Dysregulated DNA methylation in the pathogenesis of Fabry disease

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of α-galactosidase A and subsequent accumulation of glycosphingolipids with terminal α-D-galactosyl residues. The molecular process through which this abnormal metabolism of glycosphingolipids causes multisystem dysfunction in Fabry disease is not fully understood. We sought to determine whether dysregulated DNA methylation plays a role in the development of this disease. In the present study, using isogenic cellular models derived from Fabry patient endothelial cells, we tested whether manipulation of α-galactosidase A activity and glycosphingolipid metabolism affects DNA methylation. Bisulfite pyrosequencing revealed that changes in α-galactosidase A activity were associated with significantly altered DNA methylation in the androgen receptor promoter, and this effect was highly CpG loci-specific. Methylation array studies showed that α-galactosidase A activity and glycosphingolipid levels were associated with differential methylation of numerous CpG sites throughout the genome. We identified 15 signaling pathways that may be susceptible to methylation alterations in Fabry disease. By incorporating RNA sequencing data, we identified 21 genes that have both differential mRNA expression and methylation. Upregulated expression of collagen type IV alpha 1 and alpha 2 genes correlated with decreased methylation of these two genes. Methionine levels were elevated in Fabry patient cells and Fabry mouse tissues, suggesting that a perturbed methionine cycle contributes to the observed dysregulated methylation patterns. In conclusion, this study provides evidence that α-galactosidase A deficiency and glycosphingolipid storage may affect DNA methylation homeostasis and highlights the importance of epigenetics in the pathogenesis of Fabry disease and, possibly, of other lysosomal storage disorders.

1. Introduction

Fabry disease is an X-linked lysosomal storage disorder (LSD) caused by deficient activity of α-galactosidase A (α-gal A) [1]. This enzyme deficiency results in an inability of the cells to catabolize glycosphingolipids (GSLs) with terminal α-D-galactosyl residues, mainly globotriaosylceramide (Glo). GSLs accumulation occurs in the lysosomes of almost all cell types; however, it is particularly prominent in vascular endothelial and smooth muscle cells [2]. Major clinical manifestations include neuropathic pain, progressive renal insufficiency, cardiac disease and strokes. Male patients typically present with the most severe form of the disease [3]. Clinical abnormalities in heterozygous female patients are generally milder, but can occasionally be as severe as those observed in classically affected male patients.

A number of genes have been reported to be abnormally expressed in Fabry patients and a mouse model of the disease [4–9], which may contribute to the pathogenesis of the disease. However, the molecular mechanisms by which α-gal A deficiency causes the noted aberrant gene expression remains unclear.

DNA methylation represents one of the most important epigenetic mechanisms for gene regulation. Aberrant DNA methylation has been implicated in cancers, neurodegenerative and autoimmune disorders [10]. However, the association between DNA methylation and Fabry disease and other LSDs is largely unknown. Echevarria et al. [11] analyzed X-chromosome inactivation (XCI) in 56 female Fabry patients, and found that most patients had random inactivation and that, in
patients with skewed XCI (29%), disease progression was correlated with the direction of skewing. Rossanti et al. [12] reported that skewed XCI resulting in predominant inactivation of the normal allele was observed only in one out 9 females patients with Fabry disease that were analyzed. Recently, De Riso et al. [13] studied XCI process using epiallele distribution analysis approach, and demonstrated substantial concordance in direction of the methylation imbalance between androgen receptor (AR) and GLA genes. Hossain et al. [14,15] directly analyzed methylation of the GLA gene in female Fabry patients, and showed that methylation of the non-mutated allele correlated with disease severity. These studies highlighted the potential effect of methylation of GLA gene on α-gal A activity and disease phenotype in heterozygous females (for a review on the role of DNA methylation in Fabry disease, see Di Risi et al. [16]). However, the role of epigenetic modifications as a downstream mediator in disease pathogenesis has not been studied. We hypothesized that Fabry disease affects DNA methylation homeostasis, which might contribute to dysregulated gene expression and development of the disease. In this study, we investigated whether α-gal A deficiency and abnormal GSL metabolism cause aberrant DNA methylation.

2. Materials and methods

2.1. Cell culture and treatments

IMFE1 (a microvascular endothelial cell line established from a skin biopsy of a male Fabry patient) [17] and its derivatives were cultured in EGM-2MV medium (Lonza). IMFE1(α-gal+) and IMFE1(mock) cells were generated by stably transducing IMFE1 cells with a retroviral vector encoding normal α-gal A CDNA or an ‘empty’ control retrovirus, respectively [6]. IMFE1(α-gal+) and IMFE1(mock) cells were maintained in parallel to avoid passage-related variations between the 2 cell lines. Both cell lines used in this study had been passaged 9 times post-infection with α-gal A-expressing retrovirus or control vector, which is equivalent to at least 15 population doublings. Cell morphology and growth rate of this pair of cells were indistinguishable.

For the short-term treatment experiment, IMFE1 parental cells were treated with either 500 μM 1-deoxygalactonojirimycin (DGJ) or 0.5 μM GZ161 [18] for 12 days. The culture media were changed every 2–3 days with media containing freshly added compounds. Mock-treated control cells were cultured in parallel, and PBS was used in place of the DGJ or GZ161. There was no detectable cytotoxicity in drug-treated cells. Cell morphology and growth rates were indistinguishable in sham-, DGJ- and GZ161-treated groups. During the treatment, cells grew actively and underwent 4–5 population doublings.

