



Article

# Rno\_circ\_0001004 Acts as a miR-709 Molecular Sponge to Regulate the Growth Hormone Synthesis and Cell Proliferation

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**Abstract:** (1) Background: As a novel type of non-coding RNA with a stable closed-loop structure, circular RNA (circRNA) can interact with microRNA (miRNA) and influence the expression of miRNA target genes. However, circRNA involved in pituitary growth hormone (GH) regulation is poorly understood. Our previous study revealed protein kinase C alpha (*PRKCA*) as the target gene of miR-709. Currently, the expression and function of rno\_circRNA\_0001004 in the rat pituitary gland is not clarified; (2) Methods: In this study, both bioinformatics analysis and dual-luciferase report assays showed a target relationship between rno\_circRNA\_0001004 and miR-709. Furthermore, the rno\_circRNA\_0001004 overexpression vector and si-circ\_0001004 were constructed and transfected into GH<sub>3</sub> cells; (3) Results: We found that rno\_circRNA\_0001004 expression was positively correlated with the *PRKCA* gene and GH expression levels, while it was negatively correlated with miR-709. In addition, overexpression of rno-circ\_0001004 also promoted proliferation and relieved the inhibition of miR-709 in GH<sub>3</sub> cells; (4) Conclusions: Our findings show that rno\_circ\_0001004 acts as a novel sponge for miR-709 to regulate GH synthesis and cell proliferation, and are the first case of discovery of the regulatory role of circRNA\_0001004 in pituitary GH.

**Keywords:** pituitary; rno\_circ\_0001004; miR-709; GH; proliferation



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## 1. Introduction

Growth hormone (GH) is a key hormone secreted from the anterior pituitary, and has received much attention as it regulates key physiological functions such as growth and development [1]. Studies have shown that microRNAs (miRNAs) can regulate the synthesis and secretion of GH [2–4]. MiRNAs are small single endogenous RNAs that regulate post-transcriptional silencing of target genes by binding to the 3'-untranslated region (UTR) or open reading frame (ORF) region of target mRNAs [5]. More and more evidence has demonstrated that miRNA functions broadly in development, physiology, and pathology by influencing cell proliferation, cell differentiation, cell migration, apoptosis, metabolism and signal transduction [6–11]. We have previously shown that the miR-709 is highly expressed in the pituitary and inhibits the GH synthesis and suppresses the viability of GH<sub>3</sub> cells [12] by targeting Protein Kinase C alpha (*PKCA*). Protein Kinase C (PKC) is a class of phospholipid-dependent kinases that participate in regulation of protein secretion including GH and luteinizing hormone (LH) [13,14], as well as regulating cell proliferation [15].

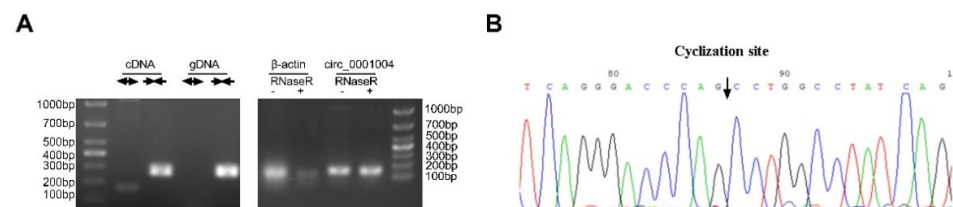
Circular RNA (circRNA) is a special type of non-coding RNA (ncRNA) molecule that, unlike traditional linear RNA, forms covalently closed loop structures generated by pre-mRNA back splicing. CircRNAs are highly stable, abundant and conserved molecules with the characteristics of cell tissue specificity [16] and have received more attention due to their multiple regulation functions in animals and plants [17–19]. Similar to other regulatory ncRNAs, circRNAs play important roles in various biological processes, such as acting as a scaffold in the assembly of protein complexes [20–22], regulating alternative RNA splicing or transcription and RNA-protein interactions [23,24] and functioning as competing endogenous RNA (ceRNA) [25] or microRNA (miRNA) sponges [26–31]. However, reports of circRNAs involved in pituitary GH regulation have been very scarce up to the present [32,33].

The rno\_circ\_0001004 was firstly discovered in the rat anterior pituitary by using Illumina sequencing [34]. It is generated from exons 9 to 11 of the Wnk2 gene, with a length of 888 bp. However, the underlying regulatory role of rno\_circ\_0001004 in the pituitary remains unknown. Thus, in the present study, the role of rno\_circ\_0001004 in the regulation of GH and cell proliferation and the circRNA-miRNA-mRNA network were explored and identified.

