The Glucocorticoid Receptor Regulates the ANGPTL4 Gene in a CTCF-Mediated Chromatin Context in Human Hepatic Cells

Masafumi Nakamoto1,2, Ko Ishihara3*, Takehisa Watanabe1, Akiyuki Hirosue1,2, Shinjiro Hino1, Masanori Shinohara2, Hideki Nakayama2, Mitsuyoshi Nakao1,4*

1 Department of Medical Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan, 2 Department of Oral and Maxillofacial Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan, 3 Priority Organization for Innovation and Excellence, Kumamoto University, Kumamoto, Japan, 4 Core Research for Evolutionary Science and Technology (CREST), Japan Agency for Medical Research and Development, Tokyo, Japan

* mnakao@gpo.kumamoto-u.ac.jp (MN); kishihar@kumamoto-u.ac.jp (KI)

Abstract

Glucocorticoid signaling through the glucocorticoid receptor (GR) plays essential roles in the response to stress and in energy metabolism. This hormonal action is integrated to the transcriptional control of GR-target genes in a cell type-specific and condition-dependent manner. In the present study, we found that the GR regulates the angiopoietin-like 4 gene (ANGPTL4) in a CCCTC-binding factor (CTCF)-mediated chromatin context in the human hepatic HepG2 cells. There are at least four CTCF-enriched sites and two GR-binding sites within the ANGPTL4 locus. Among them, the major CTCF-enriched site is positioned near the ANGPTL4 enhancer that binds GR. We showed that CTCF is required for induction and subsequent silencing of ANGPTL4 expression in response to dexamethasone (Dex) and that transcription is diminished after long-term treatment with Dex. Although the ANGPTL4 locus maintains a stable higher-order chromatin conformation in the presence and absence of Dex, the Dex-bound GR activated transcription of ANGPTL4 but not that of the neighboring three genes through interactions among the ANGPTL4 enhancer, promoter, and CTCF sites. These results reveal that liganded GR spatiotemporally controls ANGPTL4 transcription in a chromosomal context.

Introduction

The glucocorticoid receptor (GR) is a member of a family of transcription factors that regulate biological processes, such as basal and stress-associated homeostasis, energy metabolism, and the immune response in a cell-type and condition-dependent manner [1, 2]. In the absence of ligand, GR is present in the cytoplasm in a complex with chaperons such as heat-shock proteins. Upon ligand-induced activation, GR dissociates from the complex and translocates to the nucleus, typically by binding to the glucocorticoid response elements (GREs) to activate or repress transcription of target genes. After the gene control, GR dissociates from its ligand or is degraded [2].
Although evidence indicates that the GR regulates gene expression through binding to promoter regions [3–5], recent genome-wide studies reveal that the GR mainly binds to distal enhancer regions [6] to regulate target-gene activity through long-range interactions between the promoter and enhancer [7–9]. The GR target Lcn2 encoding the acute-phase protein lipocalin-2 is co-regulated through long-range interactions with Ciz1 located approximately 30-kb upstream from the Lcn2 GRE [7]. Further, genomic interaction profiling revealed that numerous GREs interact with the Lcn2 GRE before GR binding [10]. Moreover, the contact loci were enriched in DNase I-hypersensitive sites, including the consensus motif for the CCCTC-binding factor (CTCF). FKBP51, which encodes the GR chaperon FKBP51, is regulated by GR-responsive enhancers far from the transcription start site [9]. FKBP51 and these enhancers are bordered by CTCF-binding sites, which likely contribute to long-range interactions and loop formation. However, whether CTCF contributes to the regulation of GR target genes remains to be determined.

The angiopoietin-like 4 gene (ANGPTL4) is a primary target of GR and is dominantly expressed in the liver and adipose tissue [11–16]. ANGPTL4 is a secreted protein that inhibits extracellular lipoprotein lipase (LPL), which hydrolyzes triglycerides (TGs) to free fatty acids and glycerol [17–19]. Plasma TG levels are reduced in ANGPTL4-null mice and are increased in mice that overexpress ANGPTL4 in the liver [20]. ANGPTL4-null mice exhibit reductions in glucocorticoid excess-induced changes associated with hypertriglyceridemia, hepatic steatosis, and visceral obesity [15]. These findings establish the role of ANGPTL4 in GR-mediated lipid metabolism. However, the mechanism of the regulation of ANGPTL4 by CTCF as well as GR has not been investigated.

Higher-order chromosome conformations, such as chromatin looping, mediate long-range physical interactions between distal regulatory elements and their target genes [21]. The chromatin insulator is a genomic boundary element that controls enhancer activity and the formation of chromatin loops [22]. CTCF is an insulator-binding protein that cooperates with the cohesin complex and other chromatin proteins [23–27]. Genome-wide studies show that CTCF binds several tens of thousands of sites in the mammalian genome. Approximately 50% of the CTCF-binding sites reside within intergenic regions, and the others are present near promoters and within gene bodies [28, 29]. Moreover, CTCF-binding sites are frequently located on the border between transcriptionally active and repressed genes and between different histone modification domains [30]. Although most CTCF-binding sites are conserved among tissues, some of the cell and tissue type-specific sites overlap with transcriptional enhancers, suggesting that CTCF adjusts the interaction between the enhancer element and promoter [29]. For example, CTCF/cohesin-mediated higher-order chromatin mediates basal and inducible gene expression [31–34].

