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

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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.

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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

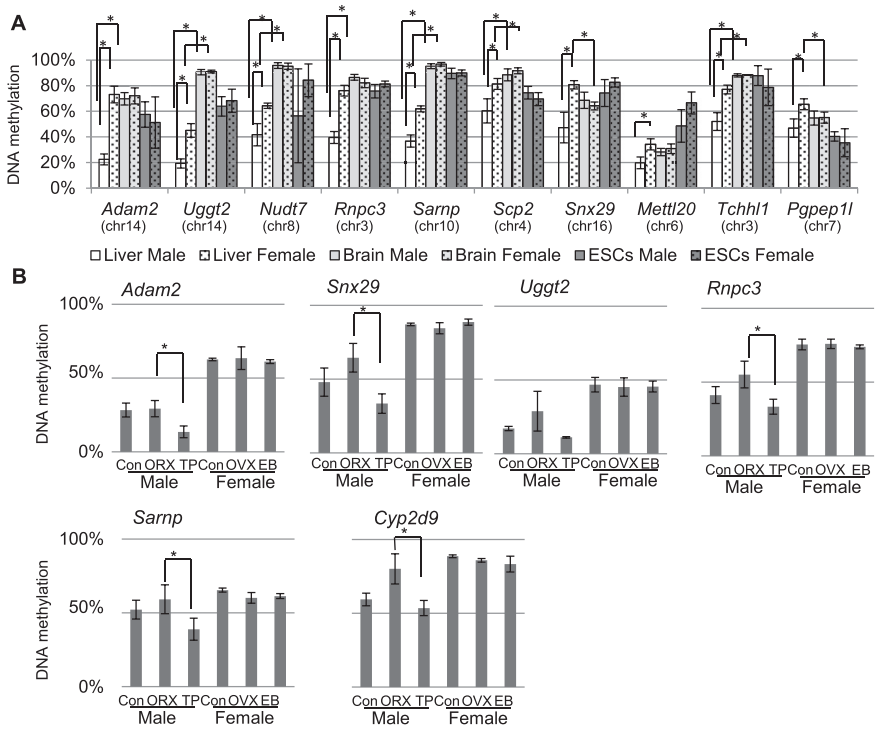

Fig. 1. Identification of differentially methylated S-DMRs which are under control of testosterone in male and female mouse livers. A) DNA methylation status of male/female liver, brain, and ES cells (ESCs), analyzed using COBRA (n = 3, biological triplicate). Numbers in parenthesis indicate the chromosome number for each gene. Student's *t*-tests were performed to compare male and female livers, male liver and brain, and female liver and brain tissues. *p < 0.05. B) Effect of sex steroids on DNA methylation status in S-DMRs (n = 3, biological triplicate). Con: Control, ORX: Orchidectomy, TP: Orchidectomy and injection of testosterone propionate, OVX: Ovariectomy, EB: Ovariectomy and injection of estradiol benzoate Student's *t*-tests were performed for each condition for male livers (*p < 0.05).

STAT5 binding signals (Fig. S2). *Cyp2d9* S-DMRs in GH-dependent dimorphic genes were also located in STAT5 ChIP signals, and the other S-DMRs did not overlap with STAT5 binding signals, indicating that certain S-DMRs are independent of the GH-STAT5 pathway.

3.2. Testosterone induces hypomethylation of male hypo-S-DMRs

To examine the effect of sex steroids on male hypo-DMRs, we analyzed DNA methylation in six selected S-DMRs in ORX or OVX mice in the presence and absence of sex steroid treatment (Fig. 1B). ORX moderately increased DNA methylation at male hypo-S-DMRs at *Snx29*, *Uggt2*, *Rnpc3* and *Cyp2d9*, and injection of testosterone decreased DNA methylation levels significantly except for *Uggt2*.

In contrast, neither OVX or estrogen administration affected DNA methylation at these S-DMRs, suggesting that testosterone should have an impact on DNA methylation of these S-DMRs The fact that testosterone-decreased methylation of S-DMRs that overlapped with STAT5 binding regions at *Cyp2d9* and *Snx29* suggested that there are S-DMRs in STAT5 binding sites that are under the control of testosterone.

