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B-cell translocation gene 2 promotes hepatic hepcidin production via induction of Yin Yang 1

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ABSTRACT

Hepcidin is a peptide hormone secreted in the liver and plays a key role in maintaining iron homeostasis. Here, we demonstrate that B-cell translocation gene 2 (BTG2) is a key player in hepatic hepcidin regulation via induction of Yin Yang 1 (YY1). Hepatic hepcidin gene expression significantly enhanced by fasting states and glucagon exposure led to induction of gluconeogenic gene expression, and elevated serum hepcidin production in mice. Notably, overexpression of BTG2 using adenoviral system (Ad-BTG2) significantly elevated serum hepcidin levels via a significant induction of YY1 gene transcription. Immunoprecipitation studies demonstrated that BTG2 physically interacted with YY1 and recruited on the hepcidin gene promoter. Finally, ablation of hepatic BTG2 gene by gene silencing markedly attenuated the elevation of serum hepcidin production along with YY1 and hepcidin mRNA expression in fasting state. Likewise, forskolin (FSK)-stimulated hepcidin promoter activity was dramatically disrupted by endogenous BTG2 knockdown. Overall, our current study provides a novel molecular mechanism of BTG2-mediated induction of hepcidin gene expression, thereby contributing to a better understanding of the hepatic hepcidin production involved in iron homeostasis.

1. Introduction

Hepcidin is a small peptide hormone mainly produced by hepatocytes in the liver that plays a key role in maintaining mammalian iron homeostasis. It represses dietary iron absorption in the enterocytes, recycling by macrophages, and the release of storage iron from hepatocytes by binding and/or triggering the internalization and degradation of ferroportin, which causes a reduction in extracellular iron concentration [1]. Hepatic hepcidin production is elevated under various physiological conditions, including infection, inflammation, and severe iron overload, whereas this phenomenon is attenuated by hypoxia, iron-deficiency anemia, erythropoietin, growth factors [1,2]. Moreover, hepcidin gene expression is increased in response to stimulatory signals such as bone morphogenetic proteins (BMP)/SMAD axis, interleukin (IL-6)/signal transducer and activator of transcription 3 (STAT3), cAMP responsive element binding protein H (CREBH), whereas it is suppressed by hypoxia inducible factor (HIF), growth differentiation factor (GDF15), twisted gastrulation protein homolog 1 (TWSG1) [1–3]. A recent report has shown that gluconeogenic signals control mammalian iron homeostasis via induction of hepcidin in starving mice [4]. Although gluconeogenic signals regulate hepcidin production in mice, the basic molecular mechanism of hepatic hepcidin regulation caused by gluconeogenic system has not been fully investigated.

Yin Yang 1 (YY1) is a multifunctional transcriptional factor of the polycomb group protein family, and exerts as a transcriptional repressor and activator by binding to consensus sequences (CCATNTT). YY1 is widely expressed in diverse tissues and involved in embryogenesis, replication, cellular proliferation, and

Abbreviations: Ad, adenovirus; BTG2, b-cell translocation gene 2; ChIP, chromatin immunoprecipitation; Dis, distal region; FSK, forskolin; GFP, green fluorescent protein; GLU, glucagon; IB, immunoblot; IP, immunoprecipitation; MT, mutant form; Pro, proximal region; qPCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA; WT, wild type; YY1, Yin Yang 1.

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differentiation [5]. Moreover, YY1 also associates with a diverse protein regulating cellular proliferation and apoptosis, such as p53, murine double minute 2 (Mdm2), retinoblastoma (Rb), histone deacetylase complex (HDAC), c-Myc, and other transcription factors [6]. Recently, YY1 promotes gluconeogenesis and steatosis by controlling glucocorticoid receptor and farnesoid X receptor in the livers of mice [7,8]. However, the link between YY1 and hepcidin production involved in the regulation of iron homeostasis remains unexplored.

BTG2 (B-cell translocation gene 2) is a member of BTG/Tob gene family of anti-proliferative genes that acts as a key regulator of the cellular machinery, including cell growth, differentiation, death, and survival [9]. Especially, BTG2 is stimulated by hypoxia, genotoxic stress, metabolic changes, retinoic acid, and attenuated by insulin, estrogen, growth factors [10]. Previously, we have elucidated that BTG2, a transcriptional co-activator, regulates the transcription of several target genes, such as gluconeogenic genes, insulin [11,12]. However, the potential role of BTG2 in the regulation of hepatic hepcidin production and its subsequent role in maintaining iron homeostasis have not been investigated.

In the current study, we demonstrate that BTG2 acts as a novel partner of hepatic hepcidin regulation and reveals a basic molecular mechanism that links to YY1 by driving hepatic hepcidin production in gluconeogenic pathway.