## 2. Results

### 2.1. Characterization of Rno\_circ\_0001004 in GH<sub>3</sub> Cells

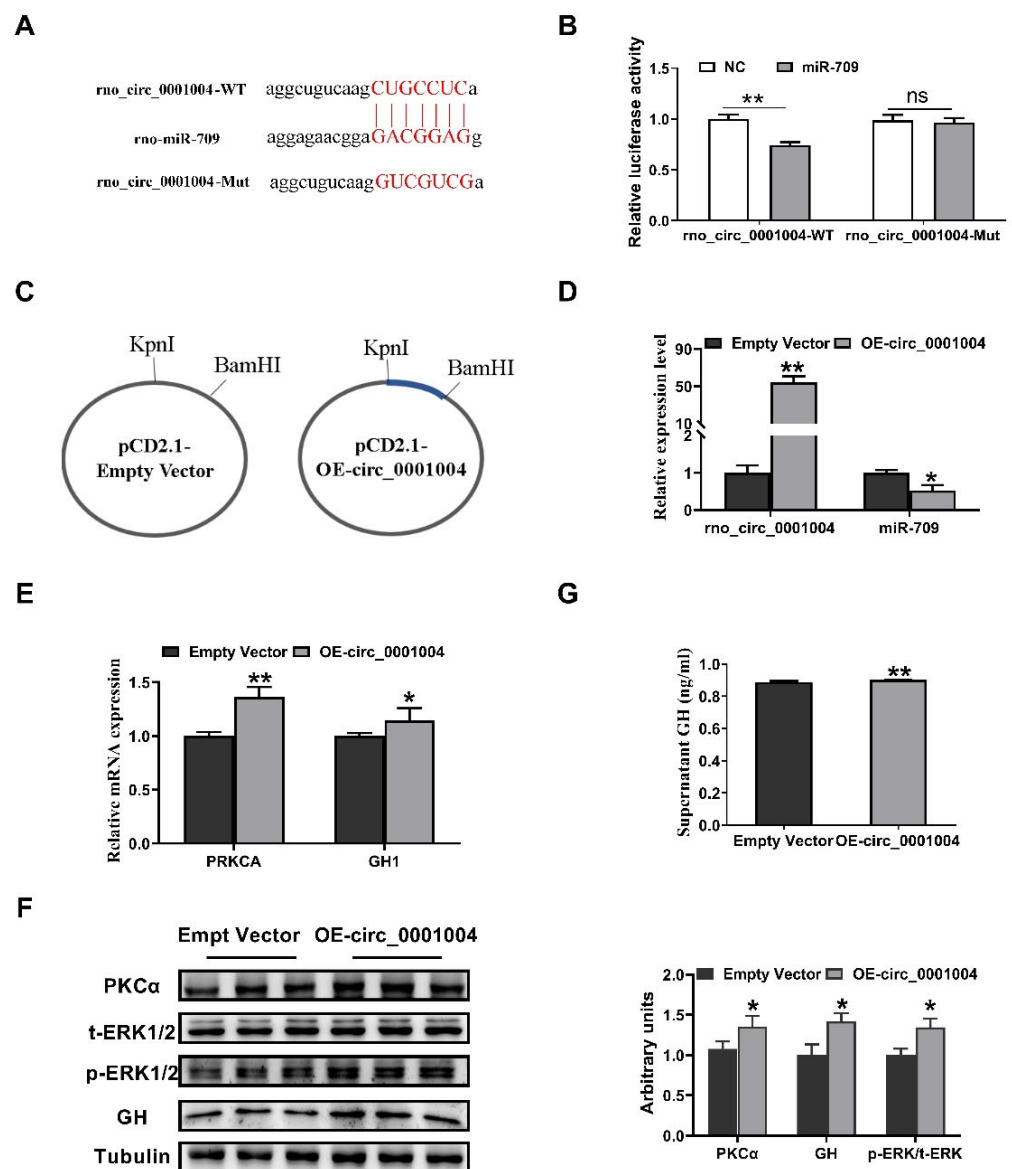
To characterize rno\_circ\_0001004 in GH<sub>3</sub> cells, we firstly detected the expression of rno\_circ\_0001004. Convergent and divergent primers were designed to amplify the linear or back-splicing products and total RNA from GH<sub>3</sub> cells with or without RNase R treatment was subjected to RT-PCR. As expected, endogenous circ\_0001004, but not pre-mRNA, was resistant to RNase R digestion (Figure 1A). Sanger sequencing validated the back-spliced junction of rno\_circ\_0001004 (Figure 1B).



**Figure 1.** Characterization of rno\_circ\_0001004 in a GH<sub>3</sub> cell. (A) PCR analysis for rno\_circ\_0001004 in the cDNA and gDNA of a GH<sub>3</sub> cell. (left) Total RNA from GH<sub>3</sub> cells with or without RNase R treatment was subjected to RT-PCR. (right) (B) The back-splice junction of rno\_circ\_0001004 was identified by Sanger sequencing.

### 2.2. Rno\_circ\_0001004 Antagonizes miR-709-Mediated Repression of GH Synthesis and Secretion

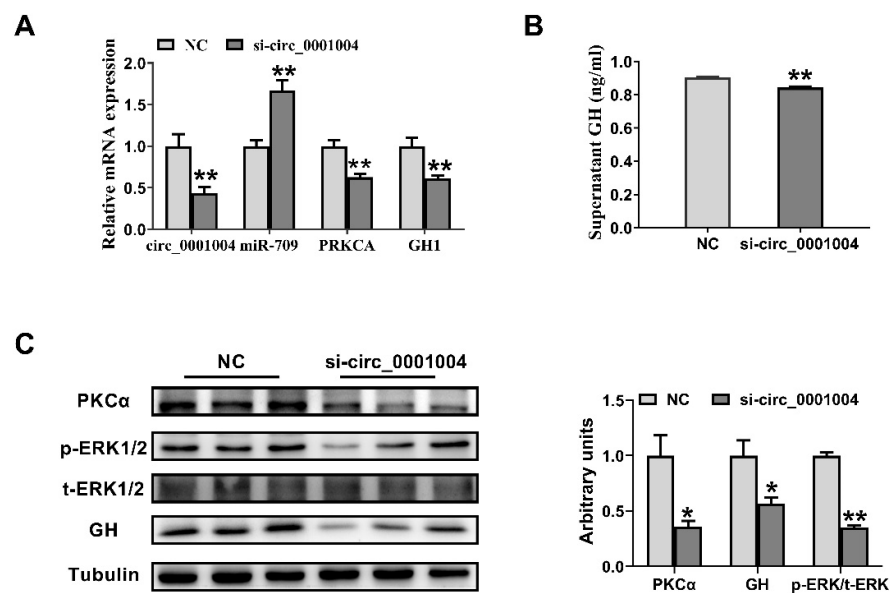
Bioinformatics analysis with RNAhybird and miRanda was performed and indicated that rno\_circ\_0001004 has miR-709 binding sites (Figure 2A). Then, a dual-luciferase reporter assay showed that miR-709 overexpression was able to down-regulate the luciferase activity compared to miR-NC, and this inhibition was eliminated when the rno\_circ\_0001004 binding site was mutated (Figure 2B). To confirm their target relationship, the rno\_circ\_0001004 overexpression vector was constructed (Figure 2C), and after transfection into a GH<sub>3</sub> cell, rno\_circ\_0001004 was overexpressed and miR-709 was correspondingly down-regulated (Figure 2D). The results above suggest that rno\_circ\_0001004 is a molecular sponge for miR-709. Our previous studies demonstrated that miR-709 significantly inhibited the GH synthesis by targeting PKC $\alpha$  [12]. Thus, we further detected the change in PKC $\alpha$  pathway and GH expression and release. Interestingly, our results show that rno\_circ\_0001004 obviously increases the mRNA and protein levels of PKC $\alpha$ , the phosphorylation of ERK1/2 (Figure 2E,F) and GH protein within cell as well as supernatant, compared to empty vectors (Figure 2F,G). These results confirm that rno\_circ\_0001004 antagonizes miR-709-mediated repression of the GH synthesis and secretion through the PKC $\alpha$  pathway.