Here, we report that GR regulates ANGPTL4 in a CTCF-mediated chromatin context in the human hepatic carcinoma cell line HepG2. We identified one GR-binding enhancer site adjacent to a liver-specific CTCF-enriched site. Moreover, in HepG2 cells treated with dexamethasone (Dex), CTCF was required for induction and subsequent silencing of ANGPTL4, likely via an enhancer–promoter interaction. Therefore, liganded GR can target ANGPTL4 but not three neighboring genes. Further, induction of ANGPTL4 transcription was decreased by long-term glucocorticoid treatment. These results indicate that liganded GR spatiotemporally controls the transcription of ANGPTL4 depending on the chromosomal context.

**Materials and Methods**

**Cell Culture**

Human hepatocellular carcinoma HepG2 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). HepG2 cells were cultured in DMEM
supplemented 10% (v/v) fetal bovine serum (FBS). The cells were treated with
100 nM Dex (Sigma-Aldrich) after the cells were cultured for 24 to 48 h in DMEM (Sigma-
Aldrich) containing 10% (v/v) dextran-coated charcoal (DCC)-treated FBS.

Quantitative Real-time PCR (qRT-PCR)
Total RNA was isolated from cultured cells with TRIzol (Invitrogen). For cDNA synthesis, 500
ng of total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription
Kit (Applied Biosystems), according to the manufacturer’s instructions. We used an ABI
Prism 7300 (Applied Biosystems) and SYBR Green to perform qRT-PCR. Each experiment
was performed at least three times. Expression levels were normalized to those of 36B4 mRNA
encoding ribosomal protein, large, P0. Primer sequences are listed in S1 Table.

Gene Knockdown
GL3 (control) and CTCF-specific siRNAs were used as previously reported [31]. The RAB11-
B-AS-siRNA sequence is as follows: 5´-GACCAAAUAACUA AUGAGA(dT)(dT)-3´ and 5´-UCUCAUUGUUUAGUC( dU)(dG)-3´. Cells were transfected for 48 h with siRNAs in the
presence of Lipofectamine RNAiMAX (Invitrogen).

Chromatin Immunoprecipitation Sequencing (ChIP-Seq)
HepG2 cells (2 × 10⁷) were treated with Dex (100 nM) for 3 h and then cross-linked with 1%
formaldehyde at room temperature for 10 min. The cells were lysed with the RIPA buffer sup-
plemented with protease inhibitors. The lysates were sonicated using a Branson Bioruptor to
yield 200–500 bp DNA fragments. ChIP was performed using anti-glucocorticoid receptor
(GR) antibodies (sc-8992; Santa Cruz Biotechnology, Inc.). DNA fragments were collected
using Dynabeads Protein A/G (Life Technologies). Purified DNA (20 ng) was used to con-
struct libraries for sequencing using an Ion Fragment Library Kit (Life Technologies). High-
throughput sequencing was performed using Proton semiconductor sequencers (Life Technolo-
gies) according to the manufacturer’s instructions. Proton sequence data were aligned to the
human reference genome hg19. Peaks were detected using the MACS algorithm included with
Avadis NGS software (Agilent Technologies). GR binding sites were detected according to a
cut-off value of P = 10⁻⁵ and their enrichment by a factor of 10 vs input. ChIP-seq data for
CTCF and histone modifications in HepG2 cells were obtained from the ENCODE/Broad
Institute via the UCSC Genome Browser website (http://genome.ucsc.edu/). Gene Expression
Omnibus (GEO) accession numbers are as follows: GSM733645, CTCF; GSM733743,
H3K27Ac; GSM798321, H3K4me1; and GSM733737, H3K4me3.

ChIP-qPCR Analysis
HepG2 cells were cross-linked with 1% formaldehyde at room temperature for 10 min.
Nuclear lysates were sonicated using the Bioruptor to yield DNA fragments of approximately
200–500 bp. ChIP was performed using the antibodies as follows: anti-CTCF (07–729, Milli-
pore), anti-GR (sc-8992, Santa Cruz Biotechnology, Inc.), anti-H3K27ac (ab4729, Abcam),
anti-RNA Pol II serine-5 phosphorylation (provided by Dr. Hiroshi Kimura). Rabbit IgG (sc-
2027, Santa Cruz Biotechnology, Inc.) served as a control. Purified DNA was used in quantita-
tive PCR analyses performed using an ABI Prism 7300 System (Applied Biosystems) and
SYBR Green. The threshold was set to intersect the linear segment of the PCR amplification
curve, and the number of cycles (Ct) required to reach the threshold was counted. We gener-
ated a standard curve using the input sample and calculated the enrichment by applying the Ct
value of each sample. Primer sequences are listed in S1 Table. ChIP analysis of RNA Pol II serine-5 phosphorylation included the addition of a phosphatase inhibitor to the reaction.

