



Article

Functional Analysis of Steroidogenic Factor 1 (sf-1) and 17 α -Hydroxylase/Lyase (cyp17 α) Promoters in Yellow Catfish *Pelteobagrus fulvidraco*

Wu-Hong Lv , Guang-Hui Chen , Mei-Qin Zhuo, Yi-Huan Xu, Yi-Chuang Xu and Xiao-Ying Tan *

Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Fishery College, Huazhong Agricultural University, Wuhan 430070, China; lvwuhong@webmail.hzau.edu.cn (W.-H.L.); cgh0626@webmail.hzau.edu.cn (G.-H.C.); zmq@mail.hzau.edu.cn (M.-Q.Z.); xuyihuan@webmail.hzau.edu.cn (Y.-H.X.); xuyichuang@webmail.hzau.edu.cn (Y.-C.X.)
* Correspondence: tx7933@mail.hzau.edu.cn; Tel.: +86-27-8728-2113

Abstract: The present study was performed to clone and characterize the structures and functions of steroidogenic factor 1 (sf-1) and 17 α -hydroxylase/lyase (cyp17 α) promoters in yellow catfish *Pelteobagrus fulvidraco*, a widely distributed freshwater teleost. We successfully obtained 1981 and 2034 bp sequences of sf-1 and cyp17 α promoters, and predicted the putative binding sites of several transcription factors, such as Peroxisome proliferator-activated receptor alpha (PPAR α), Peroxisome proliferator-activated receptor gamma (PPAR γ) and Signal transducer and activator of transcription 3 (STAT3), on sf-1 and cyp17 α promoter regions, respectively. Overexpression of PPAR γ significantly increased the activities of sf-1 and cyp17 α promoters, but overexpression of PPAR α significantly decreased the promoter activities of sf-1 and cyp17 α . Overexpression of STAT3 reduced the activity of the sf-1 promoter but increased the activity of the cyp17 α promoter. The analysis of site-mutation and electrophoretic mobility shift assay suggested that the sf-1 promoter possessed the STAT3 binding site, but did not the PPAR α or PPAR γ binding sites. In contrast, only the PPAR γ site, not PPAR α or STAT3 sites, was functional with the cyp17 α promoter. Leptin significantly increased sf-1 promoter activity, but the mutation of STAT3 and PPAR γ sites decreased leptin-induced activation of sf-1 promoter. Our findings offered the novel insights into the transcriptional regulation of sf-1 and cyp17 α and suggested leptin regulated sf-1 promoter activity through STAT3 site in yellow catfish.

Keywords: sf-1; cyp17 α ; steroidogenesis; transcriptional regulation; *Pelteobagrus fulvidraco*



Citation: Lv, W.-H.; Chen, G.-H.; Zhuo, M.-Q.; Xu, Y.-H.; Xu, Y.-C.; Tan, X.-Y. Functional Analysis of Steroidogenic Factor 1 (sf-1) and 17 α -Hydroxylase/Lyase (cyp17 α) Promoters in Yellow Catfish *Pelteobagrus fulvidraco*. *Int. J. Mol. Sci.* **2021**, *22*, 195. <https://dx.doi.org/10.3390/ijms22010195>

Received: 28 November 2020
Accepted: 23 December 2020
Published: 27 December 2020

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Steroid hormones are involved in the regulation of many physiological processes, such as embryonic development, sex differentiation, metabolism and reproduction in vertebrates [1], and their biosynthesis was regulated by key transcription factors and enzymes, such as steroidogenic factor 1 (sf-1) and 17 α -hydroxylase (cyp17 α) [2]. The sf-1 is an important transcription factor with critical regulatory roles in the transcription of steroidogenic genes [3,4]. cyp17 α is the only enzyme responsible for the conversion of C21 steroids to C19 steroids in the pathway of steroidogenesis [1,2]. At present, studies have been conducted on the function of cyp17 α and sf-1 on the mRNA and protein levels in several physiological processes [5,6]. In mammals, several transcriptional factors, such as nuclear transcription factor Y (NF-Y), specificity protein 1 (SP1) and cAMP-response element binding protein (CREB), positively regulate the activities of sf-1 promoter [7–9]. However, information associated with the structure and transcriptional regulation of sf-1 and cyp17 α promoters were very scarce in fish.

Peroxisome proliferator-activated receptor alpha and gamma (PPAR α and PPAR γ) are the two important transcription factors that modulate the expression of many target genes involved in numerous physiological processes, including steroidogenesis [10–12].

Considering that *cyp17 α* and *sf-1* are also the key enzyme and transcription factor in regulating steroidogenesis, we hypothesize that PPAR α and PPAR γ regulated steroidogenesis by targeting *cyp17 α* and *sf-1*.

Leptin belongs to a member of the cytokines and plays a vital role in reproduction [13]. Leptin has also important function in digestion (e.g., in birds), the connection with digestion was revealed by expression and immunohistochemistry analysis by Seroussi et al. [14]. Several studies reported the effects of leptin on the control of steroidogenesis in animals and cells [15,16], but the results were not consistent. For example, several studies reported that leptin attenuated gonadotropin or growth factor-stimulated steroidogenesis in isolated theca and granulosa cells [17,18], but other studies pointed out that leptin induced the synthesis of estrogen in ovary and ovarian follicles [19,20]. Signal transducer and activator of transcription 3 (STAT3) is identified as the most important factor to transduce the leptin signal [21–23]. In mammals, studies have suggested that leptin modulated steroidogenic by STAT3 [13,19]. Wu et al. [22] demonstrated that leptin regulated the transcriptional activities of lipid metabolism-related target genes by STAT3 in yellow catfish. However, whether leptin could directly mediate the transcriptional activities of genes involved in steroidogenesis, such as *sf-1* and *cyp17 α* , remained unknown.

Yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater teleost, has been cultured widely in China and other Asian countries due to the successful technology of artificial propagation. However, further investigation remained into the regulation of steroidogenesis during the propagation. It is well-known that the application of luciferase assay is a well-established technique in molecular biology for analysis of cloned promoter DNA fragments [24,25]. Thus, the present study was performed to characterize the regions of *sf-1* and *cyp17 α* promoters and investigated their transcriptional regulation in yellow catfish. Our study elucidated innovative insights into the regulatory mechanism for the biosynthesis of steroid hormones.

2. Results

2.1. Cloning and Sequence Analysis of *sf-1* and *cyp17 α* Promoters

In the present study, the transcription start sites (TSS) of *sf-1* and *cyp17 α* were identified, and 1981 bp and 2034 bp sequences were cloned for *sf-1* and *cyp17 α* promoters, respectively. Several core promoter elements closed to TSS were identified on the *sf-1* promoter, such as one CCAAT box from –38 bp to –27 bp and three SP1 binding sites located at –130 bp to –120 bp, –118 bp to –108 bp and –16 bp to –7 bp, respectively (Figure S1). One PPAR α binding site (at –413 bp/–396 bp), one PPAR γ binding site (at –1396 bp/–1388 bp) and one STAT3 binding site (at –1106 bp/–1096 bp) were predicted on the region of *sf-1* promoter, respectively (Figure S1).

On the *cyp17 α* promoter, we predicted one TATA box located from –150 bp to –136 bp, one GC box located at –55 bp to –42 bp and one SP1 binding site located at –53 bp to –43 bp, one PPAR α binding site (at –881 bp/–864 bp), one PPAR γ binding site (at –89 bp/–70 bp) and one STAT3 binding site (at –1669 bp/–1660 bp), respectively (Figure S2).

2.2. The Relative Luciferase Activity of 5'-Deletion Assay of the Regions of *sf-1* and *cyp17 α* Promoters

Deletion of the sequences from –1981 bp to –1421 bp of *sf-1* promoter significantly enhanced the relative luciferase activity. There is no significant difference after the deletion of the sequences from –1421 bp to –921 bp. The deletion of the sequences from –921 bp to –489 bp significantly reduced the relative luciferase activity of *sf-1* promoter (Figure 1A). Deletion of the sequences from –2034 bp to –1402 bp of *cyp17 α* promoter significantly decreased the relative luciferase activity. Subsequent deletion to –687 bp significantly increased *cyp17 α* promoter activity, but the deletion of the sequences from –687 bp to –330 bp showed no significant difference on *cyp17 α* promoter activity (Figure 1A).