3.3. S-DMRs in liver of core Tg mice

In the HCC model, Core Tg mice express core protein in their livers, resulting in changes to the histopathological state of the liver and aging [3]. In addition, exogenously expressed core protein stimulates the androgen-receptor signaling pathway and affects the lipid metabolism of liver cells [21]. We analyzed the DNA methylation status of S-DMRs in livers from 2-, 6-, and 16-month-old male Core Tg mice (Fig. S1B). DNA methylation in liver tissue was similar between Core Tg mice at 2 months and C57BL/6 mice at 14 weeks of age. The DNA methylation status in the *Snx29* and *Scp2* S-DMRs, which resided in the STAT5 ChIP region, changed in an age-dependent manner. The DNA methylation status of *Tchhl1* S-DMR within the STAT5 ChIP region in livers of 2-month-old Core Tg mice was similar to that of the 6- and 16-month-old mice. Furthermore, the DNA methylation status of *Adam2* and *Uggt2* S-DMRs that were free from STAT5 signals in Core Tg mouse livers were also similar between each of the ages. These data indicated that S-DMRs without STAT5 signals are stable lifelong marks in male mice.

3.4. The range of male-hypo S-DMRs were very narrow

GH-driven S-DMRs were observed over 1 kb [5]. We next analyzed DNA methylation near the *Adam2* male hypo-S-DMR to determine the range of the S-DMRs (Fig. 2A). DNA methylation status proximal to S-DMR was hypermethylated in both sexes under all conditions. Promoter regions of the highly expressed neighboring liver gene *Gulo* (Fig. S4B) were hypomethylated in both sexes, excluding region VI, which was hypermethylated in both male and female mice (Fig. 2A). Bisulfite sequencing revealed that differential methylation occurred at one CpG in *Adam2* and *Uggt2* S-DMRs (Fig. 2B). With respect to *Snx29*, male-specific hypomethylation was observed at two CpGs, indicated by black and gray arrowheads 34 bp apart in Fig. 2B. These results indicated that the range of these S-DMRs was limited.

3.5. S-DMR DNA methylation correlates with chromatin assembly but not gene expression

To examine the relationship between DNA methylation status in S-DMRs and expression of neighboring genes, we analyzed