**Figure 2.** Rno\_circ\_0001004 antagonizes miR-709-mediated repression the synthesis and secretion of GH. (A) Bioinformatics target prediction. (B) Dual luciferase reporter gene assay verified. (C) Construction of rno\_circ\_0001004 overexpression vector. (D) Expression of rno\_circ\_0001004 along with miR-709 in empty vector and overexpression vector in GH<sub>3</sub> by qRT-PCR. (E) mRNA level of PKCA and GH1 after transfection of OE-circ\_0001004. (F) Western blot evaluation results of PKC $\alpha$ , t-ERK1/2, p-ERK1/2 and GH protein expression following transfection with rno\_circ\_0001004 in GH<sub>3</sub> cells. (G) changes in supernatant GH level: average expression rose from 0.87 to 0.9 ng/mL. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

### 2.3. Knockdown of Rno\_circ\_0001004 Suppresses the GH Synthesis and Secretion in GH<sub>3</sub> Cell

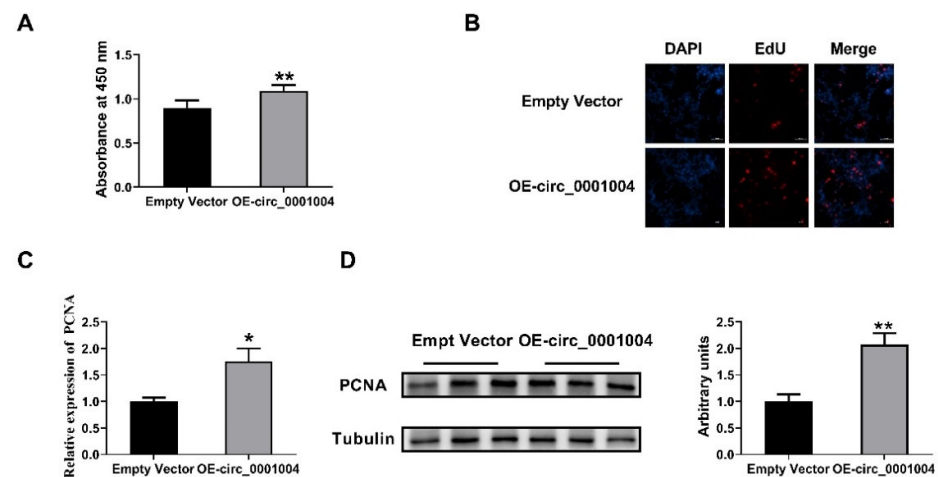
In order to further verify the effect of rno\_circ\_0001004 on GH, we transfected si-circ\_0001004 to GH<sub>3</sub> cells. As expected, si-circ\_0001004 significantly decreased the expression of circ\_0001004, while it correspondingly increased the miR-709 expression level, followed by the inhibition of mRNA in *PRKCA* and *GH1* (Figure 3A). Furthermore, a Western blot revealed that the protein expression of PKC $\alpha$ , the phosphorylation of ERK1/2, and GH both in cell and supernatant all decreased with the inhibition of circ\_0001004 (Figure 3B,C). The above results further confirm that rno\_circ\_0001004 regulates the synthesis and secretion of GH by acting as a molecular sponge for miR-709.



**Figure 3.** Knockdown of *rno\_circ\_0001004* promoted the GH synthesis and suppressed GH<sub>3</sub> cells. (A) The mRNA level of PKCA and GH1 along with *circ\_0001004* and miR-709 expression after transfection si-*circ\_0001004*. (B) The changes in supernatant GH level after transfection si-*circ\_0001004*. (C) The PKCα, t-ERK1/2, p-ERK1/2 and GH protein expression levels in GH<sub>3</sub> cells after transfection si-*circ\_0001004*. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

#### 2.4. *Rno\_circ\_0001004* Promoted the Viability of GH<sub>3</sub> Cells

Our previous study showed that miR-709 suppressed the viability of GH<sub>3</sub> cells [12]. Thus, we performed CCK8 and EdU assays to determine the effect of *rno\_circ\_0001004* on GH<sub>3</sub> cell proliferation. Intriguingly, *circ\_0001004* significantly promoted GH<sub>3</sub> cell proliferation (Figure 4A,B). Moreover, PCNA, the key marker of cell proliferation, was markedly up-regulated both in mRNA and protein levels (Figure 4C,D).