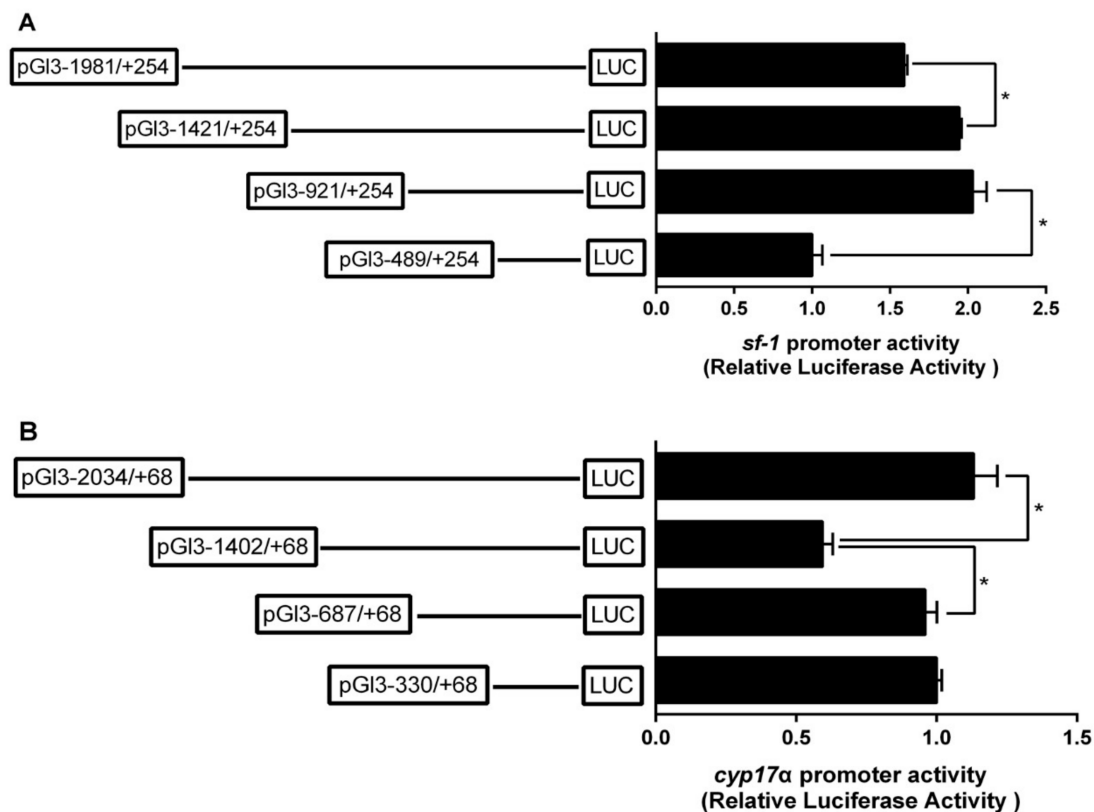


Figure 1. 5' unidirectional deletion assays of the *sf-1* and *cyp17 α* promoters of yellow catfish. Schematic diagram of truncated promoters are shown at the left panel. The results for corresponding luciferase reporter assay are shown at right panel. **(A)** A series of plasmids containing 5' unidirectional deletions of the *sf-1* promoter regions (pGI3-1981, –1421, –921, and –489) fused in frame to the luciferase gene were transfected into HEK293T cells; **(B)** A series of plasmids containing 5' unidirectional deletions of the *cyp17 α* promoter regions (pGI3-2034, –1402, –687, and –330) fused in frame to the luciferase gene were transfected into HEK293T cells. Values represent the ratio between firefly and Renilla luciferase activities. Results are expressed as the mean \pm SEM arbitrary units of three independent experiments. Symbol (*) indicates significant differences between the two groups ($p < 0.05$).

2.3. Overexpression Analysis of Yellow Catfish PPAR α , PPAR γ , and STAT3 of the Regions of *sf-1* and *cyp17 α* Promoters in HEK293T Cells

To reveal the mechanism of PPAR α , PPAR γ , and STAT3 regulating *sf-1* and *cyp17 α* in yellow catfish, we transfected PPAR α , PPAR γ , and STAT3 plasmids into HEK293T cells for 24 h, and found that they were successfully over-expressed in HEK293T cells (Figure 2).

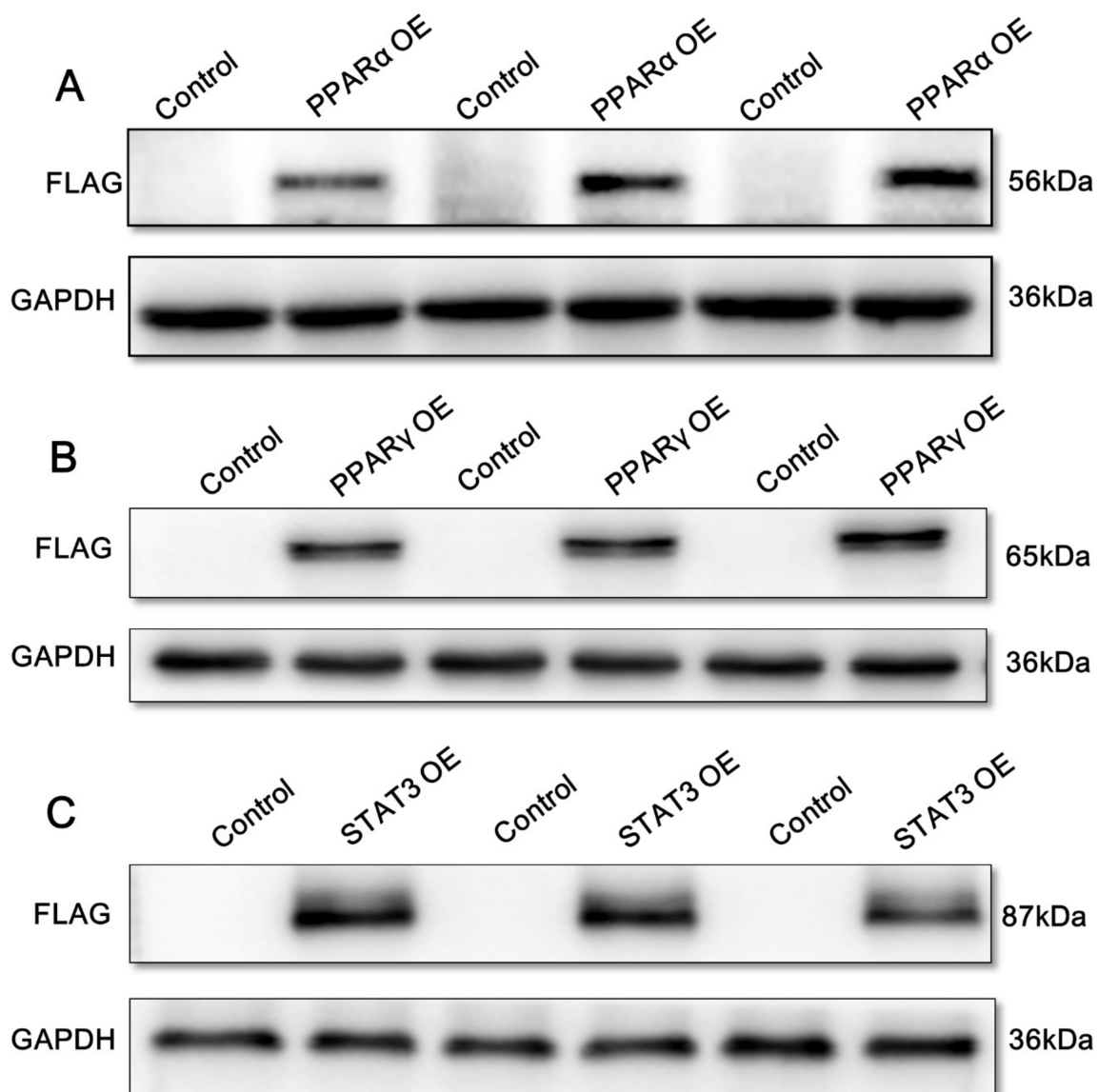


Figure 2. Overexpression (OE) of yellow catfish PPAR α , PPAR γ , and STAT3 in HEK293T cells after Western blot analysis using FLAG antibody. (A) PPAR α over-expression (OE); (B) PPAR γ OE; (C) STAT3 OE. GAPDH represents glyceraldehyde-3-phosphate dehydrogenase. ($n = 3$ represents three independent biological experiments).

To study the activities of *sf-1* and *cyp17 α* promoters induced by PPAR α , PPAR γ , and STAT3, we co-transfected PPAR α , PPAR γ , and STAT3 plasmids along with the promoter constructs into HEK293T cells for 24 h and performed the 5'-deletion assay of *sf-1* and *cyp17 α* promoters, respectively. Overexpression of PPAR α resulted in a marked reduction in *sf-1* promoter activity by 39%, compared to the control. The sequence deletion between -1981 bp and $+254$ bp presented no significant influences on PPAR α -induced promoter activity, indicating that negative response element to PPAR α existed on $-489/+254$ bp region of *sf-1* promoter (Figure 3A). Overexpressed PPAR γ significantly increased the *sf-1* promoter activity compared to the control, and showed no effect on the sequence deletion between -1981 bp and $+254$ bp on PPAR γ -induced promoter activity, indicating that active response element to PPAR γ might exist on $-489/+254$ bp region of *sf-1* promoter (Figure 3B). Overexpression of STAT3 significantly decreased the promoter activity of *sf-1*, compared to the control, The sequence deletion between -1981 bp and -1421 bp completely abolished the inhibitory effect by STAT3, and sequence deletion between -1421 bp to -921 bp increased the promoter activity by STAT3. Nonetheless, the sequence deletion between -489 bp and $+254$ bp region of *sf-1* promoter completely abolished

the STAT3-induced upregulation, reflecting that the negative response elements existed at $-1981/-1421$ bp and $-1421/-921$ bp, and the positive response element existed at $-921/-489$ bp regions of *sf-1* promoter to STAT3 (Figure 3C).

