the Dex-upregulated genes. Regarding steroid hormone production, several genes associated with steroidogenesis were downregulated primarily in the *in vivo* study (Fig. 4). No specific trend was observed in genes associated with nuclear receptors or cholesterol metabolism. Most genes in these two categories did not change or were minimally affected in the *in vitro* treatment groups.

## Dynamic response of nuclear receptors to Dex treatment *in vivo* and *in vitro*

It is interesting that the downregulation of genes was associated with steroidogenesis particularly in the *in vivo* treatment groups. This notion was in accordance with the long-term understanding that Dex can suppress the endogenous secretion of GCs via the negative feedback loop. In the *in vivo* treatment groups, *Nr5a1,* which is a nuclear receptor known as the master regulator of steroidogenesis, is downregulated (Fig. 5A). The expression of several genes known as the *Nr5a1*-induced genes (e.g. *Fdx1*, *Alas1*, and *Akr1b7*) was also downregulated (Table 1) (25, 26, 27). Moreover, the downregulation of *Nr5a1* may explain the overall suppression of steroidogenesis-related genes (Fig. 4). *Nr0b2, Nr1d2, Nr4a1, Nr4a3, Nr1h4,* and *Pparg*, in addition to *Nr5a1*, were the nuclear receptors considered DEGs (Fig. 5B, C, D, E, F and G). Notably, *Nr4a1* and *Nr4a3* were the two TFs that exhibited a transient upregulation in Y-1 cells during Dex treatment for 1 h (Fig. 5D and E). qPCR confirmed the transient differential expression of *Nr4a1* (Fig. 5H). Although some qPCR data points showed a transient upregulation of *Nr4a3* during Dex treatment for 1 h, the results did not significantly

differ (Fig. 5I). The difference in *Nr4a3* expression might be attributed to the extremely low *Nr4a3* expression in Y-1 cells (FPKM of <4).

## Discussion

### Rapid transcriptional effects of GCs

A typical action of GCs is that they bind with cytosolic steroid receptors, which in turn migrate to the nucleus and regulate the expression of target genes. For example,
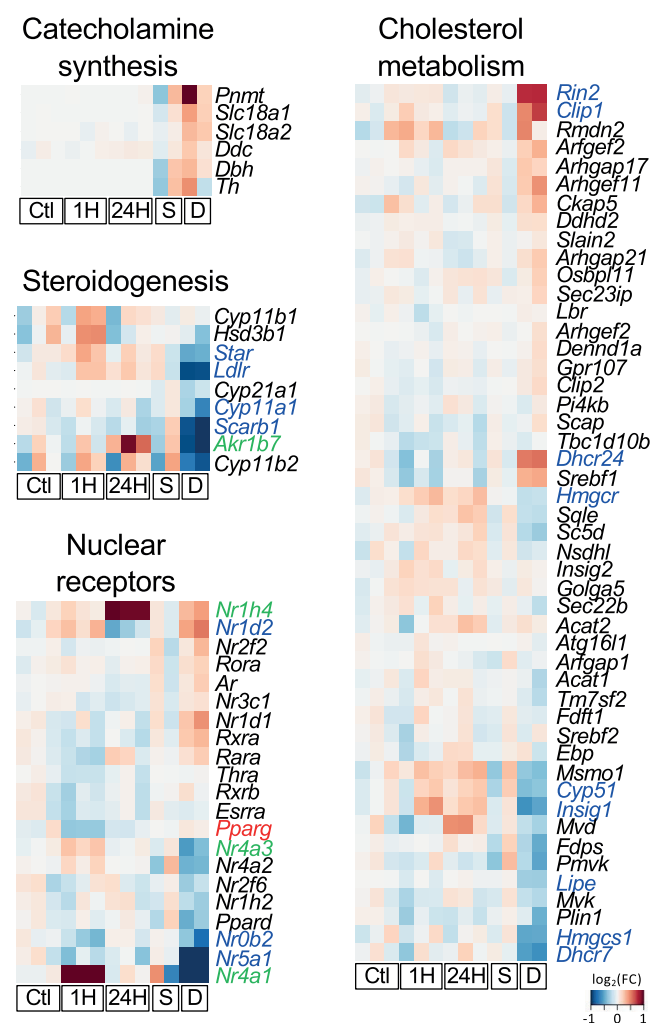