Chromosome Conformation Capture (3C) Assay

Formaldehyde cross-linked chromatin from HepG2 cells was digested with DpnII overnight, followed by ligation catalyzed by T4 DNA ligase at 16˚C for 1 h. To prepare standard curves, a bacterial artificial chromosome spanning the ANGPTL4 locus, RPCI11.C-978J4, was digested with Sau3AI, which is insensitive to Dam methylation, followed by random ligation. After reversing the cross-links, genomic DNA was purified using phenol extraction and ethanol precipitation. We used an ABI Prism 7300 (Applied Biosystems) and a Thunderbird SYBR qPCR Mix (Toyobo) to assess the ligated products. The 3C-qPCR data were normalized to the value of the loading control, using internal primers located within ANGPTL4. The relative frequencies of interactions between the reference and its physically adjacent site were normalized to 1. We used the Student t test to evaluate the results of more than three independent experiments. Primer sequences are listed in S1 Table.

Statistical Analysis

Differences between two groups were analyzed using the Student t test. To compare three groups, we performed ANOVA followed by Tukey-Kramer post hoc test. P < 0.05 indicates a statistically significant difference.

Data Availability

ChIP-seq datasets of GR are deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE85343.

Results

Distribution of CTCF and GR-enriched Sites within the ANGPTL4 Locus

The ANGPTL4 locus is located on human chromosome 19p13 where KANK3, ANGPTL4, RAB11B-AS, and RAB11B are clustered within an approximately 80-kb region. Among them, ANGPTL is a direct target of GR signaling [35]. To examine the chromatin context within this locus, we used publicly available ChIP-Seq data to conduct a survey of genome-wide CTCF-enriched sites in cell lines derived from different tissues. There are at least four CTCF-enriched sites in the ANGPTL4 locus in HepG2 cells (Fig 1A), which contain a CTCF-binding consensus motif (S1A Fig). We designated AC1 to AC4. The AC3 site is unique to HepG2 cells (S2 Fig), suggesting that AC3 plays a cell-type-specific role in regulating ANGPTL4 transcription.
We performed ChIP-Seq analysis of HepG2 cells treated with 100 nM Dex for 3 h to identify genome-wide GR binding sites (Fig 1A), and identified the GR-enriched sites (AG1 and AG2) in the ANGPTL4 locus, which possess GREs (S1A Fig). ChIP-Seq data indicated that the AG1 and AG2 sites were marked with acetylated lysine residue 27 and mono-methylated lysine residue 4 of histone H3 (H3K27Ac and H3K4me1, respectively), suggesting that they are potential enhancers. These findings are consistent with the evolutionary conservation of the AG2 sequence among rats, mice, and humans, which exhibits GR-responsive enhancer activity in rat H4IIE cells [15]. Further, AG2 and AC3 reside within an approximately 100-bp contiguous region (S1B Fig), suggesting that these sites cooperate in the GR-dependent expression of ANGPTL4.

Dexamethasone Induces ANGPTL4 Expression

We next tested the expression levels of ANGPTL4 in HepG2 cells treated with Dex. Before Dex addition, the cells were grown in DMEM medium supplemented with 10% DCC-treated FBS for the indicated times to minimize the effects of basal hormone levels. The level of ANGPTL4 mRNA immediately increased by a factor of 15 at 3 h after Dex addition and decreased from 3 to 24 h (Fig 1B). In contrast, Dex treatment did not affect the levels of the three neighboring genes (S3A Fig), indicating that ANGPTL4 is a specific GR target. Western blot analysis showed that ANGPTL4 protein was maximally induced at 3 h after Dex addition and decreased from 3 to 24 h (S3B Fig).

To determine whether ANGPTL4 was directly regulated by liganded GR, we analyzed HepG2 cells treated with the GR antagonist mifepristone (100 μM for 1 h) before Dex treatment. Mifepristone completely inhibited ANGPTL4 expression 3 h after Dex addition (Fig 1C), indicating that ANGPTL4 is the only GR target in this chromosomal region.

Dexamethasone Treatment Alters Chromatin Status at the ANGPTL4 Locus

We performed ChIP-qPCR analyses to assess the effect of Dex on the chromatin status of the ANGPTL4 locus (Fig 2). We first examined the levels of enrichment of GR at AG1 and AG2. GR was not detected at either AG site under basal conditions (Dex 0 h), while GR bound these AG sites 3 h after Dex addition (Dex 3 h) (Fig 2A). GR was similarly retained 24 h after Dex treatment (Dex 24 h), although the levels of ANGPTL4 mRNA decreased at this time (Fig 1B). In contrast, CTCF-enrichment did not significantly change at AC1 to AC4 after Dex treatment (Fig 2B), suggesting that GR does not compete with CTCF for DNA binding at the AC3 and AG2 sites, despite their proximity.

