INTRODUCTION

Acne vulgaris is a very common skin condition caused by abnormal keratinocyte proliferation and desquamation, androgen-induced sebum production, and Propionibacterium acnes (P. acnes) proliferation and resulting inflammation. Safe and effective treatment options targeting one or more of the steps in pathogenesis are needed. Nicotinamide (NAM) has been used for treating acne since 1975. NAM is an amide form of vitamin B3 and the precursor...
for nicotinamide adenine dinucleotide (NAD) and the phosphorylated derivative, NADP⁺, in the body.² NAM is an essential water-soluble nutrient in many foods and hasn’t been subjected to a rigorous evaluation of its safety.² It is used to treat a variety of skin conditions in addition to acne vulgaris including melasma and atopic dermatitis.³ NAM is used in various forms such as gel, emulsion and oral tablets (e.g., oral Nicomide, oral NicAzell).³ The treatment dosage for an adult varies from 20 mg/d to 3.5 g/d (0.2–350 mmol/L),³ depending on the severity of the condition being treated. Although current studies suggest that topical or oral use of NAM has a low incidence of side effects and a wide therapeutic index, there are no studies on the effect of oral NAM on acne vulgaris as a single-agent and these variable pharmacological doses require more research.

Nicotinamide treatment may target several steps in the above pathogenesis process. It has been demonstrated that topical NAM treatment reduces the sebum excretion rate and decreases sebum levels.⁶ It also protects the natural barrier of the skin from P. acnes infection.⁷ Moreover, NAM has an anti-inflammatory effect through inhibiting cytokine secretion and leukocyte chemotaxis.⁸ However, the effect of NAM in regulating androgen production during acne vulgaris treatment remains unknown.

The zona reticularis of the adrenal cortex is one of the primary androgen producing centres in the body.⁹ It is reported that adrenal androgens contribute to approximately 50% of the pool of circulating androgens in healthy women.¹⁰ Cholesterol is converted to pregnenolone (Preg) via the side chain cleavage system consisting of the enzyme 11-hydroxylase (CYP11A1), and subsequently synthesizes mineralocorticoids, glucocorticoids, or sex steroids by the specific expression and activities of catalytic enzymes, including cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) and 3β-hydroxysteroid dehydrogenase type II (HSD3β2). NCI-H295R cells are derived from the adrenal cortex of a 46-year-old female. Several in vitro studies using the NCI-H295R cell model revealed that starving human adrenocortical cells causes an increase in androgen production.¹¹ Hence, starved NCI-H295R cells are an ideal model for the study of androgen regulation.

Using human NCI-H295R cells, the present study investigated the effect of NAM on testosterone biosynthesis. We also explored the network changes during NAM treatment. We found that NAM inhibited androgen production not only by reducing the expression of CYP17A1 and HSD3β2 in starved NCI-H295R cells, but also by functioning through other transcription factors and classical signalling pathways.

## 2 | RESULTS

### 2.1 | NAM inhibited the cell viability of human adrenal NCI-H295R cells

To determine the effects of NAM on human adrenal NCI-H295R cells, the cells were treated with NAM at a concentration ranging from 1 to 25 mmol/L for 24 hours. CCK8 assays were then performed. The results showed that NAM significantly decreased the viability of NCI-H295R cells with the highest dose (25 mmol/L) but had no effect at lower doses (Figure 1A).

### 2.2 | NAM inhibited testosterone production of human adrenal NCI-H295R cells

To assess the effect of NAM on steroidogenesis in NCI-H295R cells, we measured testosterone levels after incubating cells with different concentrations of NAM. Steroid analysis showed lower testosterone

![Figure 1](image-url)

**Figure 1** Effects of NAM on human adrenal NCI-H295R cell viability and testosterone synthesis. The NCI-H295R cells were treated for 24 h with increasing concentrations of NAM ranging from 1 to 25 mmol/L. The cell viability (A) and the testosterone production in the cell culture supernatants (B) were measured. Bar represent mean ± SD. One-way ANOVA and Turkey-type multiple comparison test were used. *Significantly different from the compared group at P < .01
production under NAM treatment when compared to untreated cells. Moreover, NAM inhibited testosterone synthesis in a dose-dependent manner (Figure 1B).

2.3 | Transcriptome profile of human adrenal NCI-H295R cells under NAM treatment

RNA-sequencing was conducted on starved NCI-H295R cells treated with or without 25 mmol/L NAM for 24 hours. A total of 2835 genes were identified as significantly differential expressed genes (DEGs; fold change > 2, FDR < 0.01) after NAM treatment, of which 1631 genes were up-regulated, and 1204 genes were down-regulated. A heatmap was created for visualization of these data (Figure 2A). Principal component analysis (PCA) of all DEGs demonstrated large differences between the cells with or without NAM treatment (Figure 2B).