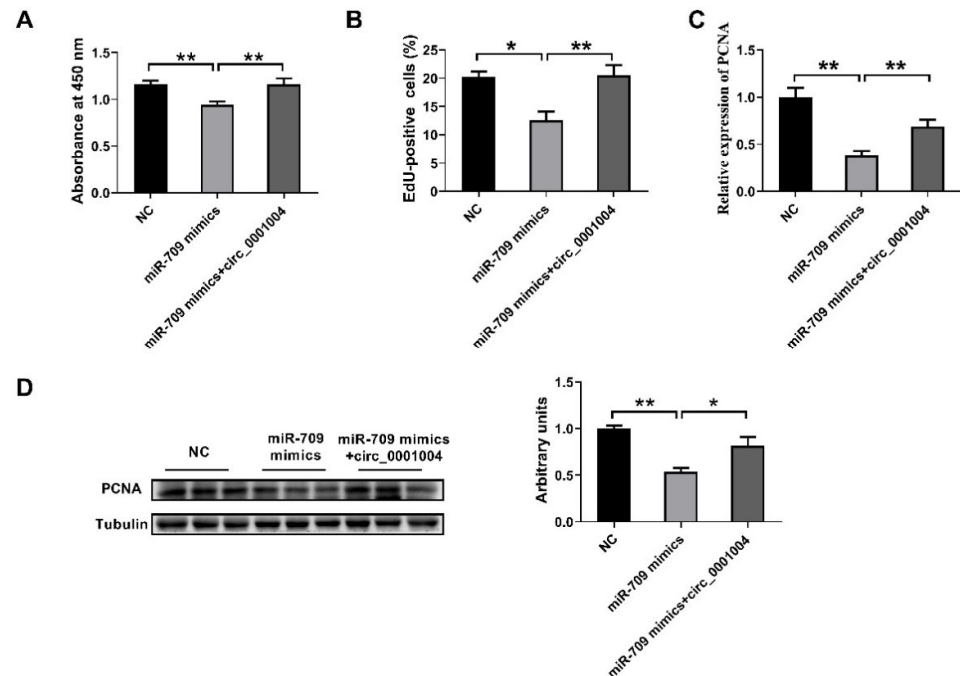


**Figure 4.** *Rno\_circ\_0001004* promoted the viability of GH<sub>3</sub> cells (A) The GH<sub>3</sub> proliferation was evaluated with CCK-8 kits. (B) Cell proliferation tested by an EdU assay. (C) PCNA mRNA expression quantified by qRT-PCR. (D) Western blot assay for PCNA protein expression. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

#### 2.5. *Rno\_circ\_0001004* Reversed the Inhibition of Cell Proliferation by miR-709

In order to further confirm the idea that *rno\_circ\_0001004* serves as a ceRNA for miR709 to regulate the viability of GH<sub>3</sub> cells, we next co-transfected miR-709 mimics and *circ\_0001004* into GH<sub>3</sub> cells. The results demonstrates that overexpression of miR-709

obviously inhibits the cell proliferation of GH<sub>3</sub>, and this inhibition is perfectly rescued by overexpression of rno\_circ\_0001004, as shown by CCK8 assay (Figure 5A), EdU assay (Figure 5B) and PCNA expression (Figure 5C,D). These results provide more profound evidence that rno\_circ\_0001004 is a sponge ceRNA for miR-709.



**Figure 5.** Effects of rno\_circ\_0001004 in reversing the inhibition of cell proliferation by miR-709. (A) The GH<sub>3</sub> proliferation was evaluated with CCK-8 kits. (B) EdU-positive cells. (C) PCNA mRNA expression quantified by qRT-PCR. (D) Western blot assay for PCNA protein expression. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

### 3. Discussion

The pituitary gland, called the ‘master gland’ of the endocrine system, is the central regulator for growth, reproduction, metabolism and stress response [35]. The anterior pituitary accounts for 80% of the entire pituitary gland and secretes six major hormones, which are crucial to our physiological well-being [36,37]. As a major hormone in the pituitary gland, GH plays an important role in regulating the growth and metabolism of organisms [38,39].

MiRNAs are a class of small ncRNA with a length of about 22 nucleotides that post-transcriptionally regulate gene expression [40]. MiRNA generally functions primarily by binding to the 3′ untranslated region (UTR) of the target mRNA [41]. They play important roles in essential processes such as cell proliferation, cell apoptosis and cell differentiation [42,43]. miRNA was reported to participate in regulating GH. MiR-34b, miR-326, miR-432, miR-548c and miR-570 were found to regulate pituitary cell proliferation [44]. miR-126 played an important role in the development of GH-secreting pituitary adenomas [2]. Our previous study showed that miR-709 inhibited the GH synthesis and suppressed the viability of GH<sub>3</sub> cells by targeting PRKCA [12].

CircRNAs are derived from the exon or intron region of genes [45]. There are currently three hypothetical models for the mechanism of circRNA formation, including lariat-driven circularization, intron pairing-driven circularization, and RNA binding protein (RBP)-mediated circularization [46]. CircRNAs containing multiple competing miRNA binding sites are likely to act as ceRNA in reducing miRNA activity and up-regulating the expression of miRNA-related target genes [47]. Thousands of strongly and stably expressed circRNAs have been detected [26]. CircFGFR4 promotes the differentiation of myoblasts by binding miR-107 [48]. Circ0005276 can promote the proliferation and migration of



prostate cancer cells [49]. Circ-ZNF609 regulates nasopharyngeal carcinoma cell growth via modulating miR-188 expression [50]. Some studies have identified circRNAs in the pituitary gland [51–54]. At present, there have been few reports of circRNA regulating GH.

In the present study, we firstly noted that rno\_circ\_0001004 has the potential to act as miR-709 binding sites. Then, characterization of rno\_circ\_0001004 was carried out and the target relationship with miR-709 was validated using a dual luciferase assay. To further probe the influence of rno\_circ\_0001004 on GH, the overexpression of a rno\_circ\_0001004 vector and si-circ\_0001004 were constructed. Next, the genes and proteins in the pathway of miR-709 regulation of GH were all evaluated. The results show that the expression trend of rno\_circ\_0001004 is almost opposite to that of miR-709, but is consistent with the expression trend of PKC $\alpha$ , GH and the phosphorylation of ERK1/2. Thus, our findings firstly clarify the molecular mechanism by which rno\_circ\_0001004 can act as a sponge for miR-709 in regulating the synthesis and secretion of GH, providing novel insight into the regulatory mechanism of GH.

Our prior study also found that miR-709 repressed the viability of GH<sub>3</sub> cells [12]. Thus, we explored whether rno\_circ\_0001004 affected the proliferation of GH<sub>3</sub> cells. Compared with the empty vector, the rno\_circ\_0001004 overexpression group can significantly promote the proliferation of GH<sub>3</sub> cells, as determined by the CCK-8 assay, PCNA expression and the EdU incorporation assay. Moreover, rno\_circ\_0001004 was able to perfectly reverse the inhibitory effect of miR-709 on the proliferation of GH<sub>3</sub> cells. Therefore, our study reveals that rno\_circ\_0001004 is able to positively regulate GH<sub>3</sub> cell viability. These results indicate that rno\_circ\_0001004 plays an essential role in the regulation GH and pituitary cell proliferation. Furthermore, they lay a foundation for further study to explore the importance of circ\_0001004 in regulation of animal and human growth and development.