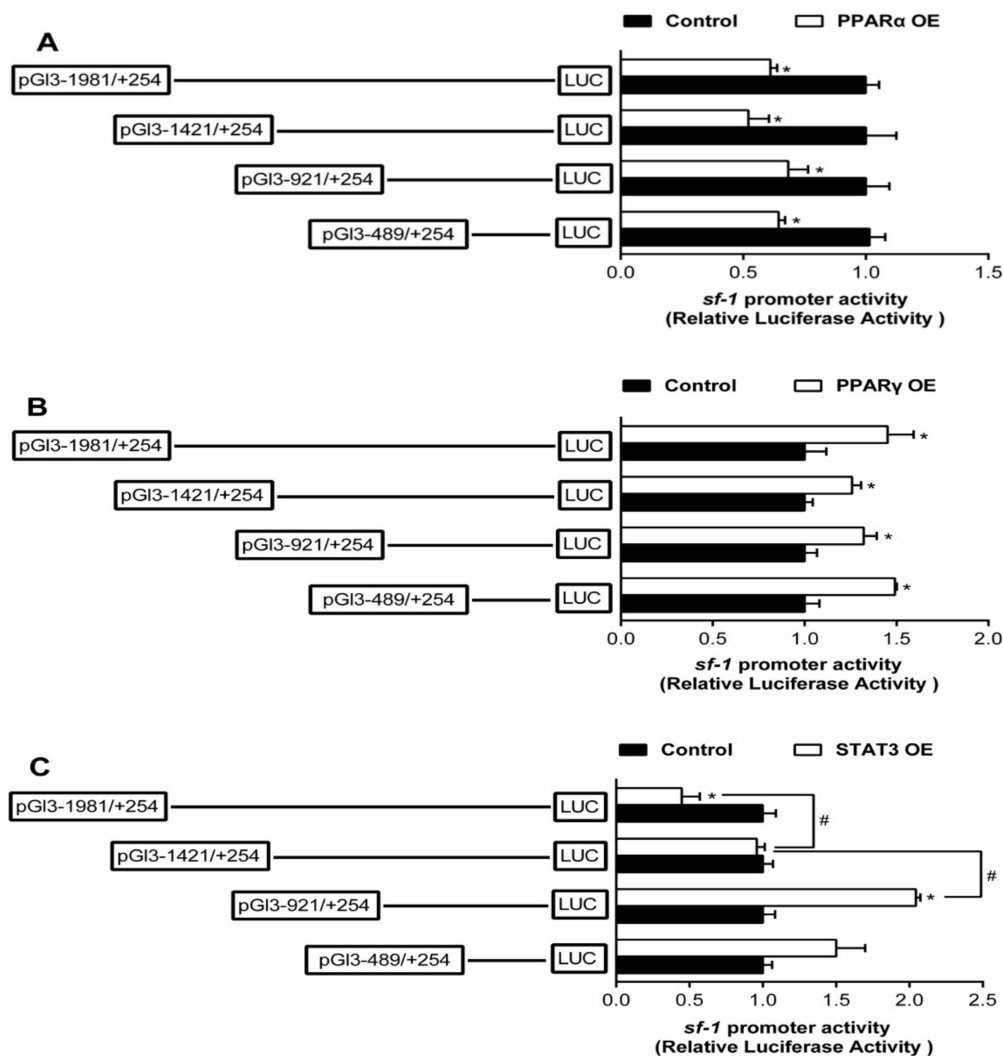


Figure 3. Overexpression (OE) analysis of 5' unidirectional deletion assays of the *sf-1* promoters of yellow catfish. (A) PPARα OE; (B) PPARγ OE; (C) STAT3 OE. Values are presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences in relative luciferase activities between the PPARα-, PPARγ-, and STAT3-OE group and the control ($p < 0.05$). Hash symbol (#) indicates significant difference between the same OE groups with different deletion regions ($p < 0.05$). The relative luciferase activity was presented as the fold activated by PPARα, PPARγ, and STAT3 compared with the control, respectively.

Overexpression of PPARα markedly reduced the promoter activity of *cyp17α* compared to the control. The inhibitory effect by PPARα was completely abolished when the sequence was deleted from -1402 to -687 bp. However, deleting the sequence between -687 bp and -330 bp recovered the inhibitory effect by PPARα, indicating that there are two negative response elements at $-1402/-687$ bp and $-330/+68$ bp, and one positive response element at $-687/-330$ bp region of *cyp17α* promoter to PPARα (Figure 4A). Compared to the control, overexpression of PPARγ increased the promoter activity of *cyp17α*. However, deleting the sequence between -2034 bp and -330 bp presented no significant effects on PPARγ-induced *cyp17α* promoter activity, indicated that there is one positive responsive element at $-330/+68$ bp region of *cyp17α* promoter to PPARγ (Figure 4B). Overexpression of STAT3 enhanced the *cyp17α* promoter activity by 3.1-fold compared to the control, and deleting the region between -2034 bp and -1402 bp of

cyp17 α promoter further up-regulated the stimulative effect of STAT3 overexpression, but the subsequent sequence deletion from -1402 bp to -687 bp alleviated this stimulative effect. These results indicated there are two positive responsive elements at $-1402/-687$ bp and $-330/+68$ bp region of *cyp17 α* promoter to STAT3 (Figure 4C).

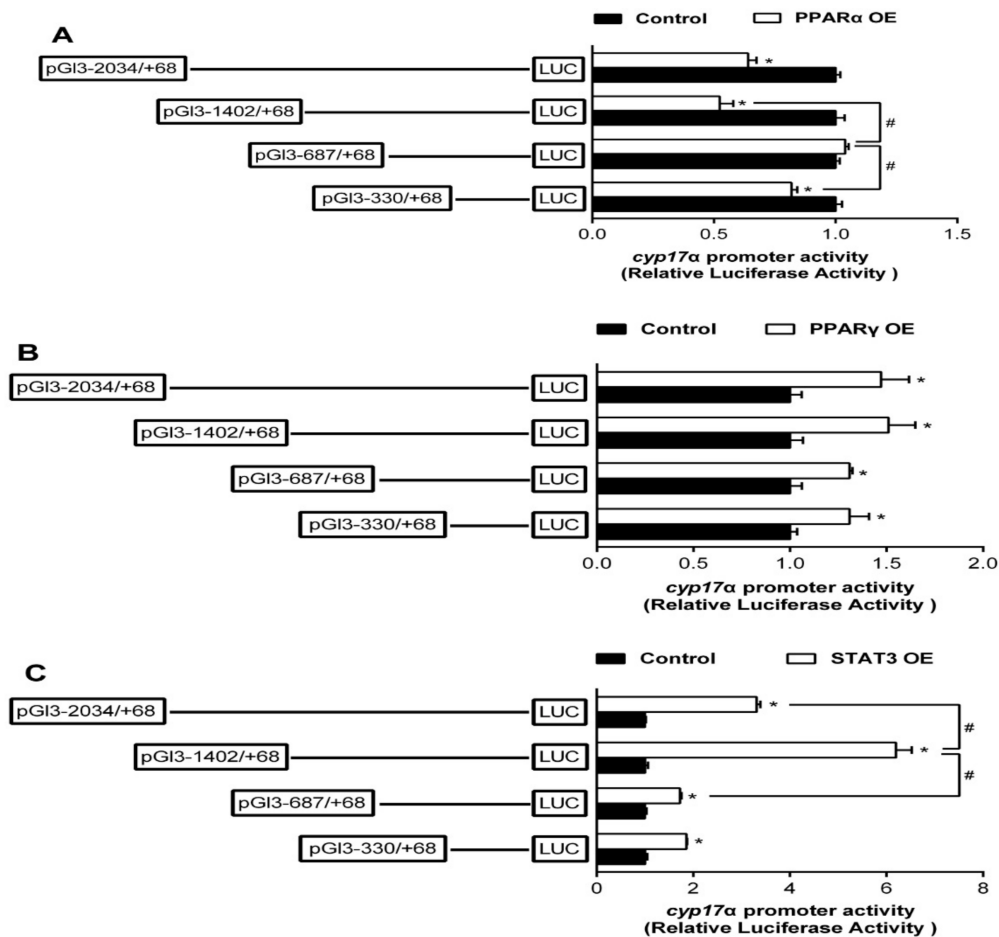


Figure 4. Overexpression (OE) analysis of 5' unidirectional deletion of the *cyp17 α* promoters of yellow catfish. (A) PPAR α OE; (B) PPAR γ OE; (C) STAT3 OE. Values are presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences in relative luciferase activities between the PPAR α -, PPAR γ -, and STAT3-OE group and the control ($p < 0.05$). Hash symbol (#) indicates significant difference between the same OE groups with different deletion regions ($p < 0.05$). The relative luciferase activity was presented as the fold activated by PPAR α , PPAR γ , and STAT3 compared with the control, respectively.

To investigate the response of promoters induced by leptin, we used 200 ng/mL leptin to incubate HEK293T for 24 h and performed the 5' deletion assays. Leptin incubation significantly increased sf-1 promoter activity. In the leptin-treated groups, the sequence deletion between -1981 bp and -921 bp of sf-1 promoter showed no significant influences on luciferase activity. However, further deletion from -921 to -489 bp significantly down-regulated leptin-induced sf-1 promoter activity (Figure 5A). Leptin incubation show no effect on the *cyp17 α* promoter activity, and no differences were found in relative luciferase activity of *cyp17 α* promoter among different deletion plasmids and between control and leptin treatment (Figure 5B).