**Figure 4**

Heatmaps of the genes related to adrenal gland functions, cholesterol metabolism, and nuclear receptors in the adrenal gland. Heatmaps showed the fold change of Dex-treated groups vs control groups. The fold change was calculated based on FPKM +10 to reduce the influence of low expressing genes. Genes must have an original FPKM > 10 in at least one sample to be included in these heatmaps. Gene names in blue, red, or green were identified as DEGs *in vivo*, *in vitro*, or both groups, respectively. Ctl, vehicle control (*in vitro*); 1H, 1-h Dex-treated group (*in vitro*); 24H, 24-h Dex-treated group (*in vitro*); S, saline-treated group (*in vivo*); D, Dex-treated group (*in vivo*).
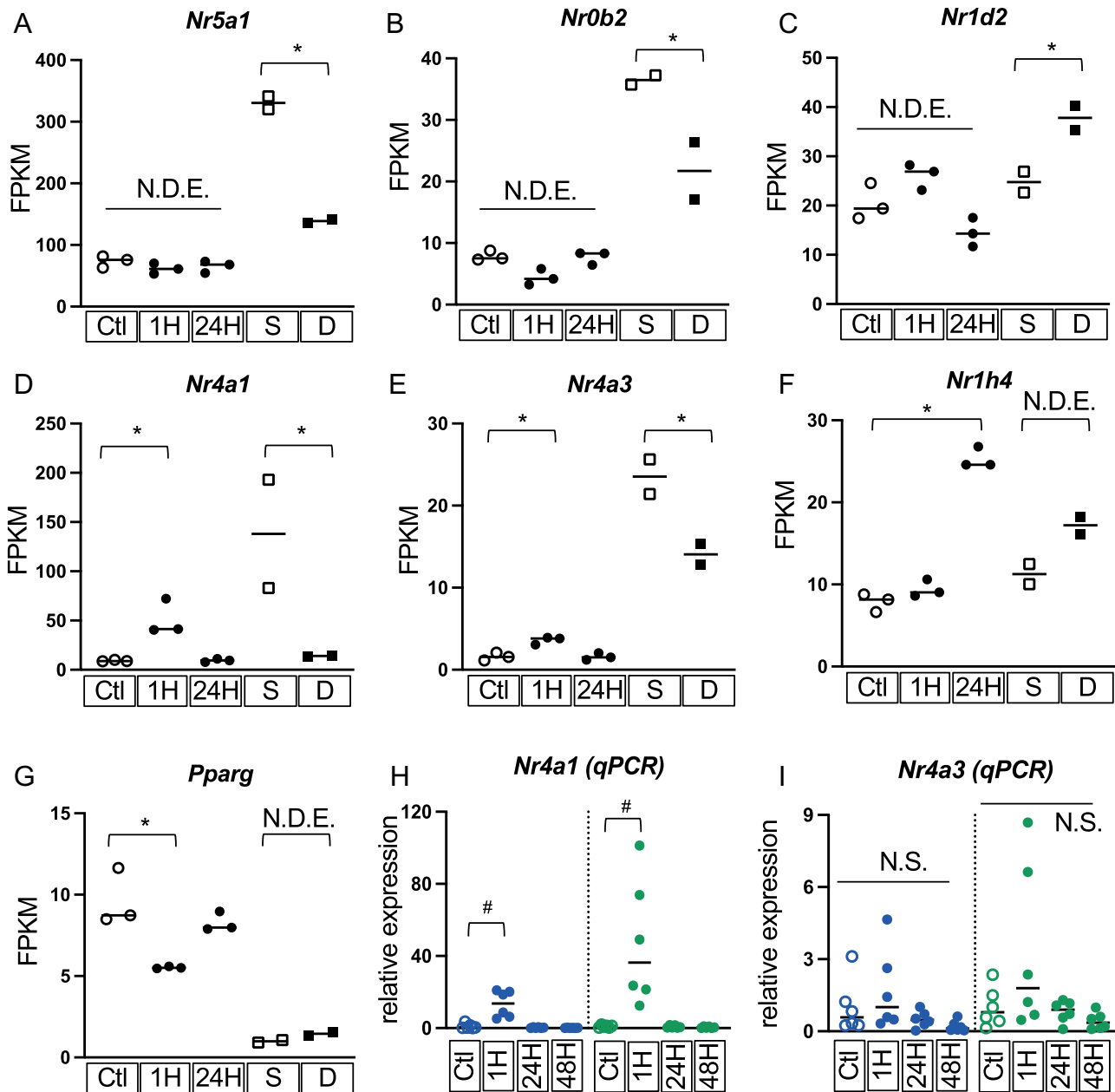
**Figure 5**