## 4. Materials and Methods

### 4.1. Cell Culture and Transfection

GH<sub>3</sub> cell line (ATCC) was cultured in F12 (Gibco, New York, NY, USA) medium supplemented with 2.5% fetal bovine serum (FBS) (Gibco, New York, NY, USA), 15% horse serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco, New York, NY, USA). Hela cells were cultured in PRMI 1640 (Gibco, New York, NY, USA) culture medium with 10% FBS and 1% penicillin/streptomycin. GH<sub>3</sub> cells were transfected with miR-709 mimic, rno\_circ\_0001004 or si-circ0001004 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 4.2. RAN Isolation, cDNA Synthesis, RT-PCR and Sanger Sequencing

Total RNA was isolated from GH<sub>3</sub> cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNAs were obtained by Color Reverse Transcription Kit (with gDNA remover) (EZBioscience, Roseville, CA, USA). Genomic DNA (gDNA) was extracted using a Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). Quantification of mRNA, miRNA, circRNA and gDNA was performed by using a SBRY Green PCR Kit (Takara, Tokyo, Japan), primers and Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) The circRNA and mRNA levels were normalized to those of  $\beta$ -actin, while the miR-709 levels were normalized to the U6 and determined by 2-DDCt method. The primer sequences for the amplification of specific primers are listed in supplementary Table S1. Sanger sequencing (chain termination sequencing) is a method of DNA sequencing based upon the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) during in vitro DNA replication [55].

### 4.3. Vector Construction

The sequence for exons 9–11 of Wnk2 was PCR amplified using primers F (5'-GGGGTA CCTGAAATATGCTATCTTACAGCCTGGCCTATCAGTGGGC-3') and R (5'-CGGGATCCT CAAGAAAAATATATTCACCTGGGTCCCTGAGGCAGC-3'), then cloned into KpnI

and BamHI restriction sites of a circular expression vector, the pcd2.1-ciR (GENESEED, Guangzhou, China), by digestion to create rno\_circ\_0001004-overexpressing vector.

#### 4.4. Dual-Luciferase Reporter Assay

Hela cells were seeded in 96-well cell culture plates. When their confluence reached about 80%, the miR-709 mimic and rno\_circ\_0001004-Wt or rno\_circ\_0001004-Mut were co-transfected into cells using Lipofectamine 2000. After incubation for 48 h, the cells were washed with PBS and the luciferase activity was measured by the Dual-GLO luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

#### 4.5. Evaluation of GH<sub>3</sub> Proliferation

GH<sub>3</sub> proliferation was assessed by the cell counting kit-8 (CCK-8) method, 5-ethynyl-2'-deoxy uridine (EdU) incorporation assay and proliferating cell nuclear antigen (PCNA) expression. Firstly, the rate of GH<sub>3</sub> proliferation was determined with the CCK-8 kit (Bioss, Beijing, China) according to the manufacturer's instructions. The number of viable cells was assessed by measuring the absorbance at 450 nm using a Synergy 2 Multi-Mode Reader (Bio Tek Instruments, Inc., Winooski, VT, USA). Secondly, DNA synthesis was examined with EdU incorporation assay (YF<sup>®</sup> 555 Click-iT EdU Imaging Kit, Suzhou US EVERBRIGHT, Suzhou, China) to evaluate GH<sub>3</sub> proliferation. The EdU positive cells were counted and normalized by the total number of Hoechst 33,342 stained cells. Lastly, GH<sub>3</sub> proliferation was evaluated by PCNA expression, which is the auxiliary component of DNA polymerase  $\delta$  and constitutes a useful proliferation marker.

#### 4.6. Western Blot Analysis

GH<sub>3</sub> cells were lysed in a RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1mM phenyl methane sulfonyl fluoride (PMSF). The concentration of protein was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Equal amounts of total protein were separated by SDS-PAGE and transferred to a PVDF membrane in a tris-glycine methanol buffer. The primary antibodies used in this study were as follows: GH monoclonal antibody (sc-374266, Santa Cruz, CA, USA), PKC $\alpha$  polyclonal antibody (BS1577, Bioworld, St. Louis Park, MN, USA), ERK1/2 monoclonal antibody (4695, CST, Danvers, MA, USA), Phospho-ERK1/2 monoclonal antibody (Tyr204) (4370, CST, Danvers, MA, USA), PCNA monoclonal antibody (200947-2E1, ZEN BIO, Chengdu, China) and Tubulin polyclonal antibody (AP0064, Bioworld, St. Louis Park, MN, USA), HRP conjugated goat anti-rabbit IgG (BS13278, Bioworld, St. Louis Park, MN, USA) and HRP conjugated goat anti-mouse IgG (BS12478, Bioworld, St. Louis Park, MN, USA) were used as secondary antibodies. The membranes were incubated with Immobilon<sup>TM</sup> Western Chemiluminescent HPR Substrate (Millipore, Burlington, WA, USA) and scanned with a FlourChem M Fluorescent Western Imaging System (Protein Simple, Santa Clara, CA, USA). The protein band density was determined by the software Image J and normalized with a corresponding Tubulin intensity.

#### 4.7. Quantification of Secretory GH by ELISA

The concentration of GH in a cell medium of GH<sub>3</sub> cells transfected with rno\_circ\_0001004 and si-circ\_0001004 was determined using the reagents in the Rat Growth Hormone ELISA kit (Enzyme-linked Biotechnology, Shanghai, China) according to the manufacturer's protocols. Color alterations in the wells were read using the 96-well microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA).