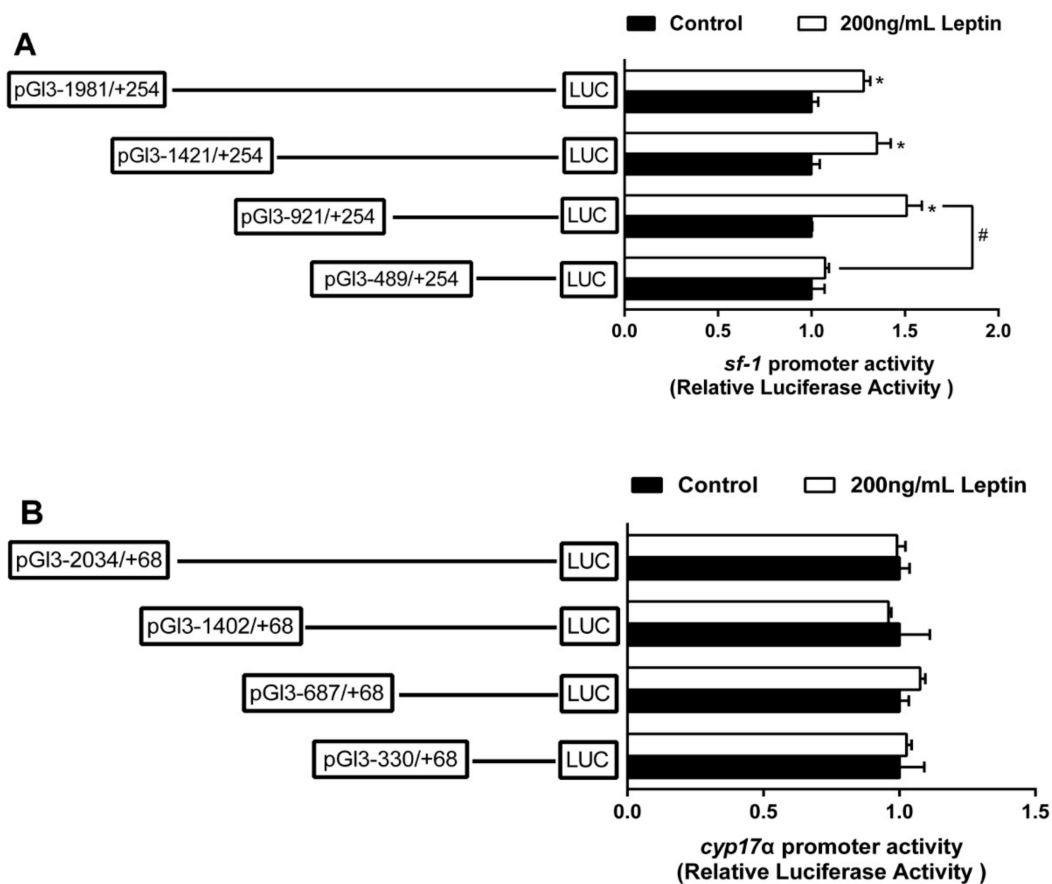


Figure 5. 5' unidirectional deletion assays for *sf-1* (A) and *cyp17α* (B) promoters after 200 ng/mL leptin treatment for 24 h. Values showed the ratio of activities of firefly to Renilla luciferase, normalized to the control. Results were presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences between two treatments in the same plasmid ($p < 0.05$). Hash symbol (#) indicates significant differences between two 5' unidirectional deletion plasmids under the same treatment ($p < 0.05$).

2.4. Site-Mutation Analysis of PPAR α , PPAR γ , and STAT3 on the Promoters of *sf-1* and *cyp17α*

To further elucidate whether the regions of *sf-1* and *cyp17α* promoters possessed PPAR α , PPAR γ , and STAT3 response elements, we performed the site-directed mutation at these regions of *sf-1* and *cyp17α* promoters. Overexpressed PPAR α reduced *sf-1* promoter activity by 42% compared to the control, and its inhibitory effect was completely abolished when this site (−413/−396 bp) was mutated, suggesting that *sf-1*-PPAR α (−413/−396 bp) site might inhibit PPAR α -induced *sf-1* transcription (Figure 6A). The *sf-1* promoter activity was enhanced by 1.6-fold by overexpressed PPAR γ , and mutation of *sf-1*-PPAR γ (−1396/−1388 bp) site completely abolished the stimulatory effect of PPAR γ , indicating that *sf-1*-PPAR γ (−1396/−1388 bp) site enhanced PPAR γ -induced *sf-1* transcription (Figure 6B). STAT3 overexpression inhibited the promoter activity of *sf-1* by 59% compared to the control. However, *sf-1* promoter activity increased by 1.7-fold by mutation of *sf-1*-STAT3 (−1106/−1096 bp) site, suggesting that *sf-1*-STAT3 (−1106/−1096 bp) site down-regulated STAT3-induced *sf-1* transcription (Figure 6C).

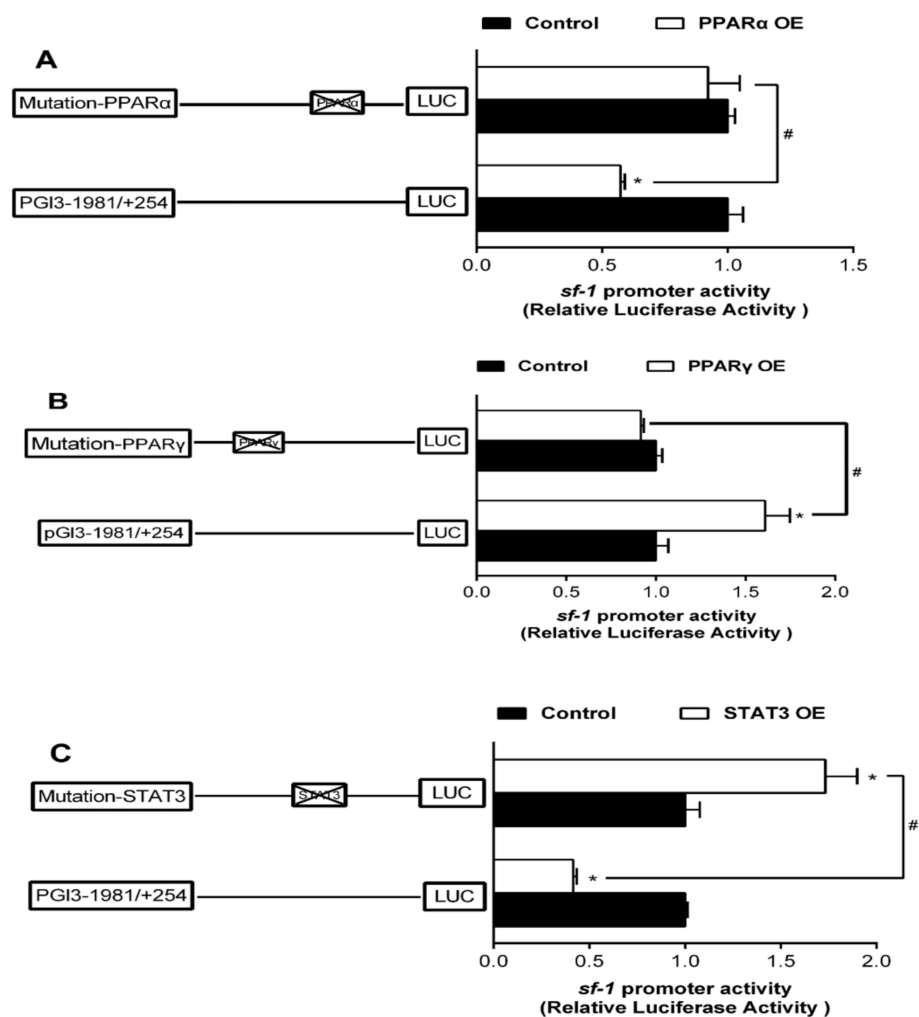


Figure 6. Assays of *sf-1* promoter activities by mutagenesis on predicted PPAR α , PPAR γ , and STAT3 binding sites at 24 h. (A) Site mutagenesis of PPAR α on $-1981/+254$ *sf-1* promoter; (B) Site mutagenesis of PPAR γ on $-1981/+254$ *sf-1* promoter; (C) Site mutagenesis of STAT3 on $-1981/+254$ *sf-1* promoter. Values are presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences between PPAR α , PPAR γ , and STAT3 overexpression and the control ($p < 0.05$). Hash symbol (#) indicates significant differences between two 5' unidirectional deletion plasmids under the same treatment ($p < 0.05$).

Overexpressed PPAR α significantly reduced the *cyp17 α* promoter activity by 48% compared to the control, whereas mutation of *cyp17 α -PPAR α* ($-881/-864$ bp) site significantly enhanced *sf-1* promoter activity, suggesting that *cyp17 α -PPAR α* site ($-881/-864$ bp) down-regulated PPAR α -induced *cyp17 α* transcription (Figure 7A). Overexpressed PPAR γ significantly increased *sf-1* promoter activity, and mutation of *cyp17 α -PPAR γ* ($-89/-70$ bp) site completely abolished the stimulatory effect of PPAR γ , indicating that *cyp17 α -PPAR γ* ($-89/-70$ bp) site played a positive regulatory role in *cyp17 α* transcription (Figure 7B). Mutation of *cyp17 α -STAT3* ($-1669/-1660$ bp) significantly down-regulated STAT3-induced increase in *cyp17 α* promoter activity, suggesting that *cyp17 α -STAT3* up-regulated STAT3-induced *cyp17 α* transcription (Figure 7C).