We next determined the enrichment of the active chromatin mark H3K27Ac in the ANGPTL4 promoter and enhancer elements AG1 and AG2 (Fig 2C). After Dex treatment for 3 h (Dex 3 h), the H3K27Ac mark increased by a factor of approximately 2 at these sites compared with the basal condition and was detected 24 h after Dex addition. Further, we determined the enrichment...
of RNA polymerase II (Pol II) at the ANGPTL4 promoter and enhancers (Fig 2D). Active Pol II was enriched at the ANGPTL4 promoter by approximately a factor of 4 at 3 h after Dex treatment and then decreased to the basal level at 24 h. In contrast, enrichment of Pol II at AG1 and AG2 was moderately detected 3 h after Dex addition, indicating that liganded GR bound to distal enhancers and activated transcription mediated by the ANGPTL4 promoter.

Long-term Dexamethasone Treatment Attenuates ANGPTL4 Induction and Down-regulates GR Expression

An excess of endogenous or exogenous glucocorticoid causes hypertriglyceridemia, insulin resistance, fatty liver, hepatic steatosis, and obesity as well as Cushing’s syndrome [36]. These changes may be associated, in part, with ANGPTL4 levels. Therefore, we examined whether Long-Term Dex Treatment (shortly LTDT; 100 nM for 14 days) altered ANGPTL4 expression (Fig 3A). Control HepG2 cells were cultured in DMEM medium supplemented with 10% DCC-treated FBS and 100 nM dexamethasone (Dex) for 14 days. Black arrows show sampling times. (B) Decrease in ANGPTL4 induction in LTDT cells. (C) Decreased enrichment of GR after Dex treatment of LTDT cells. ChIP-qPCR analysis was performed using an anti-GR antibody and an anti-rabbit IgG (control), followed by quantitative PCR using specific primers for each AG site and the control (NC). (D) CTCF enrichment in control and LTDT cells. ChIP-qPCR analysis was performed using an anti-CTCF antibody and anti-rabbit IgG (control), followed by quantitative PCR using specific primers for each AC site. (E) Expression of CTCF and GR after Dex treatment of control and LTDT cells. The amount of GR decreased in most LTDT cells. The relative level of GR normalized to that of β-tubulin is shown below. Uncropped image of western blot analysis is shown in S6 Fig. Asterisks indicate statistically significance between control and LTDT cells at each time point. **P < 0.01, ***P < 0.005.

doi:10.1371/journal.pone.0169225.g003
A

relative CTCF mRNA levels

siCont
siCTCF

relative PAB1114 AS mRNA levels

siCont
siRAB11B AS

(h after Dex)

(h after Dex)

B

CTCF

GR

β-tubulin

(h after Dex)

(kDa)

120
95
55

C

relative ANGPTL4 mRNA levels

siCont
siCTCF
siRAB11B AS

(h after Dex)

(h after Dex)

D

relative ANGPTL4 mRNA levels

siCont
siCTCF

(h after Dex)

(h after Dex)

E

CTCF

CHIP-qPCR

IgG

CTCF

IgG

CTCF

IgG

CTCF

(h after Dex)

(h after Dex)

GR

IgG

GR

IgG

GR

(h after Dex)

(h after Dex)

NC

AC3

NC

AG1

AG2
Loss of CTCF Deregulates *ANGPTL4* Expression Induced by DEX

To determine the role of CTCF in the regulation of *ANGPTL4* transcription, we performed RNA interference-mediated knockdown of CTCF expression in HepG2 cells. We conducted qRT-PCR and western blot analyses to confirm the reduction in the levels of CTCF mRNA and protein, respectively (Fig 4A, left and 4B). In addition, the depletion of CTCF did not affect GR expression at the protein level (Fig 4B and S5A Fig). When the CTCF knockdown cells were treated with Dex, *ANGPTL4* mRNA levels significantly increased 3 h after Dex addition and induction was maintained for 24 h (Fig 4C). In contrast, *ANGPTL4* expression increased 3 h after treatment and then decreased in control cells. Similarly, the increased level of *ANGPTL4* mRNA in the CTCF-knockdown cells was detected in LTDT cells (Fig 4D). Moreover, we confirmed that the expression of another GR-target gene *FKBP5* was hardly affected by CTCF knockdown (S5A Fig).