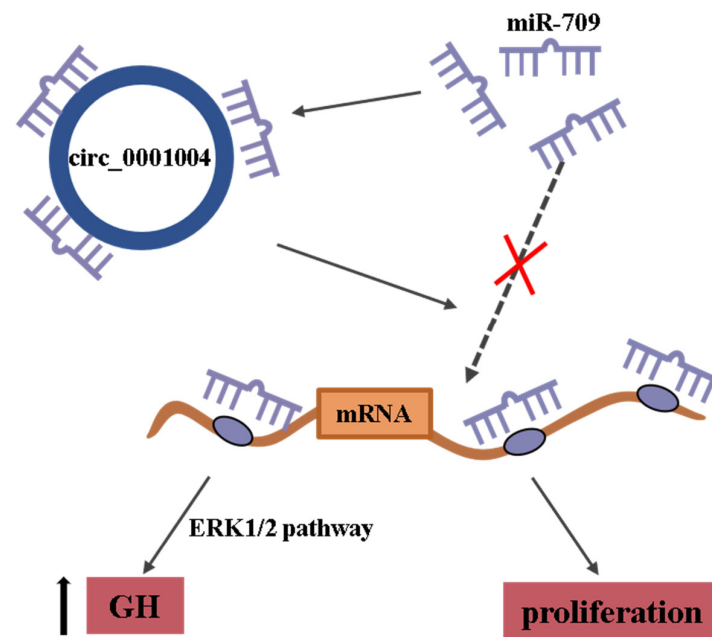
#### 4.8. Statistics Analysis

All experimental results are presented as the mean  $\pm$  S.E.M, with at least three independent replications. Statistical analysis was performed using SPSS 17.0 software. The

statistically significant differences among groups were tested by one-way analysis of variance (ANOVA).  $p < 0.05$  was considered as statistically significant. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

## 5. Conclusions

In summary, our study reveals that rno\_circ\_0001004 competitively binds miR-709 to regulate the GH synthesis and cell proliferation in rat pituitary cells (Figure 6). To the best of our knowledge, our findings are the first case to illustrate regulation of GH by circRNA\_0001004 and provide novel evidence on the circRNA-miRNA-mRNA network in pituitary cells.



**Figure 6.** Rno\_circ\_0001004 has 3 miR-709 binding sites and can act as a miR-709 molecular sponge to regulate GH synthesis and cell proliferation.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23031413/s1>.

**Author Contributions:** Methodology, Y.Z. and Q.X.; software, J.S.; validation, J.X., H.Z. and Y.W.; data curation, Y.C.; writing—original draft preparation, J.X.; writing—review and editing, Y.Z.; supervision, J.L. and T.C.; project administration, Y.Z. All authors have read and agreed to the published version of the manuscript.

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## References

- Nyberg, F. Growth Hormone in the Brain: Characteristics of Specific Brain Targets for the Hormone and Their Functional Significance. *Front. Neuroendocrinol.* **2000**, *21*, 330–348. [[CrossRef](#)] [[PubMed](#)]
- Mao, Z.G.; He, D.S.; Zhou, J.; Yao, B.; Xiao, W.W.; Chen, C.H.; Zhu, Y.H.; Wang, H.J. Differential expression of microRNAs in GH-secreting pituitary adenomas. *Diagn. Pathol.* **2010**, *5*, 79. [[CrossRef](#)] [[PubMed](#)]