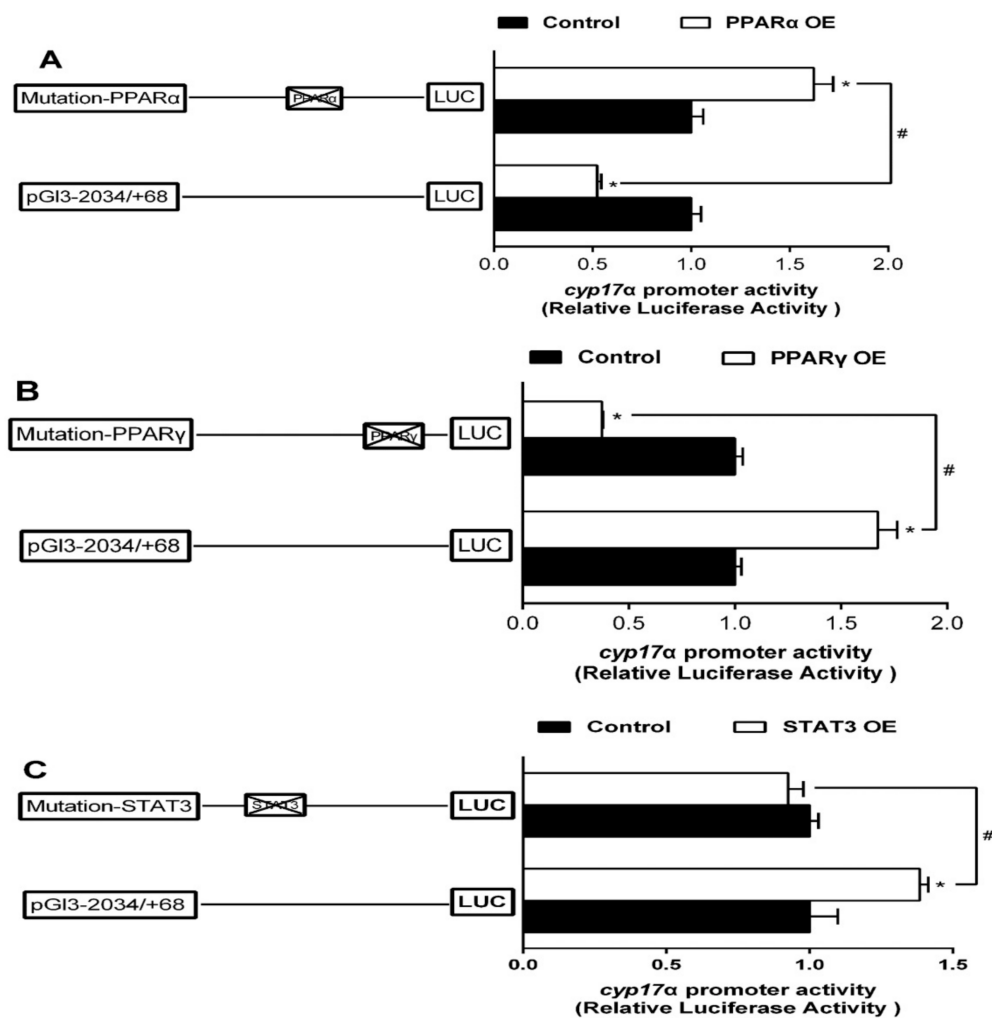


Figure 7. Assays of *cyp17 α* promoter activities of mutagenesis on predicted PPAR α , PPAR γ , and STAT3 binding sites at 24 h. **(A)** Site mutagenesis of PPAR α on $-2034/+68$ *cyp17 α* promoter; **(B)** Site mutagenesis of PPAR γ on $-2034/+68$ *cyp17 α* promoter; **(C)** Site mutagenesis of STAT3 on $-2034/+68$ *cyp17 α* promoter. Values are presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences between PPAR α , PPAR γ , and STAT3 overexpression and the control ($p < 0.05$). Hash symbol (#) indicates significant differences between two 5' unidirectional deletion plasmids under the same treatment ($p < 0.05$).

To further determine whether the leptin-induced increase in *sf-1* expression could be mediated by the STAT3 or PPAR γ , we performed the site-directed mutation at the regions of *sf-1* promoter and used leptin to incubate. Leptin incubation significantly increased *sf-1* promoter activity. However, compared to the wild type pGI3-1981/+254 vector, the leptin-induced stimulatory effect was completely abolished after the mutation of *sf-1*-PPAR γ ($-1396/-1388$ bp) site, *sf-1*-STAT3 ($-1106/-1096$ bp) site or *sf-1*-PPAR γ &STAT3 ($-1396/-1388$ bp and $-1106/-1096$ bp) sites, respectively (Figure 8).

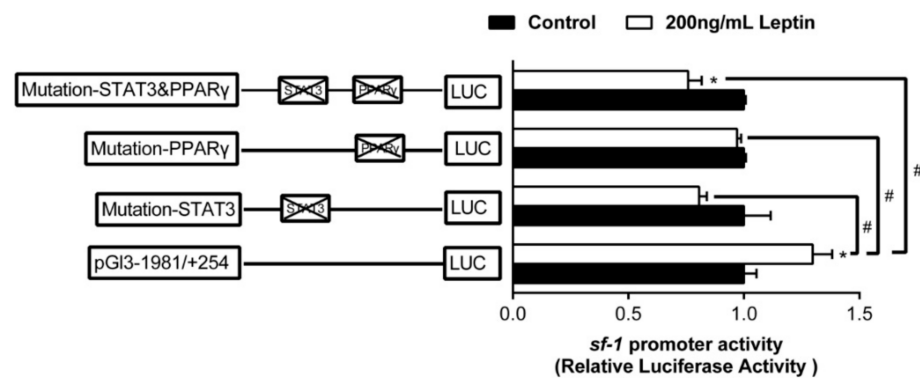


Figure 8. Assays of sf-1 promoter activity of site mutagenesis on predicted PPAR γ and STAT3 after leptin treatment at 24 h. Values are presented as mean \pm SEM ($n = 3$ independent biological experiments). Asterisk (*) indicates significant differences between different treatments in the same plasmid ($p < 0.05$). Hash symbol (#) indicates significant differences between different 5' unidirectional deletion plasmids under the same treatment ($p < 0.05$).

2.5. EMSA Analysis of Binding Sequence of Transcription Factors

We further used EMSA assays to explore whether PPAR α , PPAR γ , and STAT3 directly interact with sf-1 and cyp17 α promoters. We made a series of probes by using biotin to label the sf-1-PPAR α (−413/−396 bp), sf-1-PPAR γ (−1396/−1388 bp), sf-1-STAT3 (−1106/−1096 bp), cyp17 α -PPAR α (−881/−864 bp), cyp17 α -PPAR γ (−89/−70 bp), and cyp17 α -STAT3 (−1669/−1660 bp)-sequence, respectively. The results indicated that the sf-1-PPAR α (−413/−396 bp) and sf-1-PPAR γ (−1396/−1388 bp)-sequence did not compete for binding with the nuclear protein (Lane 3, Figure 9A,B), indicating that the sf-1-PPAR α (−413/−396 bp) site and the sf-1-PPAR γ (−1396/−1388 bp) site could not bind with the nuclear protein. For the sf-1-STAT3 site of sf-1 promoter, the unlabeled sf-1-STAT3 (−1106/−1096 bp)-sequence competed for the labeled probe (Lane 3, Figure 9C). Meanwhile, STAT3 treatment reduced the brightness of bands (Lane 5, Figure 9C), suggesting that the sf-1-STAT3 (−1106/−1096 bp) site could interact with sf-1 promoter and STAT3 weakened the binding process between STAT3 and the sf-1-STAT3 (−1106/−1096 bp) site of sf-1 promoter. Similarly, the unlabeled cyp17 α -PPAR α and cyp17 α -STAT3 sites of cyp17 α promoter did not compete for the labeled probe (Lane 3, Figure 9D,F), indicating that cyp17 α -PPAR α (−881/−864 bp) site and cyp17 α -STAT3 (−1669/−1660 bp) site of cyp17 α promoter were not bound with PPAR α and STAT3, respectively. In addition, the unlabeled cyp17 α -PPAR γ (−89/−70 bp) site competed for the labeled probe (Lane 3, Figure 9E), while PPAR γ treatment markedly increased the brightness of the binding band (Lane 5, Figure 9E), confirming that cyp17 α -PPAR γ (−89/−70 bp) was a binding site for PPAR γ and PPAR γ promoted the binding process between cyp17 α -PPAR γ and this binding site on cyp17 α promoter. Taken together, our results demonstrated that sf-1 was the target gene of STAT3, and cyp17 α was the target gene of PPAR γ .