Several lines of evidence suggest that antisense transcripts influence sense-strand transcription [37]. Further, it has been reported that CTCF has large RNA interactions in mammalian cells [38, 39]. In the *ANGPTL4* locus, the direction of *RAB11B-AS* transcript is opposite of that of *ANGPTL4* and *RAB11B* (Fig 1A and S5B Fig) and extends to the AC3/AG2 sites in the 3’-region of *ANGPTL4* gene (S5C Fig). To test whether the *RAB11B-AS* transcripts influenced *ANGPTL4* expression, we inhibited the expression of *RAB11B-AS* in HepG2 cells (Fig 4A, right) and found that Dex-induced *ANGPTL4* expression was unchanged (Fig 4C).

We next performed ChIP-qPCR analysis of CTCF-knockdown HepG2 cells to determine whether depletion of CTCF affected GR binding to the AG1 and AG2 sites. (Fig 4E). We detected a significant reduction of CTCF enrichment at AC3, and the loss of CTCF caused an increase in GR binding to AG1 and AG2 by a factor of approximately 2 at 3 h after Dex treatment. These data suggest that CTCF negatively regulated the induction of *ANGPTL4* transcription by reducing the amount of liganded GR bound to AG sites. Further, at 24 h after Dex addition, GR enrichment at the AG sites was equivalent in the control and CTCF-knockdown cells, although *ANGPTL4* was expressed at a high level when CTCF was depleted (Fig 4C). Moreover, the decrease in the level of *ANGPTL4* mRNA coexisted with a high level of GR binding to the AG sites 24 h after Dex addition (Figs 1B and 2A). Together, these results suggest that Dex-induced *ANGPTL4* expression was regulated by the CTCF-mediated chromatin context as well as by GR binding to the enhancers. We also observed that the expression level of nearby genes of *ANGPTL4* was partly increased by CTCF knockdown (S5D Fig), suggesting that the activity of the *ANGPTL4* enhancer may spread to nearby genes in the CTCF-knockdown cells. Thus, CTCF was required for proper induction and subsequent silencing of *ANGPTL4* in cells treated with Dex.

Dexamethasone-induced Chromatin Conformation Changes in the *ANGPTL4* Locus

To investigate higher-order chromatin regulation in the *ANGPTL4* locus, we performed the chromosome conformation capture (3C) assay of Dex-treated HepG2 cells. The experiments
were performed under the conditions as follows: basal condition (Dex 0 h), Dex treatment for 3 h (Dex 3 h) and 24 h (Dex 24 h). There are at least four CTCF-enriched sites (AC1–AC4) and two GR-binding enhancer sites (AG1 and AG2) in the \textit{ANGPTL4} locus (Figs 1A and 5).

DpnII was used to convert these sites and the \textit{ANGPTL4} promoter into distinct fragments, except for the composite AC3/AG2 site. Using quantitative PCR analyses of the intramolecular ligation products, we first tested the interaction frequency of AC3/AG2 as a reference, which is the counterpart of a previously demonstrated enhancer element [15], with the other 11 DpnII fragments. The interaction frequency between AC3/AG2 and the \textit{ANGPTL4} promoter was consistently high under basal and Dex conditions (Fig 5A). The interaction between AC3/AG2 and AC2 increased by a factor of 2.5 at 3 h after Dex addition and then decreased to the basal level at 24 h. Dex treatment induced a significant dynamic change at AC2, because the interaction frequencies of AC3/AG2 with the other sites were largely constant.

To further evaluate the higher-order chromatin conformation in this locus, we performed the 3C assay using the \textit{ANGPTL4} promoter as a reference (Fig 5B). The interaction frequency of the \textit{ANGPTL4} promoter with AC2 significantly increased 3 h after Dex addition and then decreased to the basal level at 24 h. Dex treatment induced a significant dynamic change at AC2, because the interaction frequencies of AC3/AG2 with the other sites were largely constant.

Discussion

In the present study, we show that GR regulated \textit{ANGPTL4} in a CTCF-mediated chromatin context in the human hepatic carcinoma cell line HepG2. Liganded GR selectively targeted \textit{ANGPTL4} but not the three neighboring genes via interactions among the enhancer, promoter, and CTCF sites. In CTCF-depleted cells, \textit{ANGPTL4} transcription was more inducible by Dex and was persistently up-regulated for 24 h, indicating that CTCF was required for proper induction and subsequent silencing of transcription. Further, long-term Dex treatment subsequently diminished the induction of \textit{ANGPTL4} transcription. Our results therefore identified the mechanism of the regulation of \textit{ANGPTL4} transcription by CTCF as well as GR.