3. Zhang, Z. Florez Sergio Gutierrez-Hartmann Arthur Martin James, F.; Amendt Brad, A. MicroRNAs Regulate Pituitary Development, and MicroRNA 26b Specifically Targets Lymphoid Enhancer Factor 1 (Lef-1), Which Modulates Pituitary Transcription Factor 1 (Pit-1) Expression. *J. Biol. Chem.* **2010**, *285*, 34718–34728. [[PubMed](#)]
4. Yu, Z.W.; Gao, W.; Feng, X.Y.; Zhang, J.Y.; Yuan, B. Roles of differential expression of miR-543-5p in GH regulation in rat anterior pituitary cells and GH3 cells. *PLoS ONE* **2019**, *14*, e0222340. [[CrossRef](#)] [[PubMed](#)]
5. Liu, Y.W. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **2004**, *116*, 281–297.
6. Gee, H.E.; Camps, C.; Buffa, F.M.; Colella, S.; Sheldon, H.; Gleadle, J.M.; Ragoussis, J.; Harris, A.L. MicroRNA-10b and breast cancer metastasis. *Nature* **2008**, *455*, E8. [[CrossRef](#)]
7. Kota, J.; Chivukula, R.R.; O'Donnell, K.A.; Wentzel, E.A.; Montgomery, C.L.; Hwang, H.W.; Chang, T.C.; Vivekanandan, P.; Torbenson, M.; Clark, K.R. Therapeutic delivery of miR-26a inhibits cancer cell proliferation and induces tumor-specific apoptosis. *Cell* **2009**, *137*, 1005. [[CrossRef](#)]
8. Ma, L.; Teruya-Feldstein, J.; Weinberg, R.A. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **2007**, *449*, 682–688. [[CrossRef](#)]
9. Png, K.J.; Halberg, N.; Yoshida, M.; Tavazoie, S.F. A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells. *Nature* **2012**, *481*, 190–194. [[CrossRef](#)]
10. Tay, Y.; Zhang, J.; Thomson, A.M.; Bing, L.; Rigoutsos, I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature* **2008**, *455*, 1124. [[CrossRef](#)]
11. Felekakis, K.; Touvana, E.; Stefanou, C.; Deltas, C. MicroRNAs: A newly described class of encoded molecules that play a role in health and disease. *Hippokratia* **2010**, *14*, 236–240. [[PubMed](#)]
12. Cheng, Y.; Chen, T.; Song, J.; Qi, Q.; Zhang, Y. miR-709 inhibits GHRP6 induced GH synthesis by targeting PRKCA in pituitary. *Mol. Cell. Endocrinol.* **2020**, *506*, 110763. [[CrossRef](#)] [[PubMed](#)]
13. Grey, C.L.; Chang, J.P. Differential modulation of ghrelin-induced GH and LH release by PACAP and dopamine in goldfish pituitary cells. *Gen. Comp. Endocrinol.* **2013**, *191*, 215–224. [[CrossRef](#)] [[PubMed](#)]
14. Pemberton, J.G.; Orr, M.E.; Stafford, J.L.; Chang, J.P. PI3K signalling in GnRH actions on dispersed goldfish pituitary cells: Relationship with PKC-mediated LH and GH release and regulation of long-term effects on secretion and total cellular hormone availability. *Gen. Comp. Endocrinol.* **2014**, *205*, 268–278. [[CrossRef](#)]
15. Petiti, J.P.; Gutiérrez, S.; De Paul, A.L. Andreoli: GH3B6 Pituitary Tumor Cell Proliferation is Mediated by PKC $\alpha$  and PKC $\epsilon$  via ERK 1/2-dependent Pathway. *Cell Physiol. Biochem.* **2010**, *26*, 135–146. [[CrossRef](#)]
16. Meng, S.; Zhou, H.; Feng, Z.; Xu, Z.; Ying, T.; Li, P.; Wu, M. CircRNA: Functions and properties of a novel potential biomarker for cancer. *Mol. Cancer* **2017**, *16*, 94. [[CrossRef](#)]
17. Chen, L.L.; Yang, L. Regulation of circRNA biogenesis. *RNA Biol.* **2015**, *12*, 381–388. [[CrossRef](#)]
18. Salzman, J.; Gawad, C.; Wang, P.L.; Lacayo, N.; Brown, P.O. Circular RNAs Are the Predominant Transcript Isoform from Hundreds of Human Genes in Diverse Cell Types. *PLoS ONE* **2012**, *7*, e30733. [[CrossRef](#)]
19. Ye, C.Y.; Chen, L.; Liu, C.; Zhu, Q.H.; Fan, L. Widespread noncoding circular RNAs in plants. *New Phytol.* **2015**, *208*, 88–95. [[CrossRef](#)]
20. Du, W.W.; Fang, L.; Yang, W.; Wu, N.; Awan, F.M.; Yang, Z.; Yang, B.B. Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity. *Cell Death Differ.* **2017**, *24*, 357–370. [[CrossRef](#)]
21. Du, W.W.; Yang, W.; Liu, E.; Yang, Z.; Yang, B.B. Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res.* **2016**, *44*, gkw027. [[CrossRef](#)] [[PubMed](#)]
22. Zeng, Y.; Du, W.W.; Wu, Y.; Yang, Z.; Awan, F.M.; Li, X.; Yang, W.; Zhang, C.; Yang, Q.; Yee, A.; et al. A Circular RNA Binds To and Activates AKT Phosphorylation and Nuclear Localization Reducing Apoptosis and Enhancing Cardiac Repair. *Theranostics* **2017**, *7*, 3842–3855. [[CrossRef](#)] [[PubMed](#)]
23. Ashwal-Fluss, R.; Meyer, M.; Pamudurti, N.R.; Ivanov, A.; Bartok, O.; Hanan, M.; Evantal, N.; Memczak, S.; Rajewsky, N.; Kadener, S. circRNA Biogenesis Competes with Pre-mRNA Splicing—ScienceDirect. *Mol. Cell* **2014**, *56*, 55–66. [[CrossRef](#)] [[PubMed](#)]
24. Salzman, J. Circular RNA Expression: Its Potential Regulation and Function. *Trends Genet.* **2016**, *32*, 309–316. [[CrossRef](#)]
25. Huang, M.; Zhong, Z.; Lv, M.; Shu, J.; Tian, Q.; Chen, J. Comprehensive analysis of differentially expressed profiles of lncRNAs and circRNAs with associated co-expression and ceRNA networks in bladder carcinoma. *Oncotarget* **2016**, *7*, 47186. [[CrossRef](#)]
26. Memczak, S.; Jens, M.; Elefsinioti, A.; Torti, F.; Krueger, J.; Rybak, A.; Maier, L.; Mackowiak, S.D.; Gregersen, L.H.; Munschauer, M.; et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* **2013**, *495*, 333–338. [[CrossRef](#)] [[PubMed](#)]
27. Hansen, T.B.; Jensen, T.I.; Clausen, B.H.; Bramsen, J.B.; Finsen, B.; Damgaard, C.K.; Kjems, J. Natural RNA circles function as efficient microRNA sponges. *Nature* **2013**, *495*, 384–388. [[CrossRef](#)]
28. Lei, P.; Chen, G.; Zhu, Z.; Shen, Z.; Du, C.; Zang, R.; Yang, S.; Hua, X.; Li, H.; Xu, X. Circular RNA ZNF609 functions as a competitive endogenous RNA to regulate AKT3 expression by sponging miR-150-5p in Hirschsprung's disease. *Oncotarget* **2017**, *8*, 808.
29. Li, F.; Zhang, L.; Li, W.; Deng, J.; Zheng, J.; An, M.; Lu, J.; Zhou, Y. Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/ $\beta$ -catenin pathway. *Oncotarget* **2015**, *6*, 6001. [[CrossRef](#)]
30. Wang, K.; Long, B.; Liu, F.; Wang, J.X.; Li, P.F. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Eur. Heart J.* **2016**, *37*, ehv713. [[CrossRef](#)] [[PubMed](#)]