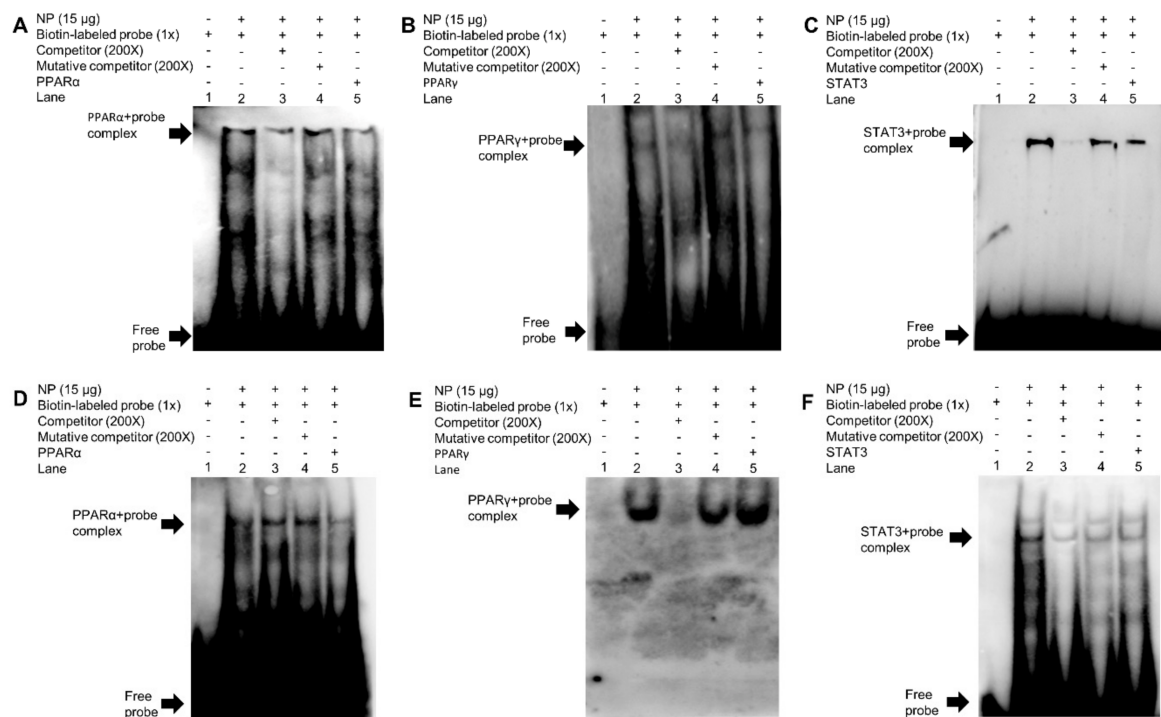


Figure 9. EMSA analysis of predicted PPAR α , PPAR γ , and STAT3 binding sf-1 and cyp17 α promoters. (A) –413/–396 bp PPAR α binding site of sf-1 (sf-1-PPAR α); (B) –1396/–1388 bp PPAR γ binding site of sf-1 (sf-1-PPAR γ); (C) –1106/–1096 bp STAT3 binding site of sf-1 (sf-1-STAT3); (D) –881/–864 bp PPAR α binding site of cyp17 α (cyp17 α -PPAR α); (E) –89/–70 bp PPAR γ binding site of cyp17 α (cyp17 α -PPAR γ); (F) –1669/–1660 bp STAT3 binding site of cyp17 α (cyp17 α -STAT3). NP represents nuclear protein.

3. Discussion

In mammals, studies have indicated that the transcription of steroidogenic enzymes and steroid hormone receptors were mediated by transcription factor PPARs and STAT3 [12,26], but the direct molecular evidences were absent. The present study, for the first time, characterized the sequences of sf-1 and cyp17 α promoters and elucidated their transcriptional regulatory mechanism in responses to PPAR α , PPAR γ , and STAT3.

In eukaryotes, the identification of the core promoter is the first step for exploring the mechanism of transcriptional initiation [27]. In the present study, the core promoters of sf-1 and cyp17 α had different structures. For example, sf-1 had CCAAT box but no GC box, while cyp17 α had GC box but no CCAAT box. Studies suggested that CCAAT box is the potential element that interacts with nuclear NF- κ B on the sf-1 promoter [7,8]. The present study found that the proximal region of cyp17 α promoter had TATA box but the proximal region of sf-1 promoter did not contain the TATA box, in agreement with other reports [2,7]. Usually, TATA-less promoters possessed various SP1 binding sites in their promoter regions [28] and similar result was found in the proximal promoter region of sf-1 in the present study. Wierstra [29] reported that SP1 directly bound with GC-rich domains and modulated transcription after various stimuli. Interestingly, we also found that the average activity of sf-1 promoter was higher than that of the cyp17 α promoter. Thus, we speculated that SP1-rich and CCAAT-rich regions probable positively regulated promoter activity. In mammals, Scherrer et al. [8] reported that SP1 and CCAAT box positively regulated sf-1 promoter activity.

Identification of transcription factor binding sites (TFBS) plays an important role in deciphering the mechanisms of gene regulation [30]. PPAR α and PPAR γ are the two important nuclear transcription factors that regulate steroidogenesis [31,32]. In the present study, we found that PPAR α significantly decreased the transcriptional activity of sf-1 and cyp17 α promoter, while PPAR γ up-regulated sf-1 and cyp17 α transcription. These results

indicated that PPAR α and PPAR γ differentially regulated steroidogenesis by targeting the sf-1 and cyp17 α in yellow catfish. However, the EMSA analysis showed that PPAR α and PPAR γ sites from the sf-1 promoter were not functional, but PPAR γ site from the cyp17 α promoter was functional binding site, suggesting that PPAR α and PPAR γ indirectly mediated transcriptional activity of sf-1 and that PPAR γ directly mediated transcriptional activity of cyp17 α . STAT3 is considered the most important factor that transmits the leptin signal [21,22]. The activated STAT3 is capable of translocating to the nucleus to regulate gene transcription [33], and responsible for steroidogenic secretion [34]. In the present study, we found that overexpression of STAT3 negatively regulate sf-1 promoter activity. Similarly, studies suggested that STAT3 negatively regulated expression of sf-1 [35,36]. Furthermore, we also found that STAT3 positively regulated cyp17 α promoter activity in yellow catfish. Roumaud and Martin [36] suggested that STAT3 negatively regulated expression of the cytochrome p450 side chain cleavage enzyme (p450scc) in leydig cells. However, the EMSA analysis showed that only STAT3 could bind with the sf-1 promoter but not with the cyp17 α , suggesting that STAT3 directly mediated transcriptional activity of sf-1 and but indirectly mediated transcriptional activity of cyp17 α .

Fish leptin is largely diverged from mammalian and it is well-known that the amino acid identity between yellow catfish leptin and human leptin is markedly low (almost 23%). However, as far as we know, fish leptin is hardly available commercially at present. Therefore, recombinant human leptin has widely been used to investigate the function of fish leptin [22,23,37,38]. Thus, in the present study, recombinant human leptin was used to study promoters function of sf-1 and cyp17 α in yellow catfish. Leptin is known to have a significant impact on steroidogenesis in mammals. For example, Dhillon et al. [39] suggested that leptin directly activated sf-1 in mice. Similarly, our study indicated that 200 ng/mL leptin treatment up-regulated the transcription activity of sf-1 promoter indicated that leptin might regulate sf-1 promoter activity through STAT3 or PPAR γ sites in yellow catfish. However, as another possibility, the obtained results in this study might be due to a pharmacological action of the “human” leptin [40,41]. Furthermore, we found that mutation of STAT3 binding site suppressed the leptin-induced increase in luciferase activity, suggesting that leptin might regulate sf-1 promoter activity through STAT3 site in yellow catfish. In addition, we also found that both of the mutation of STAT3 binding site and PPAR γ binding site suppressed the leptin-induced increase in luciferase activity, suggesting that both STAT3 and PPAR γ binding sites positively mediated sf-1 transcriptional response to leptin. Landry et al. [13] also suggested that the expression of steroidogenic genes was regulated by leptin through STAT in MA-10 leydig cells. Moreover, Reshma et al. [42] demonstrated a stimulatory effect of leptin on the mRNA expression of sf-1 in the ovarian tissues of Water Buffalo. However, we found that leptin had no effect on cyp17 α promoter. To our best knowledge, currently no reports were available on the effects of leptin incubation on cyp17 α expression and activity, and accordingly further investigation was needed to elucidate the mechanism.

In summary, the present study cloned and identified the different structure and function of sf-1 and cyp17 α promoters. We found that three transcription factors (PPAR α , PPAR γ , and STAT3) regulated the transcription activities of sf-1 and cyp17 α promoters and that the transcriptional activation of sf-1 was mediated by STAT3 and PPAR γ under leptin signal.

4. Materials and Methods

Ethical statement: All animal experiments followed the ethical guidelines of Huazhong Agricultural University (HZAU) and were approved by the Animal Experimentation Ethics Committee of our university (Wuhan, Hubei, China) (identification code: Fish-2018-0827, Date: 27 August 2018).

4.1. Experimental Animals and Reagents

The yellow catfish individuals used for DNA and RNA extraction were bought from a local commercial farm (Wuhan, China). HEK293T cell lines were obtained from the Cell Resource Center of Fishery College of HZAU. Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS) and 0.25% trypsin-EDTA were purchased from Gibco (USA). Recombinant human leptin (HPLC class), Penicillin, streptomycin and other reagents were purchased from Sigma (USA).

4.2. Promoter Cloning and Plasmids Construction

We identified the 5' complementary DNA (cDNA) sequences and the (TSS) of *sf-1* and *cyp17 α* genes of yellow catfish by RNA ligase-mediated rapid amplification of 5' cDNA ends (RLM-5'RACE) method. The protocols of promoter cloning following those in Xu et al. [43]. For generating the luciferase reporter constructs, we subcloned different plasmids with *sf-1* and *cyp17 α* promoters into pGl3-Basic vectors (Promega, USA) by using Sac I and Hind III restriction sites. The plasmids of PPAR α , PPAR γ , and STAT3 overexpression were subcloned into pcDNA3.1 (+) vector with FLAG-tag sequence inserted into the C-terminus of PPAR α , PPAR γ , and STAT3 sequences. We used ClonExpress II One Step Cloning Kit (Vazyme, Piscataway, NJ, USA) to ligate all of the products. Primers used in 5'RACE and plasmid construction are presented in Tables S1 and S2.

On the basis of the distance from its TSS, we named the plasmids as pGl3–1981/+254 *sf-1* promoter and pGl3–2034/+68 *cyp17 α* promoter, respectively. Using the templates of pGl3–1981/+254 of *sf-1* vector, we produced the plasmids of pGl3–489/+254, pGl3–921/+254, pGl3–1421/+254, and pGl3–1981/+254 of *sf-1*. Similarly, the plasmids of pGl3–330/+68, pGl3–687/+68, pGl3–1402/+68, and pGl3–2034/+68 of *cyp17 α* were generated through using pGl3–2034/+68 of *cyp17 α* vector as the template.

4.3. Sequence Analysis

We compared nucleotide sequences of *sf-1* and *cyp17 α* promoters with DNA sequences from the GenBank database. In order to analyze *sf-1* and *cyp17 α* promoters, we predicted putative TFBS by MatInspector online (<http://www.genomatix.de/>) and the JASPAR database (<http://jaspar.genereg.net/>). We conducted sequence alignments with the Clustal-W multiple alignment algorithm.