\textbf{Fig 6} shows a model of the regulation of \textit{ANGPTL4} transcription and the conformation of higher-order chromatin in cells treated with Dex. In its basal state, \textit{ANGPTL4} is not activated in the absence of glucocorticoid. However, the higher-order chromatin structure of the locus is prearranged. In the transcriptionally active state (Dex 3 h), liganded GR binds to AG sites and increases the affinities of the interactions among enhancer, promoter, and CTCF sites.
Fig 6. Model of ANGPTL4 gene regulation and higher-order chromatin in cells treated with Dex. In its basal state, ANGPTL4 is not activated in the absence of Dex-bound GR within the preexisting higher-order chromatin structure. When ANGPTL4 is transcriptionally active (Dex 3 h), liganded GR binds to AG sites and enhances interactions between enhancer, promoter, and CTCF sites to selectively induce ANGPTL4 but not the neighboring KANK3, RAB11B-AS, and RAB11B genes. In the silenced state (Dex 24 h), the positions of enhancer, promoter, and CTCF sites are restored, leading to down-regulation of ANGPTL4 transcription. CTCF is required for proper induction and subsequent silencing of the ANGPTL4 transcription.

doi:10.1371/journal.pone.0169225.g006
ANGPTL4, which specifically activate ANGPTL4 but not the neighboring genes KANK3, RAB11B-AS, and RAB11B. Since AC2 and AC3 show convergent orientations to the CTCF-binding sites, these may facilitate the formation of a chromatin loop [40]. In the silenced state (Dex 24 h), the positions of the enhancer, promoter, and CTCF sites are restored to the basal state when ANGPTL4 transcription is down-regulated. During these processes, CTCF is required for both induction and subsequent silencing of the ANGPTL4 gene.

Glucocorticoids are essential regulators of lipid homeostasis and energy metabolism, mainly via GR signaling. Dex treatment of HepG2 cells showed that ANGPTL4 was induced after 3 h and was silenced after 24 h. During this reaction, ANGPTL4 locus maintained a higher-order chromatin conformation during basal and induced states, together with specific interactions between the ANGPTL4 enhancer and promoter that were required for selective transcriptional activity. In contrast, under long-term Dex treatment, ANGPTL4 induction was significantly inhibited, at least in part, by the decrease in the level of GR. When the levels of glucocorticoids are up-regulated in vivo [41] under fasting or starvation, lipolysis in adipose tissue releases TGs and free fatty acids to other tissues, including the liver. Glucocorticoids may induce ANGPTL4 transcription to promote the catabolism. In contrast, long-term elevation of endogenous or exogenous glucocorticoids causes hypertriglyceridemia, insulin resistance, fatty liver, and obesity [36]. Such a long-term response is likely to produce cellular memory against glucocorticoid exposure. These changes may partly correlate with the level of ANGPTL4, because we show here that this protein was down-regulated in LTDT cells.

The siRNA-mediated inhibition of CTCF expression in HepG2 cells shows that ANGPTL4 transcription was highly induced by 3 h and was subsequently up-regulated for 24 h after Dex addition. Since there was no difference in liganded GR binding 24 h after Dex addition to control and CTCF-depleted cells, enhancer-promoter interactions in ANGPTL4 may contribute to persistent activation. Moreover, CTCF-binding is known to be affected by DNA methylation in the genome [42, 43], and CTCF protects adjacent sequences against de novo CpG methylation [44, 45]. Indeed, IGF2 imprinting is altered in aged and senescent human epithelial cells [46]. Our previous study shows that CTCF is down-regulated during cellular senescence [33]. Thus, the age-dependent increase of abnormal lipid metabolism may be linked to a reduction in CTCF-mediated regulation of ANGPTL4 transcription, possibly caused by changes in DNA methylation and the down-regulation of CTCF levels. In conclusion, our study of the human ANGPTL4 locus sheds light on the molecular basis of the regulation of GR target genes and its biological significance.

Supporting Information

S1 Fig. The sequences of CTCF- and GR-enriched sites in the human ANGPTL4 locus. (A) The consensus motifs of four CTCF-binding sequences (AC1–AC4) and two GR-binding sequences (AG1 and AG2). (B) The close localization of AC3 with AG2 in the 3′-region of ANGPTL4.

(TIF)

S2 Fig. CTCF-enriched sites in the ANGPTL4 locus in cultured human cell lines. ChIP-Seq data reveal four major CTCF-enriched sites in the ANGPTL4 locus. The CTCF-enriched AC3 site is uniquely present in the genome of HepG2 cells.

(TIF)

S3 Fig. The expression of four genes within human ANGPTL4 locus in cells treated with dexamethasone. (A) The expression patterns the KANK3, ANGPTL4, RAB11B, and RAB11B AS genes in cluster. In cells treated with Dex, ANGPTL4 mRNA was only induced by Dex.
**P < 0.01. (B) Western blot analysis of ANGPTL4 expression in HepG2 cells under the presence or absence of Dex.

(TIF)

**S4 Fig. GR and CTCF expression in control cells and cells subjected to long-term dexamethasone treatment (LTDT). (A) Analysis of the expression of glucocorticoid receptor (GRα) and CTCF mRNAs in LTDT cells. In the presence of Dex, there were no significant differences in the levels GRα and CTCF transcripts in control and LTDT cells. *P < 0.05. (B) Western blot analysis of GR expression in LTDT cells. Uncropped image of western blot analysis is shown in S6 Fig.

