Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Novel sex-dependent differentially methylated regions are demethylated in adult male mouse livers

Shuhei Ito [a], Keiji Hirabayashi [a], Kohji Moriishi [b], Yasuhisa Matsui [c], Kyoji Moriya [d], Kazuhiko Koike [e], Yoshiharu Matsuura [f], Kunio Shiota [a, *], Shintaro Yagi [a, *]

[a] Department of Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Tokyo, Japan
[b] Department of Microbiology, Division of Medicine, Interdisciplinary Graduate School of Medicine and Engineering (Faculty of Medicine), University of Yamanashi, Japan
[c] Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan
[d] Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[e] Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[f] Department of Molecular Virology, Research Institute for Microbial Diseases Osaka University, Osaka, Japan

ARTICLE INFO

*Article history:*
Received 23 April 2015
Available online xxx

*Keywords:*
S-DMRs
Testosterone
DNA methylation
STAT5

ABSTRACT

In mammalian livers, sexual dimorphisms are observed in tissue-specific functions and diseases such as hepatocellular carcinoma. We identified sex-dependent differentially methylated regions (S-DMRs) which had been previously been characterized as growth hormone- STAT5 dependent. In this study, we performed genome-wide screening and identified ten additional hypomethylated S-DMR gene regions in male livers. Of these S-DMRs, *Uggt2* and *Sarnp* were hypomethylated in both male and female livers compared to brain and embryonic stem (ES) cells. Similarly, *Adam2*, *Uggt2*, and *Scp2* were hypomethylated in female embryonic germ (EG) cells and not in male EG cells, indicating that these S-DMRs are liver-specific male hypo-S-DMRs. Interestingly, the five S-DMRs were free from STAT5 chromatin immunoprecipitation (ChIP) signals, suggesting that S-DMRs are independent of the growth hormone-STAT5-pathway. Instead, the DNA methylation statuses of the S-DMRs of *Adam2*, *Snx29*, *Uggt2*, *Sarnp*, and *Rnpc3* genes were under the control of testosterone. Importantly, the hypomethylated S-DMRs of the *Adam2* and *Snx29* regions showed chromatin decondensation. Epigenetic factors could be responsible for the sexual dimorphisms in DNA methylation status and chromatin structure, as the expression of *Dnmt1*, *Dnmt3b*, and *Tet2* genes was lower in male mice compared to female mice and TET2 expression recovered following orchidectomy by testosterone treatment. In conclusion, we identified novel male-specific hypomethylated S-DMRs that contribute to chromatin decondensation in the liver. S-DMRs were tissue-specific and the hypomethylation is testosterone-dependent.



## 1. Introduction

In mammalian livers, sexual dimorphisms are observed in tissue-specific functions such as metabolism of sex steroids [1]. Sexual dimorphisms are more prevalent in diseases such as hepatocellular carcinoma (HCC), which is more common in men and postmenopausal women who exhibit high levels of serum testosterone [2]. Sexual dimorphism is also observed in animal models for HCC; male mice that express the hepatitis C virus (HCV) capsid protein (core protein) in the liver (male core Tg mice) preferentially develop HCC [3].

The sex differences in drug and sex steroid metabolisms result from sexual dimorphic expression of cytochrome P450 genes in the liver that are associated with differential DNA methylation of gene promoter regions [4]. *Cyp2d9*, a gene that encodes testosterone 16 alpha-hydroxylase, is highly expressed in livers of males and exhibits hypomethylation of its promoter region [4]. Such sex-dependent differentially methylated regions (S-DMRs) have been reported for several sex-dimorphic genes [5], suggesting involvement of DNA methylation in sexually dimorphic gene expression and stability.

* Corresponding authors. Yayoi 1-1-1, Laboratory of Cellular Biochemistry, Bunkyo-ku, Tokyo, 113-8657, Japan. Fax: +81 3 5841 8014.
*E-mail addresses:* ashiota@mail.ecc.u-tokyo.ac.jp (K. Shiota), alfa155shin@me.com (S. Yagi).

http://dx.doi.org/10.1016/j.bbrc.2015.04.137
0006-291X/

Another crucial mechanism in hepatic sexual dimorphism is the growth hormone -STAT5 pathway (GH-STAT5 axis) [6]. The sexually differential patterns of serum GH [7] secreted from the pituitary cause differential activation of STAT5. Deficiency of *Stat5b* [6] or hypophysectomy [8] results in the loss of most sexual dimorphic gene expression, which can be recovered by GH injection. Sexually dimorphic GH secretion patterns are governed by sex steroids [9].

STAT5 binding sites are often associated with decondensed chromatin structures, indicated by their enrichment in DNase hypersensitive sites (DHS) [10]. Chromatin structure is governed by epigenetic mechanisms including DNA methylation and histone modification. DNA methylation is often associated with condensed chromatin [11]. DNA methylation patterns in several male-biased genes can be modified by female-type GH administration [5]. These data suggest that the GH-STAT5 axis has an impact on local chromatin structure through an epigenetic system including DNA methylation. In addition, neonatal androgenization affects the DNA of several promoters in adult mice including those of*Cyp7b1* and *Hnf6* genes [12].

DNA methylation profiles, which consist of numerous genome-wide, tissue-dependent, differentially methylated regions (T-DMRs), change during cell differentiation and development and in response to environmental factors [13,14]. In this study, we investigated S-DMRs in the mouse liver and the effect of sex steroids on the DNA methylation profiles of adult mouse livers.