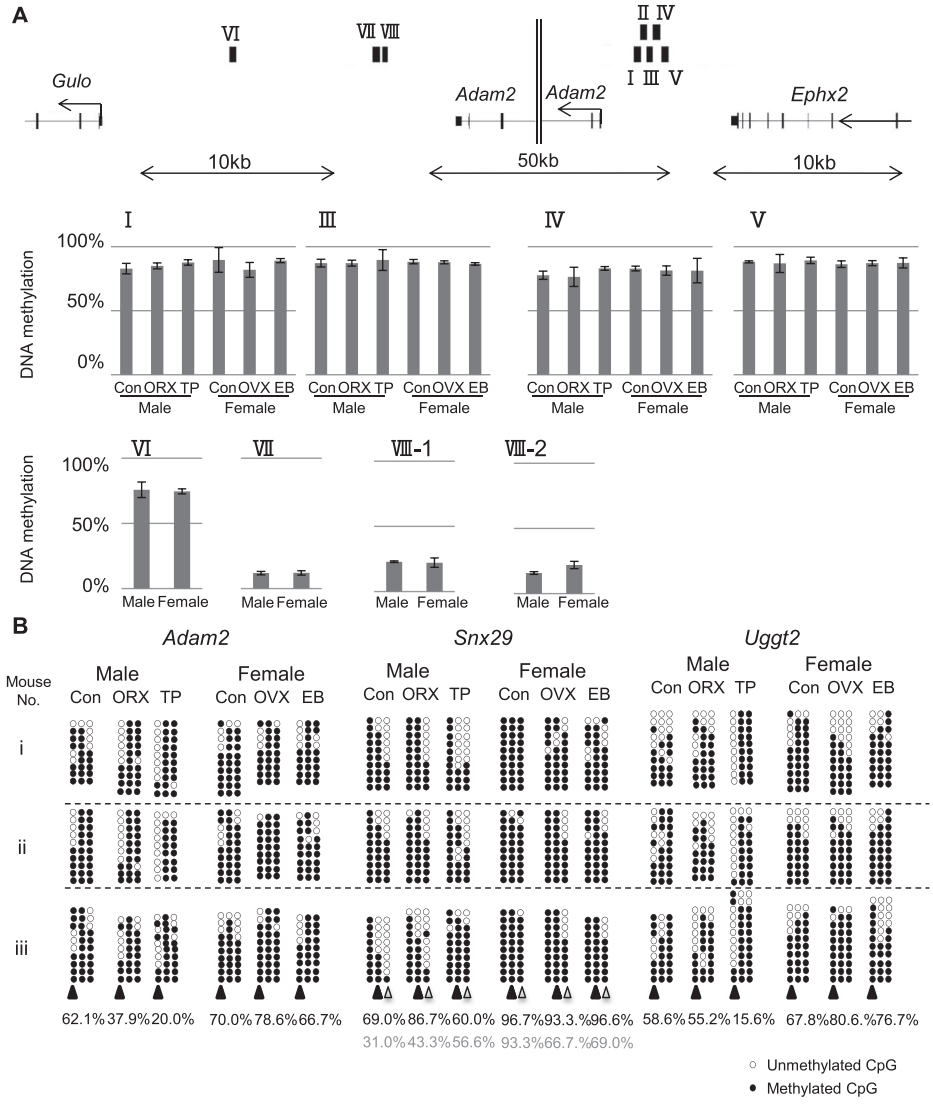

Fig. 2. S-DMRs are localized in discrete regions. A) DNA methylation status of the *Adam2* gene as analyzed using COBRA. The upper panel shows methylation sites analyzed using COBRA. Middle and lower panels show DNA methylation status. The DNA methylation status in region VIII-2 was significantly hypomethylated in male mice compared to female mice. Region II was a S-DMR detected using D-REAM. The associated COBRA data is shown in Fig. 2A. B) DNA methylation status of *Adam2*, *Uggt2*, and *Snx29* S-DMRs analyzed using bisulfite sequencing. Three individual mouse (I, II, and III) livers were analyzed. Open circles indicate unmethylated CpGs, and filled circles indicate methylated CpGs. CpGs indicated by black arrowheads were analyzed using COBRA (Fig. 1A). Percentages in black and gray font represent the ratio of unmethylated CpGs to total CpGs indicated by black and gray arrowheads, respectively.

transcripts from neighboring genes by using quantitative RT-PCR. Transcripts from previously identified STAT5-dependent genes, *Cyp2d9*, *C6*, and *Cyp7b1* [20] decreased in ORX mice and increased in ORX mice injected with testosterone (Fig. S4A). However, both *Snx29* and *Rundc2a* gene expression levels were similar between males and females and were not affected by ORX and injection of testosterone (Fig. 3A, Fig. S4B). Furthermore, *Snx29* was highly expressed in the brain where S-DMRs were highly methylated. qRT-PCR analysis of the *Adam2* gene indicated high expression in spermatids and ovaries, modest expression in the brain, and no expression in the liver. *Ephx2* and *Gulo*, which are found upstream and downstream of *Adam2,* respectively, were highly expressed in the liver of both sexes. *Ephx2* and *Gulo* were modestly expressed in ovaries and spermatids that exhibited hypermethylation at S-DMRs (Figure S4A). Expression level of the *Uggt2* gene was similar among tissues with different DNA methylation statuses at the *Uggt2* S-DMR (Fig. 3A). These results indicated that there is no correlation between DNA methylation of these S-DMRs and gene expression of their neighboring genes. We then analyzed DNase1 sensitivity [22] in S-DMRs (Fig. 3B). *Gapdh* showed a high ΔCt value, suggesting chromatin structure was relaxed at this region. Under similar conditions, *Adam2* and *Snx29* S-DMR showed higher, although not significant, DNase1 sensitivity in control and TP mice compared to ORX mice, suggesting chromatin decondensation was associated with DNA demethylation.