To validate our sequencing data, we selected seven representative genes that were altered following NAM treatment and performed qRT-PCR to assess their expression levels. Seven genes were confirmed to have lower expression in NAM-treated NCI-H295R cells compared to the untreated group (Figure 2C).

2.4 | Gene set enrichment analysis (GSEA) of genes modulated by NAM in NCI-H295R cells

As many genes had altered expression patterns following NAM treatment, we performed gene set enrichment analysis (GSEA) to investigate the enriched transcriptome changes due to NAM. GSEA was based on a hallmark database which summarized and represented specific well-defined biological processes. This analysis revealed two gene sets that were significantly up-regulated and 11 gene sets that were down-regulated in the NAM treatment group compared to the untreated group (\(P\)-value < .01, \(q\)-value < 0.1). The down-regulated gene sets were associated with E2F targets, TGF\(\beta\) signalling, androgen response, and oestrogen response (Figure 3A). The GSEA of component 2, corresponding to pathway databases, revealed several up- and down-regulated gene sets in the NAM-treated group versus the untreated group (\(P\)-value < .01, \(q\)-value < 0.1). Enrichment was seen for gene sets involved in targets of IGF1 and IGF2, steroid hormone biosynthesis, cell cycle, and mitotic related pathways (Figure 3B). In addition, GO gene set analysis in GSEA identified a significant enrichment for steroid dehydrogenase activity, ovulation cycle, and steroid biosynthesis process gene sets (\(P\)-value < .01, \(q\)-value < 0.1; Figure 3C). However, no significantly over-expressed GO gene sets (\(P\)-value > .01, \(q\)-value > 0.1; data unshown) were identified.

2.5 | Bioinformatic analysis of the DEGs

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis was performed to determine the enriched pathways of the DEGs in the NAM-treated NCI-H295R cells. The top 20 enriched KEGG pathways are shown in Figure 4A, indicating that the DEGs were involved in the TGF\(\beta\) signalling pathway, cytokine-cytokine receptor interaction, and ovarian steroidogenesis. The heatmap of DEGs of ovarian steroidogenesis was performed and several crucial enzymes during testosterone biosynthesis were significantly mis-regulated (Figure 4B).

We also performed GO annotation and enrichment analysis for DEGs (Table 1). The genes were found to be enriched in biological processes such as cell proliferation and differentiation, SMAD protein signal transduction, and cellular hormone metabolic process. Furthermore, these DEGs were enriched in molecular functions such as RNA binding and cellular component as protein-containing complex.

To investigate the interaction among the proteins of these genes, we performed a protein–protein interaction analysis of the DEGs and found associations with steroid hormone biosynthesis, TGF\(\beta\) signalling pathway, and cell proliferation in the STRING database (Figure 4C). This analysis showed some close interactions among these genes.

3 | DISCUSSION

In the present study, we demonstrated that NAM inhibited cell viability and testosterone production in human adrenal NCI-H295R cells at the concentration of 25 mmol/L. Through RNA-sequencing, we identified the potential involved genes, pathways and their interactions which may be responsible for these effects. These results indicate the underlying molecular mechanisms of NAM in treating acne vulgaris through inhibiting testosterone synthesis.

The human androgen producing tissues are the zona reticularis of the adrenal cortex and theca or Leydig cells of the gonads. The human adrenal cortex produces a variety of steroids such as dehydroepiandrosterone (DHEA) and androstenedione (A4) which have little androgenic activity, and more potent androgens including testosterone. The androgenic character makes human adrenal NCI-H295R cells a good model to study androgen biosynthesis as they produce more androgen in a starvation state. NAM plays roles in the treatment of acne vulgaris and the development of diabetes but its effects on androgen production is unknown. Using this ideal cell model, we found that NAM not only inhibits cell viability but also reduces testosterone production in starved NCI-H295R cells at high doses. The reduced cell viability might affect the total testosterone production, although the androgen synthetic ability of adrenal cells was also reduced.