Expression of differentially expressed nuclear receptors. Dot plots showed (A, B, C, D, E, F, G) FPKM levels with means. (H and I) qPCR was used to verify the transient differential expression of *Nr4a1* and *Nr4a3*. Data in blue used *Gapdh* as the internal control gene. Data in green used *Rplp0* as the internal control gene. The relative expression of the untreated control was set as one. *Identified as DEGs using DESeq2 in the RNA-seq; #Identified as genes with a significant difference with the control group (*t*-test, *P* < 0.05); N.S., no significant difference (*t*-test); N.D.E., not differently expressed using DESeq2 in the RNA-seq. Ctl, vehicle control (*in vitro*); 1H, 1-h Dex-treated group (*in vitro*); 24H, 24-h Dex-treated group (*in vitro*); 48H, 48-h Dex-treated group (*in vitro*); S, saline-treated group (*in vivo*); D, Dex-treated group (*in vivo*).

GCs can activate or suppress gene expression via GR or mineralocorticoid receptor that binds with the glucocorticoid-responsive elements. GCs could also tether/interact with other TFs to regulate gene expression (28). Although the genetic effect of steroid hormones is not commonly short-term, several data have shown that steroid hormone exposure causes transcriptional changes more rapidly than previously believed. Our *in vitro* experiments revealed that Y-1 cells had a dynamic early response to Dex treatment. The DEGs identified in the 1-h Dex-treated group are extremely different from those identified in the 24-h Dex-treated group.

The rapid cytoplasmic movement of steroid receptors toward the cell nucleus occurs within minutes after steroid