4.4. Plasmid Transfections and Assays of Luciferase Activities

We used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to transiently transfect the plasmids into HEK293T cells following the manufacturer's protocols. We used all reporter plasmids in equimolar amounts in Opti-MEM (Invitrogen, USA), and 20 ng pRL-TK were co-transfected as the control. At 4 h after the transfection, we replaced the transfection medium by DMEM (10% FBS) with or without 200 ng/mL leptin. The leptin concentrations were selected after the publications in our laboratory [22,37]. After 24 h incubation, we harvested cells to assay the luciferase activity by Dual-Luciferase Reporter Assay System (Promega, USA). The relative luciferase activities of these promoters were obtained by the ratio of Firefly to Renilla luciferase activity. We conducted all experiments in triplicates.

4.5. Protein Expression of Yellow Catfish PPAR α , PPAR γ , and STAT3 in HEK293T Cells

We used western blot to determine the protein expression of yellow catfish PPAR α , PPAR γ , and STAT3 in HEK293T cells based on the methods described in our studies [44,45]. The primary antibodies included rabbit polyclonal of anti-GAPDH (1:10,000, Abcam, Cambridge, UK) and anti-FLAG (1:10,000, Proteintech, Wuhan, China). We visualized the protein bands by Vilber Fusion FX6 Spectra imaging system (Vilber Lourmat, Collegen, France), and quantified them by Image-Pro Plus 6.0.

4.6. Site-Mutation Analysis of Binding Sites on the *sf-1* and *cyp17 α* Promoters

To identify the corresponding binding sites on the regions of *sf-1* and *cyp17 α* promoters, we used QuickChange II Site-Directed Mutagenesis Kit (Vazyme, Piscataway, NJ, USA) to perform site-directed mutagenesis analysis. pGI3-*sf-1*-1981 and pGI3-*cyp17 α* -2034 were used as templates, respectively. The primers for mutagenesis are shown in Table S3. We name these mutant constructs as Mut-*sf-1*-PPAR α , Mut-*sf-1*-PPAR γ , Mut-*sf-1*-STAT3, Mut-*sf-1*-PPAR γ &STAT3, Mut-*cyp17 α* -PPAR α , Mut-*cyp17 α* -PPAR γ , and Mut-*cyp17 α* -STAT3, respectively. Then we used Lipofectamine 2000 (Invitrogen) to co-transfect the plasmids and pRL-TK into HEK293T cells. After 4 h, we replaced the transfection medium by DMEM (10% FBS) with or without 200 ng/mL leptin. After 24 h incubation, we harvested cells to perform the analysis of the luciferase activity, based on the procedure described above.

4.7. Electrophoretic Mobility-Shift Assay (EMSA)

We conducted EMSA assays to explore the functional binding sites of PPAR α , PPAR γ , and STAT3 on the regions of *sf-1* and *cyp17 α* promoters, based on the methods of Xu et al. [43]. We transfected HEK293T cells with the same amount of pcDNA3.1, PPAR α , PPAR γ , and STAT3 plasmid, respectively. We extracted the nuclear proteins from HEK293T cells and measured the concentrations of proteins by the BCA method. All the oligonucleotide sequences for EMSA analysis are presented in Table S4.

4.8. Statistical Analysis

We used SPSS 19.0 software for all these statistical analysis. All of these data were expressed as means \pm SEM (standard errors of means). Before statistical analysis, we evaluated all data for normality using the Shapiro–Wilk test. In order to test the homogeneity of variances, we performed Bartlett’s test. We analyzed data with one-way ANOVA and Duncan’s multiple or Student’s t-test where appropriate. Significant level was $p < 0.05$.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1422-0067/22/1/195/s1>, Figure S1: Nucleotide sequence of yellow catfish *sf-1* promoter. Numbers are relative to the transcription start site (+1). The putative transcription factor binding sites are underlined. The highlighted sequences show putative transcription factor binding sites, Figure S2: Nucleotide sequence of yellow catfish *cyp17 α* promoter. Numbers are relative to the transcription start site (+1). The putative transcription factor binding sites are underlined. The highlighted sequences show putative transcription factor binding sites, Table S1: Primers used for *sf-1* and *cyp17 α* promoters cloning and 5'-deletion plasmids construction in yellow catfish, Table S2: Primers used for overexpression plasmids of yellow catfish PPAR α , PPAR γ and STAT3 into pcDNA3.1 (+) vector, Table S3: Primers used for site-mutation analysis, Table S4: Primers used for electrophoretic mobility-shift assays.

Author Contributions: W.-H.L.: Investigation, Methodology, Writing—original draft. G.-H.C. and M.-Q.Z.: Methodology, Writing—review and editing. Y.-H.X. and Y.-C.X.: Methodology, Writing—review and editing. X.-Y.T.: Funding acquisition, Project administration, Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by National Natural Science Foundation of China (grant no.: 31572605) and by the National Key R&D Program of China (Grant No. 2018YFD0900400).

Acknowledgments: We thank all the staffs in Laboratory of Molecular Nutrition for Aquatic Economic Animals, Fishery College, Huazhong Agricultural University, for their help during the experiments.

Conflicts of Interest: The authors disclosed no conflict of interest.

References

1. Tokarz, J.; Möller, G.; de Angelis, M.H.; Adamski, J. Steroids in teleost fishes: A functional point of view. *Steroids* **2015**, *103*, 123–144. [[CrossRef](#)]
2. Payne, A.H.; Hales, D.B. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr. Rev.* **2004**, *25*, 947–970. [[CrossRef](#)] [[PubMed](#)]
3. Plummer, S.; Sharpe, R.M.; Hallmark, N.; Mahood, I.K.; Elcombe, C. Time-dependent and compartment-specific effects of in utero exposure to di (n-butyl) phthalate on gene/protein expression in the fetal rat testis as revealed by transcription profiling and laser capture microdissection. *Toxicol. Sci.* **2007**, *97*, 520–532. [[CrossRef](#)] [[PubMed](#)]
4. Xie, Q.P. Steroidogenic Factor 1 (SF-1) Is Essential for Steroidogenesis, Gonadal Development and Sex Differentiation in Nile Tilapia. Ph.D. Thesis, Southwest University, Chongqing, China, 2016.
5. Qin, F.; Shen, T.; Li, J.; Qian, J.; Zhang, J.; Zhou, G.; Tong, J. SF-1 mediates reproductive toxicity induced by Cerium oxide nanoparticles in male mice. *J. Nanobiotechnol.* **2019**, *17*, 41. [[CrossRef](#)] [[PubMed](#)]
6. Thitiphuree, T.; Nagasawa, K.; Osada, M. Molecular identification of steroidogenesis-related genes in scallops and their potential roles in gametogenesis. *J. Steroid. Biochem. Mol. Biol.* **2019**, *186*, 22–33. [[CrossRef](#)] [[PubMed](#)]
7. Woodson, K.G.; Crawford, P.A.; Sadovsky, Y.; Milbrandt, J. Characterization of the promoter of SF-1, an orphan nuclear receptor required for adrenal and gonadal development. *Mol. Endocrinol.* **1997**, *11*, 117–126. [[CrossRef](#)]
8. Scherrer, S.P.; Rice, D.A.; Heckert, L.L. Expression of steroidogenic factor 1 in the testis requires an interactive array of elements within its proximal promoter. *Biol. Reprod.* **2002**, *67*, 1509–1521. [[CrossRef](#)]
9. Meinsohn, M.C.; Smith, O.E.; Bertolin, K.; Murphy, B.D. The orphan nuclear receptors steroidogenic factor-1 and liver receptor homolog-1: Structure, regulation, and essential roles in mammalian reproduction. *Physiol. Rev.* **2019**, *99*, 1249–1279. [[CrossRef](#)]
10. Puttabyatappa, M.; Vande Voort, C.A.; Chaffin, C.L. hCG-induced down-regulation of PPAR γ and liver X receptors promotes periovulatory progesterone synthesis by macaque granulosa cells. *Endocrinology* **2010**, *151*, 5865–5872. [[CrossRef](#)]
11. Gatta, V.; Tatone, C.; Ciriminna, R.; Vento, M.; Franchi, S.; d’Aurora, M.; Stuppia, L. Gene expression profiles of cumulus cells obtained from women treated with recombinant human luteinizing hormone+ recombinant human follicle-stimulating hormone or highly purified human menopausal gonadotropin versus recombinant human follicle-stimulating hormone alone. *Fertil. Steril.* **2013**, *99*, 2000–2008.
12. Tatone, C.; Benedetti, E.; Vitti, M.; Di Emidio, G.; Ciriminna, R.; Vento, M.E.; Cela, V.; Borzi, P.; Certa, G.; Lispi, M.; et al. Modulating intrafollicular hormonal milieu in controlled ovarian stimulation: Insights from PPAR expression in human granulosa cells. *J. Cell. Physiol.* **2016**, *231*, 908–914. [[CrossRef](#)] [[PubMed](#)]
13. Landry, D.A.; Sormany, F.; Haché, J.; Roumaud, P.; Martin, L.J. Steroidogenic genes expressions are repressed by high levels of leptin and the JAK/STAT signaling pathway in MA-10 Leydig cells. *Mol. Cell. Biochem.* **2017**, *433*, 79–95. [[CrossRef](#)]
14. Seroussi, E.; Knytl, M.; Pitel, F.; Elleder, D.; Krylov, V.; Leroux, S.; Morisson, M.; Yosefi, S.; Miyara, S.; Ganesan, S.; et al. Avian Expression Patterns and Genomic Mapping Implicate Leptin in Digestion and TNF in Immunity, Suggesting That Their Interacting Adipokine Role Has Been Acquired Only in Mammals. *Int. J. Mol. Sci.* **2019**, *20*, 4489. [[CrossRef](#)] [[PubMed](#)]
15. Swain, J.E.; Dunn, R.L.; Daniel, M.C.; Janis, G.M.; Smith, G.D. Direct effects of leptin on mouse reproductive function: Regulation of follicular, oocyte, and embryo development. *Biol. Reprod.* **2004**, *71*, 1446–1452. [[CrossRef](#)] [[PubMed](#)]
16. Kumar, P.A.; Sivakumar, A.V.N.; Pathipati, D.; Chakravarthi, V.P.; Brahmaiah, K.V.; Rao, V.H. Leptin induced in vitro development of ovarian follicles in sheep is related to the expression of P450 aromatase and steroidogenesis. *Theriogenology* **2019**, *136*, 1–6. [[CrossRef](#)] [[PubMed](#)]
17. Spicer, L.J.; Francisco, C.C. The adipose obese gene product, leptin: Evidence of a direct inhibitory role in ovarian function. *Endocrinology* **1997**, *138*, 3374–3379. [[CrossRef](#)] [[PubMed](#)]
18. Agarwal, S.K.; Vogel, K.; Weitsman, S.R.; Magoffin, D.A. Leptin antagonizes the insulin-like growth factor- α augmentation of steroidogenesis in granulosa and theca cells of the human ovary. *J. Clin. Endocr. Metab.* **1999**, *84*, 1072–1076. [[CrossRef](#)]
19. Ruiz-Cortes, Z.T.; Martel-Kennes, Y.; Nicolas, Y.G.; Downey, B.R.; Murphy, B.D. Biphasic effects of leptin in porcine granulosa cells. *Biol. Reprod.* **2003**, *68*, 789–796. [[CrossRef](#)]
20. Gregoraszczyk, E.L.; Rak, A. Superactive human leptin antagonist reverses leptin-induced excessive progesterone and testosterone secretion in porcine ovarian follicles by blocking leptin receptors. *J. Physiol. Pharmacol.* **2015**, *66*, 39–46.
21. Bates, S.H.; Stearns, W.H.; Dundon, T.A.; Schubert, M.; Tso, A.W.; Wang, Y.; Neel, B.G. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* **2003**, *421*, 856–859. [[CrossRef](#)]
22. Wu, K.; Tan, X.Y.; Xu, Y.H.; Chen, Q.L.; Pan, Y.X. JAK and STAT members of yellow catfish *Pelteobagrus fulvidraco* and their roles in leptin affecting lipid metabolism. *Gen. Comp. Endocrinol.* **2016**, *226*, 14–26. [[CrossRef](#)] [[PubMed](#)]
23. Wu, K.; Tan, X.Y.; Xu, Y.H.; Chen, G.H.; Zhuo, M.Q. Functional Analysis of Promoters of Genes in Lipid Metabolism and Their Transcriptional Response to STAT3 under Leptin Signals. *Genes* **2018**, *9*, 334. [[CrossRef](#)] [[PubMed](#)]
24. Solberg, N.; Krauss, S. Luciferase assay to study the activity of a cloned promoter DNA fragment. *Methods Mol. Biol.* **2013**, *977*, 65–78. [[PubMed](#)]
25. Delhove, J.M.K.M.; Karda, R.; Hawkins, K.E.; FitzPatrick, L.M.; Waddington, S.N.; McKay, T.R. Bioluminescence monitoring of promoter activity in vitro and in vivo. *Methods Mol. Biol.* **2017**, *1651*, 49–64. [[PubMed](#)]
26. De Miguel, F.; Lee, S.O.; Onate, S.A.; Gao, A.C. Stat3 enhances transactivation of steroid hormone receptors. *Nucl. Recept.* **2003**, *1*, 3. [[CrossRef](#)]