Androgen production begins with cholesterol; CYP11A1 catalyses the initial enzymatic reaction from cholesterol to pregnenolone (Preg) at the inner mitochondrial membrane. Then, the hydroxylase and 17, 20-lyase activity of CYP17A1 helps catalyse the conversion of Preg to 17\(\alpha\)-hydroxypregnenolone (17OH-Preg) and DHEA. Through the enzyme HSD3\(\beta\), DHEA is converted to...
Figure 2  Transcriptome changes in NCI-H295R cells grown under SM conditions with and without NAM (25 mmol/L) treatment. A. Total number of DEGs were showed in the heatmap (fold change > 2, FDR < 0.01). One thousand six hundred thirty-one genes were up-regulated shown in red and 1204 genes were down-regulated shown in green. In the heatmap, rows showed individual DEGs. Duplicate samples were depicted in columns. Gene expression levels were displayed for each independent sample. B. The principal component analysis (PCA) of all DEGs among four samples. The NAM-treated groups were shown in blue and the untreated groups were shown in red. C. Validation of seven representative DEGs obtained from RNA-sequencing by qRT-PCR. Analysis of the relative gene expression was performed based on $2^{\Delta\Delta CT}$ method using GAPDH and HPRT1 for normalization. Results are presented as mean ± SD. *$P < .01$. SM-starved medium. C: ■ 0 mmol/L, □ 25 mmol/L. [Correction added on 12 March 2020, after first online publication: Figure 2B has been amended]
A4 and further to testosterone by aldo-keto reductases (AKRs).\textsuperscript{15} Besides the classic pathway of androgen production, AKRs also play a major role in a backdoor pathway of androgen synthesis.\textsuperscript{16,17} RNA-sequencing analysis revealed that key enzymes of both androgen biosynthesis pathways (e.g., CYP11A1, CYP17A1, HSD3β, AKRs) were all affected by NAM. NAM transforms to NAD(P) in the cytoplasm and could affect several NAD-dependent enzymes.\textsuperscript{18,19} HSD3β and AKRs are monomeric NAD(P)H-dependent oxidoreductases, indicating that the activity of these enzymes might be altered by NAM.\textsuperscript{16}

Multiple signalling pathways appear to modulate androgen production. The adrenocorticotropic hormone (ACTH)/cAMP/PKA pathway is widely studied. ACTH binds to a G-protein coupled receptor (GPCR) and triggers the production of cAMP and activation of PKA signalling leading to the phosphorylation of various proteins and transcription factors involved in androgen synthesis.\textsuperscript{20-22} We found that the expression of the adenylyl cyclase family (ADCY1, ADCY2, ADCY5, ADCY6, ADCY10) was altered with NAM treatment, indicating that NAM might affect cAMP levels and the downstream pathways of androgen synthesis. Moreover, TGF superfamilies such as TGFβ, BMPs, and inhibitors have also been implicated in androgen production in adrenal cells through the SMAD signalling pathway.\textsuperscript{23-25} The NCI-H295R cells treated with NAM showed DEGs enriched in SMAD signalling pathways. Growth factors like insulin-like growth factor-1 (IGF-1) are a potential connection node between TGF signalling pathway and steroidogenesis in our PPI analysis. IGF-1 has also been shown to regulate androgen biosynthesis in Leydig cells and adrenal cells.\textsuperscript{26-28}

There are few reports about the potential impact of NAM on the testicular androgenesis. It has been shown that administration of NAM antagonists in neonatal male rats causes a significant reduction in the numbers of supporting cells and spermatogonia/tubular cross-section.\textsuperscript{29} In addition, NAD+ has been reported to overcome the effects of ethanol on testicular steroidogenesis.\textsuperscript{30} There are no appropriate cell models derived from the theca or Leydig cells of...
In conclusion, our studies of NAM-treated human adrenal NCI-H295R cells allowed us to identify the role of NAM in the inhibition of cell viability and androgen production. Several key enzymes of steroid biosynthesis were altered under starvation growth conditions with NAM, such as decreased CYP11A1, CYP17A1, HSD3B2 and AKR1C3 expression, which may lead to the reduction in androgen production. Meanwhile, signalling pathways related to cell cycle, cell proliferation and steroidogenesis were also altered and might play a role in the regulation of androgen synthesis. These findings can help explain the potential actions of NAM in acne vulgaris and make NAM a candidate for the treatment of other hyperandrogenic disorders.
4 | METHODS

4.1 | Cell cultures and treatments

Human adrenal NCI-H295R cells were maintained under normal growth conditions (growth medium, GM) in DMEM:F-12 medium (HyClone Corporation) with 0.1% Insulin-Transferrin-Selenium Supplement (Gibco), 2.5% Nu-Serum I (Becton Dickinson) and 100 U/mL penicillin and streptomycin (HyClone Corporation). The serum-free medium (starvation medium, SM) only contained DMEM:F-12 and 100 U/mL penicillin and streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C and the cells were divided once a week. Nicotinamide (Beyotime Biotechnology) was dissolved in distilled water and used at a final concentration of 1–25 mmol/L. Cells were subcultured in 12-well plates at a density of 5 × 10⁵ cells/well, in a volume of 1.0 mL medium. When the
4.2 | Cell viability test

NCI-H295R cells were reseeded in 96-well plates at 2000 cells/well in 100 µL complete growth medium and incubated overnight. On the second day, cells were starved and treated with 1, 5, or 25 mmol/L of NAM. Following treatment for 24 hours, the effects of NAM on cell viability were determined by the enhanced CCK8 assay (Beyotime Biotechnology) according to the manufacturer’s instructions. Each value represented the mean for at least three independent experiments.