**Table 1** Top differentially expressed genes in adrenal glands in Dex-treated male mice.

| Top upregulated genes | | | | Top downregulated genes | | | |
|---|---|---|---|---|---|---|---|
| | Average FPKM | | | | Average FPKM | | |
| Gene name[a] | Saline | Dex | Fold change | Gene name[b] | Saline | Dex | Fold change |
| Abca1 | 10.2 | 42.8 | 4.20 | Nr4a1 | 138.0 | 14.0 | 0.10 |
| Pdk4 | 3.0 | 11.7 | 3.83 | 2010003K11Rik | 16.3 | 3.2 | 0.20 |
| Zbtb16 | 6.6 | 25.4 | 3.83 | Gm13889 | 18.9 | 4.2 | 0.22 |
| Chrna7 | 3.8 | 14.2 | 3.79 | 2010109I03Rik | 14.7 | 4.1 | 0.28 |
| Ddit4 | 28.6 | 107.9 | 3.77 | Tnfrsf12a | 225.4 | 70.5 | 0.31 |
| Fam107a | 5.3 | 19.0 | 3.60 | Mob4 | 78.9 | 25.9 | 0.33 |
| Nt5e | 4.1 | 13.8 | 3.40 | Apoc4 | 11.6 | 3.9 | 0.34 |
| Fzd4 | 3.6 | 11.6 | 3.25 | Coq10b | 63.5 | 21.6 | 0.34 |
| Greb1 | 20.3 | 63.4 | 3.12 | Cryba4 | 24.9 | 9.0 | 0.36 |
| Lrg1 | 9.2 | 28.3 | 3.07 | Yam1 | 6933.1 | 2565.2 | 0.37 |
| Lnpep | 8.2 | 24.2 | 2.94 | Mapkapk3 | 13.9 | 5.3 | 0.38 |
| Maf | 4.6 | 13.3 | 2.87 | Fdx1 | 2978.8 | 1129.6 | 0.38 |
| Tsc22d3 | 28.3 | 80.4 | 2.84 | Avpi1 | 60.7 | 23.1 | 0.38 |
| Tbx20 | 4.8 | 13.6 | 2.83 | Junb | 44.5 | 17.0 | 0.38 |
| Rin2 | 6.6 | 18.2 | 2.74 | Ldlr | 31.9 | 12.3 | 0.38 |
| Vcam1 | 9.3 | 25.4 | 2.72 | Hba-a2 | 17.0 | 6.7 | 0.40 |
| AW551984 | 3.9 | 10.4 | 2.70 | Tubb6 | 121.8 | 49.0 | 0.40 |
| Dock10 | 4.1 | 11.0 | 2.68 | Ptp4a1 | 42.6 | 17.6 | 0.41 |
| Klf15 | 9.5 | 25.5 | 2.67 | Smim3 | 31.0 | 12.8 | 0.41 |
| Rab3c | 8.7 | 23.1 | 2.65 | Atf4 | 146.0 | 60.9 | 0.42 |
| Pde10a | 6.0 | 15.8 | 2.65 | Nr5a1 | 330.6 | 138.7 | 0.42 |
| Arl4d | 5.6 | 14.5 | 2.60 | Scx | 32.3 | 13.6 | 0.42 |
| Errfi1 | 14.2 | 36.6 | 2.58 | Dusp10 | 10.0 | 4.2 | 0.42 |
| Hmgcs2 | 4.8 | 12.3 | 2.54 | Hes6 | 174.1 | 75.7 | 0.43 |
| Atp7a | 5.4 | 13.7 | 2.54 | Srrd | 11.4 | 5.0 | 0.44 |
| Fkbp5 | 47.6 | 121.0 | 2.54 | Etnk2 | 22.9 | 10.1 | 0.44 |
| Bmp4 | 5.4 | 13.7 | 2.53 | Gm10275 | 16.6 | 7.5 | 0.45 |
| Ston1 | 8.1 | 20.5 | 2.52 | Alas1 | 423.9 | 191.0 | 0.45 |
| Ptprn2 | 5.4 | 13.6 | 2.52 | Tmem120a | 29.4 | 13.6 | 0.46 |
| Irs2 | 4.3 | 10.8 | 2.49 | Adamts1 | 12.6 | 5.9 | 0.46 |
| Bdkrb2 | 4.5 | 11.2 | 2.47 | Creb3l1 | 19.1 | 9.1 | 0.47 |
| Klhl24 | 6.0 | 14.9 | 2.47 | Zswim4 | 16.5 | 7.9 | 0.48 |
| Pdp1 | 11.9 | 29.3 | 2.47 | Dusp14 | 12.4 | 5.9 | 0.48 |
| Tgfbr3 | 38.4 | 93.2 | 2.43 | Fam222a | 34.9 | 16.7 | 0.48 |
| Slc9a7 | 5.0 | 12.0 | 2.41 | Akr1b7 | 1279.3 | 611.1 | 0.48 |
| Rims3 | 5.1 | 12.3 | 2.40 | Cldn5 | 11.8 | 5.7 | 0.48 |
| Osbpl3 | 19.7 | 47.1 | 2.39 | Naa38 | 20.3 | 9.8 | 0.48 |
| Cebpd | 16.7 | 39.6 | 2.36 | 2310009B15Rik | 16.1 | 7.8 | 0.48 |
| Arrdc2 | 4.4 | 10.1 | 2.32 | Agtr1b | 13.5 | 6.6 | 0.49 |
| Adcyap1r1 | 12.2 | 28.3 | 2.31 | RP24-139E15.1 | 16.4 | 8.1 | 0.49 |
| Klhl11 | 5.5 | 12.7 | 2.31 | Lsm5 | 14.5 | 7.2 | 0.49 |
| Ncoa7 | 5.8 | 13.4 | 2.30 | Esm1 | 18.9 | 9.3 | 0.50 |
| Mcf2l | 4.8 | 10.9 | 2.30 | Mir22hg | 15.2 | 7.5 | 0.50 |
| Map3k6 | 8.7 | 19.9 | 2.29 | Hbb-bt | 19.5 | 9.7 | 0.50 |
| | | | | Lars2 | 270.7 | 134.8 | 0.50 |

[a]Only list the genes that have FPKM > 10 in the Dex-treated group; [b]Only list the genes that have FPKM > 10 in the saline-treated group.

hormone administration (29). *In vitro* studies have shown that Dex-induced transcriptional changes can be a direct effect independent from other confounding external stimuli present in organismal model systems. For example, a 40-min exposure to estrogen in human umbilical vein endothelial cell culture leads to an acute PI3K-mediated upregulation of 250 genes (30). GR ChIP-seq studies have shown a significant enrichment in GR-binding sites after 1 h of Dex treatment in cells originating from different types of tissues (31, 32). RNA-seq analysis using rat primary cardiomyocytes revealed 98 differentially regulated genes after 1 h of Dex stimulation. This indicated the upregulation of several genes involved in the development of hypertrophic cardiomyopathy, which

is a common adverse effect of Dex (33). The same study revealed that the treatment with Dex in 10-week-old mice could mimic findings found in primary cardiomyocytes. The abovementioned *in vivo* and *in vitro* studies provided important evidence showing that Dex treatment causes short-term expression changes at a genome-wide level.