2. Materials and methods

2.1. Animal experiments

Male 8-week old C57BL/6 mice (Samtako, Osan, Republic of Korea) were used for this experiment, as below described [13]. For studies in the fasting and feeding conditions, mice were fed *ad libitum* and fasted for 12 h. For glucagon stimulation experiments, glucagon (Sigma–Aldrich, St. Louis, MO, USA) was injected intraperitoneally into mice at a dose of 10 μg/kg body weight for 6 h. For overexpression of BTG2, WT mice were infected with adenoviral vector expressing BTG2 (1.10^9 plaque-forming units, pfu) by tail vein injection. For ablation of BTG2 gene, WT mice were intravenously injected with lentivirus short hairpin BTG2 (sh BTG2, a single dose of 1.10^9 transducing units (TU) per ml). Livers from fast, adenovirus- and lentivirus-infected mice were utilized in the preparation of total RNA and/or protein. All animal experiments were performed in accordance with the rules and regulations of the Institutional Animal Use and Care Committee (IAUCC), Keimyung University School of Medicine.

2.2. Measurement of serum hepcidin

Serum hepcidin level was measured using an enzyme-linked immunosorbent assay kit (Uscn Life Science Inc., Hubei, China) in accordance with the manufacturer's instructions, as previously described [14].

2.3. Plasmids and DNA constructs

Mouse hepcidin gene promoter was kind gifted by Dr. Hueng-Sik Choi (Chonnam National University, Gwangju, Republic of Korea) [14]. The YY1 response element-mutated hepcidin promoter was generated using a Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and the following primers: forward, 5′-GAGTAACAGTTTTACTGAAGGCAC-3′, and reverse, 5′-GTGCCTTCAGTAAAACTGTTACTC-3′. Expression vector for BTG2 was described previously [13] and pCMVSPORT6-YY1 plasmids was kindly provided by Dr. Gap Ryol Lee (Sogang University, Seoul, Republic of Korea). All constructs were confirmed by DNA sequencing.

2.4. Cell culture and transient transfection assays

AML-12 cells (immortalized mouse hepatocytes) were cultured in DMEM/F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), insulin-transferrin-selenium (Gibco-BRL), dexamethasone (40 ng/ml, Sigma–Aldrich), and antibiotics in a humidified atmosphere containing 5% CO_2 at 37 °C [15]. Transient transfections were carried out as previously described [13,15].

2.5. Isolation and culture of primary mouse hepatocytes

Mouse primary hepatocytes were performed from the livers of 8-week-old male mice (Samtako), as previously described [13].

2.6. Preparation of recombinant adenovirus and siRNA experiments

Adenoviruses encoding full length BTG2 (Ad-BTG2), green fluorescent protein (GFP) and lentiviral BTG2-targeted short hairpin RNA (sh RNA) were described previously [13]. The siRNAs for BTG2 (si Scram and si BTG2) were chemically synthesized (Bioneer Research, Seoul, Republic of Korea), and transfected according to the manufacturer's instructions, as previously described [11].

2.7. RNA isolation and analysis

Total RNAs were isolated from liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described [13,15]. Quantitative polymerase chain reaction (qPCR) analysis were conducted using primers of BTG2, Nur77, YY1, and hepcidin as described previously [11,13,14]. The expression of all transcripts was normalized to ribosomal L32 expression.

2.8. Co-immunoprecipitation assay

Total protein isolated from mouse liver was immunoprecipitated with antibodies against BTG2 and YY1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then blotted with these antibodies [13]. Signals were detected with ECL-Plus Western blot detection kit (Amersham Bioscience, Piscataway, NJ, USA).

2.9. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously [11,13]. Briefly, after 36 h infection with Ad-BTG2 in mouse primary hepatocytes, cells were treated with FSK (10 μM) for 6 h. The cells were subsequently harvested, and ChIP assay was performed with anti-YY1. After purification, DNA samples were quantified by PCR with two pairs of primers for the proximal (−450/−250 bp) and distal (−1900/−1700 bp) region of the hepcidin gene promoter. The specific primers used for PCR are as follows: proximal, forward 5′-ATATGGTCTTCACAGTGGTC-3′ and reverse 5′-CAGTGATTGGATTGGTGAGT-3′; distal, forward 5′-TTGGGTAGGGTTCCTAGGGT-3′ and reverse 5′-CGT-GGTACAGAGGGAGGGCT-3′.

2.10. Statistical analysis

Results are expressed as means (±S.E.M.). Analysis of variance was employed to determine significant differences as detected by Student's *t* tests and/or ANOVA methods using prism program. Statistical significance was considered at $P < 0.05$.