31. Zheng, Q.; Bao, C.; Guo, W.; Li, S.; Chen, J.; Chen, B.; Luo, Y.; Lyu, D.; Li, Y.; Shi, G. Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat. Commun.* **2016**, *7*, 11215. [[CrossRef](#)] [[PubMed](#)]
32. Du, Q.; Zhang, W.; Feng, Q.; Hao, B.; Cheng, C.; Cheng, Y.; Li, Y.; Fan, X.; Chen, Z. Comprehensive circular RNA profiling reveals that hsa\_circ\_0001368 is involved in growth hormone-secreting pituitary adenoma development. *Brain Res. Bull.* **2020**, *161*, 65–77. [[CrossRef](#)] [[PubMed](#)]
33. Yu, Z.W.; Ren, W.Z.; Wang, T.; Zhang, W.D.; Yuan, B. CircAgtbp1 Acts as a Molecular Sponge of miR-543-5p to Regulate the Secretion of GH in Rat Pituitary Cells. *Animals* **2021**, *11*, 558. [[CrossRef](#)] [[PubMed](#)]
34. Han, D.X.; Sun, X.L.; Fu, Y.; Wang, C.J.; Liu, J.B.; Jiang, H.; Gao, Y.; Chen, C.Z.; Yuan, B.; Zhang, J.B. Identification of long non-coding RNAs in the immature and mature rat anterior pituitary. *Sci. Rep.* **2017**, *7*, 17780. [[CrossRef](#)]
35. Scully, K.M.; Rosenfeld, M.G. Pituitary Development: Regulatory Codes in Mammalian Organogenesis. *Science* **2002**, *295*, 2231–2235. [[CrossRef](#)]
36. Hagerjohnson, G. An Introduction to Behavioral Endocrinology. *Q. Rev. Biol.* **2011**, *2*, 273–282.
37. Tissier, P.; Hodson, D.J.; Lafont, C.; Fontanaud, P.; Mollard, P. Anterior pituitary cell networks. *Front. Neuroendocrinol.* **2012**, *33*, 252–266. [[CrossRef](#)]
38. Isaksson, O.; Lindahl, A.; Nilsson, A.; Isgaard, J. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. *Endocr. Rev.* **1987**, *8*, 426–438. [[CrossRef](#)] [[PubMed](#)]
39. Salomon, F.; Cuneo, R.C. The effects of treatment with recombinant human growth hormone on body composition and metabolism. *N. Engl. J. Med.* **1989**, *321*, 1797. [[CrossRef](#)]
40. Carthew, R.W.; Sontheimer, E.J. Origins and mechanisms of miRNAs and siRNAs. *Cell* **2009**, *136*, 642–655. [[CrossRef](#)]
41. He, L.; Hannon, G.J. MicroRNAs: Small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **2004**, *5*, 522–531. [[CrossRef](#)] [[PubMed](#)]
42. Miska, E.A. How microRNAs control cell division, differentiation and death. *Curr. Opin. Genet. Dev.* **2005**, *15*, 563–568. [[CrossRef](#)]
43. Zamore, P.; Haley, B. Ribo-gnome: The Big World of Small RNAs. *Science* **2005**, *309*, 1519–1524. [[CrossRef](#)] [[PubMed](#)]
44. Daniela, D.A.; Dario, P.; Paula, M.; Magali, R.; Anne, W.; Gerald, R.; Monica, F.; Maria, C.C.; Jacqueline, T.; Alfredo, F. Altered microRNA expression profile in human pituitary GH adenomas: Down-regulation of miRNA targeting HMGA1, HMGA2, and E2F1. *J. Clin. Endocrinol. Metab.* **2012**, *97*, E1128–E1138.
45. Xiang, L.; Li, Y.; Chen, L.L. The Biogenesis, Functions, and Challenges of Circular RNAs. *Mol. Cell* **2018**, *71*, 428–442.
46. Jeck, W.R.; Sorrentino, J.A.; Wang, K.; Slevin, M.K.; Burd, C.E.; Liu, J.; Marzluff, W.F.; Sharpless, N.E. Circular RNAs are abundant, conserved, and associated with ALU repeats. *Rna* **2013**, *19*, 141–157. [[CrossRef](#)]
47. Qu, S.; Zhong, Y.; Shang, R.; Zhang, X.; Song, W.; Kjems, J.; Li, H. The emerging landscape of circular RNA in life processes. *RNA Biol.* **2017**, *14*, 992–999. [[CrossRef](#)]
48. Li, H.; Wei, X.; Yang, J.; Dong, D.; Hao, D.; Huang, Y.; Lan, X.; Plath, M.; Lei, C.; Ma, Y. circFGFR4 Promotes Differentiation of Myoblasts via Binding miR-107 to Relieve Its Inhibition of Wnt3a. *Mol. Ther. Nucleic Acids* **2018**, *11*, 272–283. [[CrossRef](#)]
49. Feng, Y.; Yang, Y.; Zhao, X.; Fan, Y.; Zhou, L.; Rong, J.; Yu, Y. Circular RNA circ0005276 promotes the proliferation and migration of prostate cancer cells by interacting with FUS to transcriptionally activate XIAP. *Cell Death Dis.* **2019**, *10*, 792. [[CrossRef](#)]
50. Li, M.; Li, Y.; Yu, M. CircRNA ZNF609 Knockdown Suppresses Cell Growth via Modulating miR-188/ELF2 Axis in Nasopharyngeal Carcinoma. *OncoTargets Ther.* **2020**, *13*, 2399–2409. [[CrossRef](#)]
51. Guo, H.X.; Yuan, B.; Su, M.T.; Zheng, Y.; Zhang, J.B. Identification of Circular RNAs in the Anterior Pituitary in Rats Treated with GnRH. *Animals* **2021**, *11*, 2557. [[CrossRef](#)] [[PubMed](#)]
52. Han, D.X.; Wang, C.J.; Sun, X.L.; Liu, J.B.; Zhang, J.B. Identification of circular RNAs in the immature and mature rat anterior pituitary. *J. Endocrinol.* **2019**, *240*, 393–402. [[CrossRef](#)] [[PubMed](#)]
53. Li, C.; Li, X.; Ma, Q.; Zhang, X.; Cao, Y.; Yao, Y.; You, S.; Wang, D.; Quan, R.; Hou, X. Genome-wide analysis of circular RNAs in prenatal and postnatal pituitary glands of sheep. *Sci. Rep.* **2017**, *7*, 16143. [[CrossRef](#)] [[PubMed](#)]
54. Zhang, H.; Hu, B.; Xiong, J.; Chen, T.; Zhang, Y. Genomewide analysis of circular RNA in pituitaries of normal and heat-stressed sows. *BMC Genom.* **2019**, *20*, 1013. [[CrossRef](#)] [[PubMed](#)]
55. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463–5467. [[CrossRef](#)] [[PubMed](#)]