## Rapid and transient responses of the Nr4a subfamily

In Y-1 cells, Dex treatment leads to a rapid and transient upregulation of *Nr4a1* and *Nr4a3*, which are orphan nuclear receptors belonging to the Nr4a subfamily (contains *Nr4a1*, *Nr4a2*, and *Nr4a3*). Although the expression of *Nr4a1* and *Nr4a3* in Dex-treated Y-1 cells increased in the 1-h Dex-treated group, the expression was low in the 24- and 48-h Dex-treated groups. This rapid and transient upregulation of the Nr4a subfamily has been observed in several different types of cells that are stimulated. The expression of *Nr4a1* and *Nr4a3* in 3T3-L1 preadipocytes increases only within the first 4 h during the cell differentiation process from preadipocyte to adipocyte (34). The upregulation of *Nr4a1* and *Nr4a2* in T cells reaches the peak level within the first hour after antigen stimulation (35). In addition, the expression of *Nr4a1* and *Nr4a3* increases within the first hour and then decreases in macrophages if coincubated with apoptotic thymocytes (36). Interestingly, *Nr4a1* and *Nr4a3* are enriched in the GO term 'cellular response to corticotropin-releasing hormone stimulus' (GO: 0071376), which is the leading biological process GO term identified using 44 genes that were classified because of the opposite response *in vitro* and *in vivo* (Fig. 2E). Most of the 44 genes had a transient response in the *in vitro* treatment groups. Because our study does not specifically focus on the GR-dependent and/or GR-independent pathways, the data cannot confirm the possible crosstalk between GR and the Nr4a subfamily (37). However, a previous GR ChIP-seq study did not detect GR bindings around the promotor region of *Nr4a1* in a Dex-treated human cell line (38).

## Effects of Dex on steroidogenesis

GC suppresses the HPA axis activity partly by reducing the secretion of steroid hormones from the adrenal cortex. This GC-mediated suppression is commonly associated with an hour-to-day timescale. Although the short-term non-transcriptional and long-term transcriptional effects of GCs on steroid hormone secretion are known, most studies focused on time points several hours after establishing baseline transcription levels at time 0. For example, efforts

for classifying GC-associated mechanisms in Leydig MA10 cells showed suppressed cAMP-mediated *Star* transcription via NR4A1 after 4 h of Dex incubation (10). In bovine adrenocortical primary cells, 48 h of Dex treatment resulted in a 50% suppression of the adrenocorticotropic hormone (ACTH) or forskolin-induced *CYP11A* and *CYP17* expressions (9). Another study revealed that 24-h incubation with a physiological range of Dex inhibits steroidogenic gene expression including *STAR*, *CYP11A1*, and *HSD3B* in the human glial cell line via GR (11). All these *in vitro* studies have shown that the GC-mediated suppression of steroidogenesis could be direct and independent of the HPA axis. At least, immunostaining results showed that GR is expressed in all cortical zones and the adrenal medulla as well (39, 40). Although GC was found to have an inhibitor effect on steroidogenesis, seemingly contradictory results have also been reported. For example, in the human adrenocortical (H295A) cell line, 24-h Dex treatment did not affect the transcription of *CYP11A1*, *CYP17*, and *HSD3B* (41).

In the 1-h *in vivo* treatment groups, the expression of several genes involved in steroidogenesis was downregulated, thereby indicating the suppression of steroidogenesis in mouse adrenocortical cells at the transcriptional level 1 h after Dex treatment. Because the expression of most downregulated genes identified in our *in vivo* treatment groups did not change in the *in vitro* treatment groups (except *Akr1b7*, which was upregulated after 24 h of treatment), a systemic effect from the HPA axis may influence the adrenal gland at the transcriptional level. Our data did show that 1 h of Dex treatment significantly increased the expression of pituitary *Rasd1*, a gene associated with mechanisms mediating the early response of GCs to negative feedback (8). Although a systemic effect from HPA axis cannot be ruled out, a direct effect is more probable in our 1-h *in vivo* treatment study. Notably, carbon dioxide inhalation was used for sacrification in our study. Sacrification via $CO_2$ inhalation is significant stress and can lead to high plasma ACTH and corticosterone levels in rodents (42). A possible influence from acute stress may still exist even though high-dose Dex treatment should significantly suppress the HPA axis activity via the negative feedback.