3.6. Sex-dependent changes in DNMT and TET expression

We analyzed the expression of *Dnmt1, Dnmt3a, Dnmt3b, Tet2*, and *Tet3* genes, which catalyze DNA methylation and demethylation, respectively, by using qRT-PCR and western blot. qRT-PCR indicated that the levels of *Dnmt1, Dnmt3b*, and *Tet2* gene expression were lower in 14 week old male mice than female mice (Fig. S5A). Furthermore, *Tet2* gene expression increased in ORX mice and decreased by injection of testosterone. In addition, TET2 protein levels decreased following ORX and had a tendency to

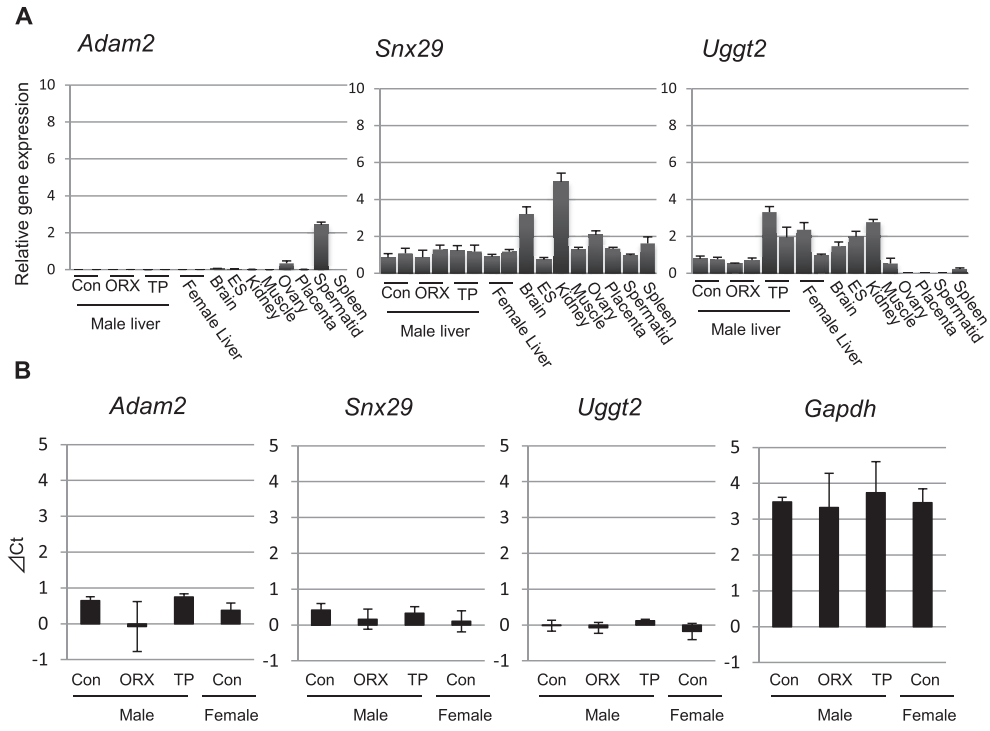

Fig. 3. Expression of genes with S-DMRs do not correlate with testosterone levels, but lead to relaxed-chromatin. A) Gene expression of *Adam2*, *Snx29*, and *Uggt2*. The graph shows relative gene expression levels standardized to *Actb* gene expression (n = 3, experimental triplicate). For each condition, two mouse livers were used for gene expression analysis. B) qPCR of DNase-digested and undigested DNA. ΔCt (Ct values of DNase digested samples − Ct values of undigested samples) are shown (n = 3, biological triplicate). Error bars shown represent the standard deviation (S.D.). Student's *t*-tests were performed, but no significant differences were detected between of the conditions in any combination.

increase after TP treatment (Fig. S5B,C). Coincident of DNA demethylation and increased TET2 protein levels in TP treatment prompted us to analyze molecular interactions of TET2 and AR with S-DMRs by ChIP. ChIP assay analyses for AR and TET2 revealed no differences in the levels of binding by these factors among all the groups of mice (Fig. S6). These results suggested that sex-dimorphisms in epigenetic factors such as *Dnmt1*, *Dnmt3b*, and *Tet2* could be involved in sexual dimorphisms in DNA methylation.