(TIF)

**S5 Fig. Expression analysis of CTCF knockdown cells. (A) qRT-PCR analysis of GRα (left) and FKBP5 (right) in siRNA-transfected HepG2 cells treated with Dex. (B) The transcribed sequence of RAB11B and RAB11B AS in the human ANGPTL4 locus. The arrow indicates the transcriptional start site. (C) Close localization of AC3/AG2 with the 3´-region of RAB11B AS. (D) qRT-PCR analysis of four genes within human ANGPTL4 locus in siRNA-transfected HepG2 cells treated with Dex. The relative expression level is indicated as a value normalized to the level of 36B4 mRNA. Asterisks indicate statistically significance between control and CTCF-knockdown cells at each time point. *P < 0.05, **P < 0.01, ***P < 0.005.

(TIF)

**S6 Fig. Uncropped images of western blot analysis. (A) Uncropped images of Fig 3E. (B) Uncropped image of S4B Fig.

(TIF)

**S1 Table. Primers used in this study.

(PDF)

**Acknowledgments

We thank Dr. H. Kimura (Tokyo Institute of Technology) for the antibody against RNA polymerase II and Dr. Takashi Seki for assistance with high-throughput sequencing, and the members of our laboratory for discussions and technical support.

**Author Contributions

Conceptualization: MaN KI MiN.

Funding acquisition: MiN.

Investigation: MaN KI MiN.

Methodology: MaN KI MiN.

Resources: TW AH SH MS HN.

Writing – original draft: MaN KI MiN.

**References

1. Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol. 2010; 120(2–3): 69–75. doi: 10.1016/j.jsbmb.2010.03.058 PMID: 20346397
2. Patel R, Williams-Dautovich J, Cummins CL. Minireview: new molecular mediators of glucocorticoid receptor activity in metabolic tissues. Mol Endocrinol. 2014; 28(7): 999–1011. doi: 10.1210/me.2014-1062 PMID: 2476614

3. Payvar F, DeFranco D, Firestone GL, Edgar B, Wrange O, Okret S, et al. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell. 1983; 35(2 Pt 1): 381–392. PMID: 6317184

4. Scheiderleit C, Geisse S, Westphal HM, Beato M. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus. Nature. 1983; 304(5928): 749–752. PMID: 6310405

5. Perlmann T, Wrange O. Specific glucocorticoid receptor binding to DNA reconstituted in a nucleosome. EMBO J. 1988; 7(10): 3073–3079. PMID: 2846275

6. John S, Sabo PJ, Johnson TA, Sung MH, Biddie SC, Lightman SL, et al. Interaction of the glucocorticoid receptor with the chromatin landscape. Mol Cell. 2008; 29(5): 611–624. doi: 10.1016/j.molcel.2008.02.010 PMID: 18342607

7. Hakim O, Sung MH, Voss TC, Splinter E, John S, Sabo PJ, et al. Diverse gene reprogramming events occur in the same spatial clusters of distal regulatory elements. Genome Res. 2011; 21(5): 697–706. doi: 10.1101/gr.111153.110 PMID: 21471403

8. Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, et al. Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. J Biol Chem. 2000; 275(37): 28488–28493. doi: 10.1074/jbc.M004029200 PMID: 10866990

9. Koliwad SK, Kuo T, Shipp LE, Backhed F, So AY, et al. Angiopoietin-like 4 (ANGPTL4, fasting-induced adipose factor) is a direct glucocorticoid receptor target and participates in glucocorticoid-regulated triglyceride metabolism. J Biol Chem. 2009; 284(38): 25593–25601. doi: 10.1074/jbc.M109.025452 PMID: 19628674

10. Koliwad SK, Gray NE, Wang JC. Angiopoietin-like 4 (Angptl4): A glucocorticoid-dependent gatekeeper of fatty acid flux during fasting. Adipocyte. 2012; 1(3): 182–187. doi: 10.4161/adip.20787 PMID: 23700531

11. Mattijssen F, Alex S, Swarts HJ, Groen AK, van Schothorst EM, Kersten S. Angptl4 serves as an endogenous inhibitor of intestinal lipid digestion. Mol Metab. 2014; 3(2): 135–144. doi: 10.1016/j.molmet.2013.11.004 PMID: 24634819

12. Zhang R. The ANGPTL3-4-8 model, a molecular mechanism for triglyceride trafficking. Open Biol. 2016; 6(4).

13. Koster A, Chao YB, Mosior M, Ford A, Gonzalez-DeWhitt PA, Hale JE, et al. Transgenic angiopoietin-like (angptl4) overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism. Endocrinology. 2005; 146(11): 4943–4950. doi: 10.1210/en.2005-0476 PMID: 16081640

14. Ong CT, Corces VG. Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat Rev Genet. 2011; 12(4): 283–293. doi: 10.1038/nrg2957 PMID: 21358745
22. Ong CT, Corces VG. CTCF: an architectural protein bridging genome topology and function. Nat Rev Genet. 2014; 15(4): 234–246. doi: 10.1038/nrg3663 PMID: 24614316