27. Goodrich, J.A.; Tjian, R. 2010. Unexpected roles for core promoter recognition factors in cell-type-specific transcription and gene regulation. *Nat. Rev. Genet.* **2010**, *11*, 549–558. [[CrossRef](#)]
28. Smale, S.T.; Kadonaga, J.T. The RNA polymerase II core promoter. *Annu. Rev. Biochem.* **2003**, *72*, 449–479. [[CrossRef](#)]
29. Wierstra, I. Sp1: Emerging roles-beyond constitutive activation of TATA-less housekeeping genes. *Biochem. Biophys. Res. Commun.* **2008**, *372*, 1–13. [[CrossRef](#)]
30. Chan, T.M.; Leung, K.S.; Lee, K.H. TFBS identification based on genetic algorithm with combined representations and adaptive post-processing. *Bioinformatics* **2007**, *24*, 341–349. [[CrossRef](#)]
31. Lovekamp-Swan, T.; Davis, B.J. Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ. Health Perspect.* **2003**, *111*, 139–145. [[CrossRef](#)]
32. Kurzynska, A.; Bogacki, M.; Chojnowska, K.; Bogacka, I. Peroxisome proliferator activated receptor ligands affect progesterone and 17 β -estradiol secretion by porcine corpus luteum during early pregnancy. *J. Physiol. Pharmacol.* **2014**, *65*, 709–717. [[PubMed](#)]
33. Gorissen, M.; de Vrieze, E.; Flik, G.; Huising, M.O. STAT genes display differential evolutionary rates that correlate with their roles in the endocrine and immune system. *J. Endocrinol.* **2011**, *209*, 175–184. [[CrossRef](#)] [[PubMed](#)]
34. Arias-Álvarez, M.; García-García, R.M.; Torres-Rovira, L.; Gonzalez-Bulnes, A.; Rebollar, P.G.; Lorenzo, P.L. Influence of leptin on in vitro maturation and steroidogenic secretion of cumulus-oocyte complexes through JAK2/STAT3 and MEK 1/2 pathways in the rabbit model. *Reproduction* **2010**, *139*, 523–532. [[CrossRef](#)] [[PubMed](#)]
35. Phillips, K.P.; Tanphaichitr, N. Mechanisms of obesity-induced male infertility. *Expert. Rev. Endocrinol. Metab.* **2010**, *5*, 229–251. [[CrossRef](#)] [[PubMed](#)]
36. Roumaud, P.; Martin, L.J. Roles of leptin, adiponectin and resistin in the transcriptional regulation of steroidogenic genes contributing to decreased Leydig cells function in obesity. *Horm. Mol. Biol. Clin. Investig.* **2015**, *24*, 25–45. [[CrossRef](#)] [[PubMed](#)]
37. Song, Y.F.; Wu, K.; Tan, X.Y.; Zhang, L.H.; Zhuo, M.Q.; Pan, Y.X.; Chen, Q.L. Effects of recombinant human leptin administration on hepatic lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*: In vivo and in vitro studies. *Gen. Comp. Endocrinol.* **2015**, *212*, 92–99. [[CrossRef](#)]
38. Song, Y.F.; Tan, X.Y.; Pan, Y.X.; Zhang, L.H.; Chen, Q.L. 2018. Fatty acid β -oxidation is essential in leptin-mediated oocytes maturation of yellow catfish *Pelteobagrus fulvidraco*. *Int. J. Mol. Sci.* **2018**, *19*, 1457. [[CrossRef](#)]
39. Dhillon, H.; Zigman, J.M.; Ye, C.; Lee, C.E.; Lowell, B.B. Leptin directly activates sf1 neurons in the vmh, and this action by leptin is required for normal body-weight homeostasis. *Neuron* **2006**, *49*, 191–203. [[CrossRef](#)]
40. Hausman, G.J.; Barb, C.R.; Lents, C.A. Leptin and reproductive function. *Biochimie* **2012**, *94*, 2075–2081. [[CrossRef](#)]
41. Broughton, D.E.; Moley, K.H. Obesity and female infertility: Potential mediators of obesity's impact. *Fertil. Steril.* **2017**, *107*, 840–847. [[CrossRef](#)]
42. Reshma, R.; Mishra, S.R.; Thakur, N.; Parmar, M.S.; Somal, A.; Bharti, M.K.; Pandey, S.; Chouhan, V.S.; Verma, M.R.; Singh, G.; et al. Modulatory role of leptin on ovarian functions in water buffalo (*bubalus bubalis*). *Theriogenology* **2016**, *86*, 1720–1739. [[CrossRef](#)] [[PubMed](#)]
43. Xu, Y.H.; Luo, Z.; Wu, K.; Fan, Y.F.; You, W.J.; Zhang, L.H. Structure and functional analysis of promoters from two liver isoforms of CPT I in grass carp *Ctenopharyngodon idella*. *Int. J. Mol. Sci.* **2017**, *18*, 2405. [[CrossRef](#)]
44. Wei, C.C.; Luo, Z.; Hogstrand, C.; Xu, Y.H.; Wu, L.X.; Chen, G.H.; Song, Y.F. Zinc reduces hepatic lipid deposition and activates lipophagy via Zn²⁺/MTF-1/PPAR α and Ca²⁺/CaMKK β /AMPK pathways. *FASEB J.* **2018**, *32*, 6666–6680. [[CrossRef](#)] [[PubMed](#)]
45. Zhao, T.; Wu, K.; Hogstrand, C.; Xu, Y.H.; Chen, G.H.; Wei, C.C.; Luo, Z. Lipophagy mediated carbohydrate-induced changes of lipid metabolism via oxidative stress, endoplasmic reticulum (ER) stress and ChREBP/PPAR γ pathways. *Cell. Mol. Life Sci.* **2020**, *77*, 1987–2003. [[CrossRef](#)] [[PubMed](#)]