## 2. Materials and methods

### 2.1. Animals and cells

C57BL/6N mice were obtained from Charles River (Yokohama, Japan). Orchidectomy (ORX) or ovariectomy (OVX) was performed in six male and female mice at 10 weeks of age; the mice were sacrificed 4 weeks later. Siliconized tubes (TP: 2 cm, EB: 0.5 cm) containing testosterone propionate (TP) (WAKO, Osaka, Japan) or estradiol benzoate (EB) (Sigma–Aldrich Japan K.K. Tokyo, Japan) were subcutaneously implanted into three ORX mice or three OVX mice, respectively and kept for 4 weeks. The effects of ORX/OVX and TP/EB were confirmed by measuring the weight of the seminal vesicles and uteruses (Fig. S3A,B). Tissue samples were collected and frozen at −80 °C until use.

Embryonic stem (ES) cells and Embyonic germ(EG) cells were maintained as previously described [15,16]. We used three ES cell lines derived from males (mB6_1, mJ1, and mB6_6) and three cell lines derived from females (fBRC6, fB6_1, and fB6_2). We used two EG cell lines EG G10 and EG G12.

### 2.2. Sexing

Sex of mice was determined by PCR with primers specific for the X and Y chromosomes (Table S1). PCR reactions were performed using BIOTAQ HS DNA polymerase (BIOLINE, London, UK) under the following conditions: 95 °C for 7 min followed by 30–31 cycles of 95 °C for 30 s, 55–60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

### 2.3. DNA methylation analysis

T-DMR profiling with restriction tag-mediated amplification (D-REAM) was performed as previously described [14] to search for male hypo-S-DMRs. In D-REAM microarray analysis was conducted using the Affymetrix GeneChip mouse promoter 1.0R arrays (Affymetrix, California, U.S.A.). The resulting data were analyzed using MAT (bandwidth, 300 bp). Data were visualized using the Integrated Genome Browser (Affymetrix, California, U.S.A.).

Bisulfite-treated genomes were used for combined bisulfite restriction analysis (COBRA) analysis **and bisulfite sequencing**. Primers are listed in Supplementary information. The thermocycling program was 94 C for 10 min, 43 cycles of 94 C for 30 s, 55–60 C for 30 s and 72 C for 30sec, and 72 C 10 min. Restriction digests were performed on the final PCR products using *Hpy*CH4IV (New England Biolabs) at 37 °C for 3 h, followed by analysis using a MultiNA (SHIMAZDU, Kyoto, Japan) microchip electrophoresis apparatus. For bisulfite sequencing minimum of nine clones were sequenced, and the methylation status of individual CpGs was determined.

### 2.4. RT-qPCR (Biomark)

The expression levels of genes with S-DMRs were analyzed using microfluidic dynamic arrays [17] (Fluidigm Japan K.K., Tokyo, Japan) using a previously described protocol [18]. Primers used for this assay are listed in Supplementary information. Data were normalized to *Actb* gene expression levels.

### 2.5. DNase assay

DNase assay was performed described in Ref. [19]. Briefly, lysis solution (ActiveMotf, California, U.S.A.) was added to 4 mg of liver. After homogenization and centrifugation (2300 × g for 10 min at 4 °C), the supernatant was moved into two tubes. The samples were incubated at 37 °C for 5 min and 20U of RQ1 RNase-Free DNase (Promega, Madison, WI, USA; M6101) was then added to one of each tube and incubated at 37 °C for an additional 10 min. The reaction was stopped by adding EDTA. Proteinase (10 mg/mL) was added and incubated at 55 °C overnight. DNA was extracted using phenol chloroform isoamylalcohol (25:24:1) and subsequent ethanol precipitation. Digested/undigested DNA were analyzed using the Applied Biosystems 7500 real-time PCR system. The ΔCt value = Ct value (DNase digested sample) - Ct value (undigested sample) was adjusted using the ΔCt of the Rho gene. Primers used are listed in Supplementary information.

## 3. Results

### 3.1. Identification of male hypo-S-DMRs

We screened S-DMRs hypomethylated in male mouse liver by using D-REAM, a microarray-based genome-wide DNA methylation analysis method, and found S-DMRs to be less methylated in male livers (male hypo-S-DMRs) than in female livers [14]. Using D-REAM, we identified a male hypo-S-DMR at *Cyp2d9* (Fig. S2), a locus previously shown to carry an identified S-DMR [4]. In addition, COBRA of the 10 identified S-DMRs indicated they were significantly hypomethylated (>10% difference in average DNA methylation levels) in the livers of male mice compared to those of female mice (Fig. 1A). Some S-DMRs (*Uggt2* and *Sarnp*) exhibited lower methylation in both male and female livers than in the brain and embryonic stem (ES) cells. In the brain or ES cells, there were no significant differences in the methylation levels of these S-DMRs between male and female mice, indicating that these S-DMRs are liver-specific male hypo-S-DMRs. We also examined DNA methylation levels in sperm and in male and female embryonic germ (EG) cells. *Adam2, Uggt2,* and *Scp2* were hypomethylated in female EG cells (Fig. S1A). Sperm was hypermethylated in almost all the S-DMRs that we analyzed, including *Adam2* (Fig. S1A), a gene that is highly expressed in spermatids (Fig. 3A).

We then compared these S-DMRs with those of known STAT5-binding regions by using published STAT5 ChIP-seq data [20]. Five S-DMRs (*Snx29, Mettl20, Scp2, Nudt7,* and *Tchhl1*) overlapped with



Please cite this article in press as: S. Ito, et al., Novel sex-dependent differentially methylated regions are demethylated in adult male mouse livers, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.04.137