23. Ishihara K, Oshimura M, Nakao M. CTCF-dependent chromatin insulator is linked to epigenetic remodeling. Mol Cell. 2006; 23(5): 733–742. doi: 10.1016/j.molcel.2006.08.008 PMID: 16949388

24. Wendt KS, Yoshida K, Itoh T, Bando M, Koch B, Schirghuber E, et al. Cohesin mediates transcriptional insulation by CCCTC-binding factor. Nature. 2008; 451(7180): 796–801. doi: 10.1038/nature06634 PMID: 18235444

25. Rubio ED, Reiss DJ, Welcsh PL, Disteche CM, Filippova GN, Baliga NS, et al. CTCF physically links cohesin to chromatin. Proc Natl Acad Sci USA. 2008; 105(24): 8309–8314. doi: 10.1073/pnas.0801273105 PMID: 18550811

26. Parelho V, Hadjur S, Spivakov M, Leleu M, Sauer S, Gregson HC, et al. Cohesins functionally associate with CTCF on mammalian chromosome arms. Cell. 2008; 132(3): 422–433. doi: 10.1016/j.cell.2008.01.011 PMID: 18237772

27. Zlatanova J, Caiafa P. CTCF and its protein partners: divide and rule? J Cell Sci. 2009; 122(Pt 9): 1275–1284. doi: 10.1242/jcs.039990 PMID: 19386894

28. Kim TH, Abdullaev ZK, Smith AD, Ching KA, Loukinov DI, Green RD, et al. Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell. 2007; 128(6): 1231–1245. doi: 10.1016/j.cell.2006.12.048 PMID: 17382889

29. Chen H, Tian Y, Shu W, Bo X, Wang S. Comprehensive identification and annotation of cell-type-specific and ubiquitous CTCF-binding sites in the human genome. PLoS One. 2012; 7(7): e41374. doi: 10.1371/journal.pone.0041374 PMID: 22829947

30. Cuddapah S, Jothi R, Schones DE, Roh TY, Cui K, Zhao K. Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. Genome Res. 2009; 19(1): 24–32. doi: 10.1101/gr.082800.108 PMID: 19056695

31. Mishiro T, Ishihara K, Hino S, Tsutsumi S, Aburatani H, Shirahige K, et al. Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster. EMBO J. 2009; 28(9): 1234–1245. doi: 10.1002/emboj.2009.81 PMID: 19322193

32. Watanabe T, Ishihara K, Hirosue A, Watanabe S, Hino S, Ojima H, et al. Higher-order chromatin regulation and differential gene expression in the human tumor necrosis factor/lymphotoxin locus in hepatocellular carcinoma cells. Mol Cell Biol. 2012; 32(8): 1529–1541. doi: 10.1128/MCB.06478-11 PMID: 22354988

33. Hirosue A, Ishihara K, Tokunaga K, Watanabe T, Saitoh N, Nakamoto M, et al. Quantitative assessment of higher-order chromatin structure of the INK4/ARF locus in human senescent cells. Aging Cell. 2012; 11(3): 553–556. doi: 10.1111/j.1474-9726.2012.00809.x PMID: 22340434

34. Narendra V, Rocha PP, An D, Raviram R, Skok JA, Mazzoni EO, et al. CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. Science. 2015; 347(6225): 1017–1021. doi: 10.1126/science.1262088 PMID: 25722416

35. Rose AJ, Herzig S. Metabolic control through glucocorticoid hormones: an update. Mol Cell Endocrinol. 2013; 380(1–2): 65–78. doi: 10.1016/j.mce.2013.03.007 PMID: 23523966

36. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature. 2000; 405(6785): 482–485. doi: 10.1038/35013100 PMID: 10839546

37. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature. 2000; 405(6785): 486–489. doi: 10.1038/35013100 PMID: 10839547
44. Butcher DT, Mancini-DiNardo DN, Archer TK, Rodenhiser Dl. DNA binding sites for putative methylation boundaries in the unmethylated region of the BRCA1 promoter. Int J Cancer. 2004; 111(5): 669–678. doi: 10.1002/ijc.20324 PMID: 15252835

45. Filippova GN, Cheng MK, Moore JM, Truong JP, Hu YJ, Nguyen DK, et al. Boundaries between chromosomal domains of X inactivation and escape bind CTCF and lack CpG methylation during early development. Dev Cell. 2005; 8(1): 31–42. PMID: 15669143

46. Fu VX, Schwarze SR, Kenowski ML, Leblanc S, Svaren J, Jarrard DF. A loss of insulin-like growth factor-2 imprinting is modulated by CCCTC-binding factor down-regulation at senescence in human epithelial cells. J Biol Chem. 2004; 279(50): 52218–52226. doi: 10.1074/jbc.M405015200 PMID: 15471867