4.3 | Hormone analysis in cell culture supernatants

For steroid hormone analysis, the testosterone concentrations in the cell culture supernatants were measured using the electrochemiluminescence immunoassay (Roche) according to the manufacturer’s instructions. The concentration of testosterone was normalized by the protein concentrations of the cell lysates. The protein concentration was measured by BCA assay (Thermo Fisher) according to the standard protocol. Each value represented the mean for at least three independent experiments.

4.4 | RNA isolation, RNA purification, and RNA-sequencing

Total RNA was extracted from cultured NCI-H295R cells grown under serum starvation and NAM conditions using TRIzol Reagent according to the manufacturer’s instructions (Takara Bio). The concentration and RNA integrity number of the total RNA were analysed by Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was then enriched using Oligo (dT) magnetic beads (Invitrogen). Following fragment screening, library building, and PCR product purification, the samples were sequenced on a BGISEQ-500 platform at the BGI Bioinformatics Corporation. A total of 203.76 mol/L raw reads was collected for all four samples. If the pair-end reads had junction contamination, or satisfied N > 5% or low quality (quality value < 10) >20%, the reads were removed. Clean data were aligned to the hg38 RefSeq (RNA sequences, GRCh38) by HISAT (v2.0.4). Finally, 92.20% of the data were mapped to the hg38 reference databases.

4.5 | Identification of differentially expressed genes (DEGs)

To analyse the expression of genes, we used Bowtie2 (v1.2.8) to align the clean data to the reference gene sequences and the gene expression levels were quantified by RSEM (v1.2.8). Differentially expressed genes (DEGs) were identified using DEGseq2. During the procedure, the significance of each observed expression changes between samples was tested and corrections for false-positives were performed using Benjamini and Storey’s false discovery rate (FDR). Genes with an FDR < 0.01 and fold change > 2 were identified as DEGs. Principal component analysis (PCA) plots were created with ClustVis (https://biit.cs.ut.ee/clustvis/clustvis). Heatmaps were generated with R (version 3.6.1) heatmap package.

4.6 | Gene set enrichment analysis (GSEA)

Gene set enrichment analysis v4.0.1 (JAVA version) was downloaded from the Gene Set Enrichment Analysis website (http://software.broadinstitute.org/gsea/downloads.jsp). The reference gene sets were downloaded from the MSigDB (Molecular Signatures Database) (http://software.broadinstitute.org/gsea/msigdb/collections.jsp). The expression dataset, phenotype class, and reference gene sets were loaded in the GSEA software. The processes were performed on NAM-treated group versus untreated group with a permutation number of 1000 according to the default weighted enrichment statistical method. Gene sets with the P-value < .01 and the FDR < 0.1 were considered to show a significant enrichment.

4.7 | Enrichment and functional data analysis

Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis of DEGs was performed using Kobas v3.0 (http://kobas.cbi.pku.edu.cn/index.php). The Benjamini-Hochberg FDR was used to correct the P-value, which was defined as q-value. The KEGG pathway plot was generated with R (version 3.6.1) ggplot package. Gene ontology (GO) enrichment was performed with Gene Ontology (http://geneontology.org/). Fisher’s exact test was used to identify the significant GO terms of the DEGs, and the P-value was corrected by FDR. The significance of the pathways and GO terms was considered by corrected P-value (q-value or FDR). The protein–protein interaction (PPI) network was performed using STRING v11.0 (https://string-db.org/).

4.8 | Quantitative real time PCR (qRT-PCR)

Total RNA was reverse transcribed to cDNA using the Prime Script RT Kit with gDNA Eraser (Takara Bio). qRT-PCR was performed using
SYBR Premix Ex Taq (Takara Bio) on a LightCycler 480 System according to the manufacturer’s instructions. The primer sequences are shown in Table S1. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used for normalization and the relative expression of mRNA was calculated based on the $2^{-\Delta\Delta C_T}$ method.

4.9 | Statistical analysis

Data were expressed as the mean ± SD of at least three independent experiments. Statistical analysis was performed using the two-tailed Students t test when comparing two samples, while group comparison was performed by one-way ANOVA followed by Tukey’s multiple comparison test. Values were considered significant at $P < .05$ and referred to two-sided probability.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Shigang Zhao for his invaluable help in the article. This work was supported by the National Key Research and Development Program of China (2018YFC1004303).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

Wei Zhang https://orcid.org/0000-0002-0747-8184

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Gao X, Yu Z, Yang J, Gao Y, Li S, Zhang W. An integrated RNA-Seq and network study reveals the effect of nicotinamide on adrenal androgen synthesis. *Clin Exp Pharmacol Physiol*. 2020;47:821–830. 
[https://doi.org/10.1111/1440-1681.13258](https://doi.org/10.1111/1440-1681.13258)