## Effects of Dex on neuronal cells

GCs are critical regulators of neuronal cell development and function (43). They upregulate the expression of PNMT in the adrenal medulla, which converts norepinephrine to epinephrine. The GC-induced PNMT expression

has been evaluated using several models including in hypophysectomized rats. Results revealed that a low PNMT expression can be restored by ACTH or supraphysiological doses of external GC (23, 44, 45). The rich vasculature within the adrenal gland allows the rapid delivery of GCs to cortical layers and the medulla (46). Endogenous GCs are supplied to the adrenal medulla via the corticomedullary portal system. This phenomenon leads to extremely high GC concentrations in the adrenal medulla and induction of PNMT expression. The lack of PNMT in *Cyp11a1* knockout embryos further confirms that a high concentration of intra-adrenal GC is required to stimulate adrenal PNMT (47). In the current study, although the heatmaps showed that the expression of several genes associated with catecholamine synthesis might be elevated in the Dex-treated groups, none of them were DEGs. One possibility is that the Dex treatment suppresses endogenous GC secretion. Thus, the effect of Dex on the medulla, at least at the acute regulation of catecholamine synthesis, is attenuated due to the reduced quantity of endogenous GC entering the adrenal medulla. This also explains why the adrenal PNMT expression increases as early as 20 min after Dex treatment in hypophysectomized rats. However, there was no change in non-hypophysectomized rats (48). Although PNMT did not significantly increase based on our data, GO analysis did show that the upregulated genes were associated with the function and development of neuronal cells. An RNA-seq study showed that the proliferation of cells is the leading functional network in hypothalamic neural-progenitor/stem cells treated with Dex (49), thereby indicating the direct effect of Dex on neuronal cell development.

## Difference between adrenal cortex and adrenocortical Y-1 cells

Notably, the Y-1 cells are adrenocortical tumor cells; therefore, the characteristics (i.e. pathways and mechanisms) of these cells significantly differ from those of normal adrenocortical cells. For example, Y-1 cells do not express 21-hydroxylase (*Cyp21a1*) and, thus, do not secrete corticosterone (50). Moreover, Y-1 cells had a significantly low expression of the lipid transport protein apolipoprotein E (encoded by *Apoe*), a key modulator of cholesterol homeostasis and corticosterone secretion in the adrenal gland (FPKM of approximately 5 in Y-1 cells vs FPKM of >500 in the whole adrenal gland RNA-seq) (51). In the current study, only 58 genes were differentially expressed during 1 h of Dex treatment both *in vivo* and *in vitro.* Notably, most Dex-responsive genes identified

in the *in vitro* treatment were not observed in the *in vivo* treatment, and vice versa. It is understandable that data on Y-1 cell line, which is an adrenocortical tumor cell line of a LAF$_1$ (C57L × A/HeJ) male mouse, differ in an *in vivo* study using the whole adrenal glands of B6 mice. The difference could also be attributed to complex physiological responses, including the HPA axis negative feedback, in living animals. Although we did not use a cell type-specific approach for our *in vivo* study, the whole adrenal gland RNA-seq still provides valuable resource data emphasizing the difference in the Dex-mediated effect between Y-1 cells and the *in vivo* mouse model at the transcriptome level.

## Sexually dimorphic adrenal gland and effects of Dex

The histology and transcriptome of the adrenal gland are sexually dimorphic in mice (19, 21, 52, 53). Moreover, Dex elicits its effect in a sex-specific manner in several aspects via complex mechanisms that are still not completely understood (54, 55, 56, 57). Several immortalized adrenocortical cell lines from transgenic mice, which include a cell line generated using female mice (58), are developed. However, the Y-1 cell line is most widely used. Because the Y-1 cell line was collected from a male mouse, only male mice were included in our study. With consideration of GR-mediated sexual dimorphism in several tissues (59, 60, 61), the Dex-mediated short-term effect in the adrenal gland and adrenocortical cells in female mice could differ from that in male mice. Further studies need to be conducted to assess the short-term effects mediated by Dex in the female adrenal gland and female-derived adrenocortical cells.

In conclusion, we assessed the early/rapid murine adrenal transcriptome response to Dex treatment *in vivo* and *in vitro*. Results showed that 1 h of Dex treatment had acute transcriptional effects in the adrenal gland and Y-1 cells. However, less than 10% of Dex-responsive genes were differentially expressed both *in vivo* and *in vitro*. Interestingly, a group of Dex-responsive genes had opposite directions of response *in vivo* and *in vitro*, indicating a dynamic early response of the mouse adrenal gland and adrenocortical Y-1 cells to Dex at the transcriptomic level.

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