4. Discussion

4.1. Testosterone affects DNA methylation of S-DMRs

Genome-wide DNA methylation analysis allowed us to identify S-DMRs that were specifically hypomethylated in male mouse livers. Although some S-DMRs exhibited tissue-specific methylation patterns (i.e., less methylation in the liver than other tissues in both sexes), levels of DNA methylation at these S-DMRs were lower in the livers of male than in female mice. We also found that testosterone had an impact on DNA methylation at the identified S-DMRs in livers of adult male mice.

Previous studies indicated sexual dimorphisms of DNA methylation at promoter regions in sexual dimorphic genes is under the control of the GH-STAT5 pathway [5]. In addition, DNA methylation at some S-DMRs in male mice can be changed by altering the GH secretion pattern in a manner similar to that in female mice [5]. We found that the S-DMR of *Cyp2d9*, an authentic dimorphic gene under the control of GH-STAT5, had altered DNA methylation levels that correlated to the levels of serum testosterone. Importantly, the S-DMRs identified in this study that overlapped with STAT5-binding signals became demethylated following administration of testosterone. Sexually dimorphic DNA methylation appears in *Cyp2d9* promoter as early as postnatal day 2 [4], and neonatal injection of testosterone results in DNA methylation of male-biased GH-STAT5 genes in adult female mice [12]. These observations illustrate that the molecular pathway regulating DNA methylation of several S-DMRs in GH-STAT5-dependent male-biased genes is regulated by male sex hormones from neonatal to adult stages.

4.2. Sex-dimorphic expression of DNMTs and TETs

Our ChIP analysis failed to identify factors binding to the liver male hypo-S-DMRs; however, we found sexual dimorphisms in molecules involved in DNA methylation and demethylation. In mice, DNA methylation is added at cytosines by three DNA methyltransferases, and is removed by pathways involving ten-eleven translocation proteins, which hydroxylate methylated cytosines [23]. DNMT1 and DNMT3B were expressed at significantly lower levels in male mice compared to female mice, and the level of TET2 protein, which was lower in male mice, decreased following ORX, suggesting that a combination of these factors contribute to the dimorphic DNA methylation profiles in adult mouse livers. Further studies will be needed to confirm this finding.

4.3. DNA methylation changes in aged mice with steatosis

Plasma testosterone levels are lower in aged animals than young animals [24], and are affected by steatosis in the rodent model [12,25,26]. Despite steatosis in livers of aged Core Tg mice, we found that two S-DMRs, which overlapped with STAT5 binding signals, had reversed DNA methylation profiles. Because DNA methylation changes occur at GH-dependent S-DMRs in aged mice, DNA methylation at S-DMRs near GH-STAT5-regulated genes are predicted to be more susceptible to environmental factors than S-

DMRs free from STAT5 binding. These findings will provide novel insight into the mechanisms of steatosis.

4.4. Sex dimorphic epigenetic factors would construct S-DMRs

Of the epigenetic factors examined, expression of the *Dnmt1*, *Dnmt3b*, and *Tet2* genes were lower in male than in female mice. However, TET2 protein was higher in male mice compared to female mice. Similarly, TET2 expression levels could be restored by ORX following testosterone treatment. Recent studies have indicated that post-transcriptional modification of DNMTs and TETs alters their intracellular localization and enzymatic activity might affect inconsitence between RNA transcripts and protein levels of TET2 [27,28]. These data indicate that epigenetic interactions consisting of several complex steps influence the epigenetic status of S-DMRs.

4.5. Sex dimorphic DNA methylation status involves in chromatin structure around S-DMR

Snx29 and *Adam2* S-DMRs were shown to be located in a narrow region of the genome. Our results illustrate there are a limited number of S-DMRs with and without STAT5 signals that are not correlated with neighboring gene expression. Differences in DNase sensitivity at *Adam2* and *Snx29* S-DMRs, suggested a relaxed chromatin structure associated with hypomethylated S-DMRs. Decondensed chromatin structures contribute to genomic instability and binding of specific transcription factors [22], providing genomic landmarks for the organization of tissue-specific genome function. Therefore, the identified S-DMRs could serve as novel genomic landmarks that can be used to investigate male-specific genome organization in the mouse liver.

Availability of supporting data

The array data from this study have been submitted to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under accession no. E-TABM-551 and no. E-TABM-789.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.137.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.137.

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