2.2. Mouse tissues

Kidneys were obtained from 6-month-old male Fabry and WT mice. Both colonies had mixed genetic backgrounds of C57BL/6 and 129 strains (~75% C57BL/6 background) as described previously [6]. Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor Scott & White Research Institute.

2.3. Gb3 immunostaining and quantitative analysis

Gb3 immunofluorescence staining was performed using a Gb3-specific mouse monoclonal antibody as previously described [6]. Quantitative measurement of Gb3 levels was performed using mass spectrometry as described [6].

2.4. Bisulfite treatment and pyrosequencing

Genomic DNA was extracted using Qiagen DNeasy kit. Bisulfite treatment was performed using EZ DNA Methylation-Gold kit (Zymo Research) with 300 ng genomic DNA as starting material. Pyrosequencing was performed using PyroMark Q96 MD (Qiagen) according to manufacturer’s instructions. Primers used were: AR Cpg hot spot 1 (forward, Biotin-AGGAATAGGTTTGGTTTTAGGTT; reverse, TCCCTCTTTTCTCCTCC; sequencing, TCTCCCTCCTCCCTCA; amplicon 132 bp); AR Cpg hot spot 2 (forward, GGGAGTATTGTTGTTGGA-GAG; reverse, Biotin-CATCTACAAAACCATCCTCTTACIT; sequencing, GAGTAGTTTTTGGAGA; amplicon 276 bp).

2.5. DNA methylation array

Data from Infinium Methylation EPIC array that covers 865,859 CpG sites was assessed for quality using Genome Studio software (detected CpGs, bisulfite conversion controls, hybridization controls). The raw data (IDAT files) that contains intensity signals was read into R using functions from minfi package [19]. MethylationEPICcytoHs-1-0_B4 manifest was used to annotate the probes from EPIC array using IlluminaHumanMethylationEPICCanno.ilm10b4.hr19 package [20]. Probe filtering was performed to remove Cpg sites that have low detection p-value, known SNPs and cross-reactive probes. After quality control filtering, 793,793 sites with reliable methylation data were used for further analysis. Intensity values were normalized using control probes to generate β and M-values. Normalized M-values were used to identify differentially methylated probes using the limma package [21]. Differentially methylated regions were analyzed using the DMRcante package [22].

Principal component analysis showed that samples in each group (n = 3 per group) clustered together, except a sham control from a ‘chemical’ model that was excluded from subsequent analysis. Methylation array data were integrated with RNA sequencing data using the following criteria: (a) differentially methylated regions (DMRs) (P < 0.01); (b) all the Cpgs in a functional methylation region are in the same direction, i.e., all hyper, or hypo-methylated; and (c) >50% of Cpg sites in the region have delta beta >0.1. We identified 519 DMRs, corresponding to 81 genes that passed CPM filter (>1) in RNA-seq. Among them, 21 genes were differentially expressed (FDR < 0.05).

2.6. RNA sequencing

Genomic DNA and RNA were extracted using AllPrep DNA/RNA kit (Qiagen). Raw reads from RNA sequencing were assessed for quality using FastQC [23]. Adapter sequences were trimmed and low quality reads (q < 20) were filtered using cutadapt [24]. Reads were aligned to the human genome (GRCh37) using hisat2 [25]. Aligned SAM files were converted to BAM format using samtools [26]. Total aligned reads were quantified for each gene using featureCounts [27].

2.7. Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [6], using pre-designed TaqMan probe/primer sets (Applied Biosystems). 18S rRNA was used as internal control.

2.8. Analysis of methionine cycle metabolites

Concentrations of methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cystathionine, choline and betaine in cultured cells and mouse tissues were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [28]. Total homocysteine (Hcy) and total cysteine (Cys) were also measured by LC-MS/MS as previously described [29].

2.9. Statistical analysis

Data were presented as mean ± SEM. Significance was determined using the Student’s t-test.
3. Results and discussion

3.1. Effect of α-gal A deficiency on the methylation status of specific CpG sites in the AR gene promoter

To determine the potential role of Fabry disease on DNA methylation status, we examined whether manipulation of α-gal A activity and/or GSL levels lead to altered methylation levels in cultured cells. To this end, we employed two models – a ‘genetic’ model and a ‘chemical’ model (Fig. 1A). These models were generated using a Fabry patient-derived microvascular endothelial cell line (IMFE1) that exhibits both endothelial characteristics and Fabry phenotype [17]. In the ‘genetic’ model, IMFE1 cells were infected with a retroviral vector encoding wild type α-gal A to generate IMFE1(α-gal+), or infected with an ‘empty’ retroviral vector to generate IMFE1(mock) control. IMFE1(α-gal+) cells showed increased α-gal A activity and decreased lysosomal Gb3 storage compared to IMFE1(mock) cells [6]. In the ‘chemical’ model, IMFE1 cells were treated with either 500 μM DGJ (a potent competitive inhibitor of α-gal A), or 0.5 μM GZ161 (an inhibitor of glucosylceramide synthase) for 12 days. IMFE1 harbors the R112H GLA mutation, and thus retains residual α-gal A activity (~15% of normal controls) [17]. The purpose of using DGJ in this experiment was to extinguish this residual activity and thereby exacerbate Gb3 accumulation. Compared to sham treated IMFE1 cells, DGJ treatment resulted in markedly increased Gb3 accumulation (Fig. 1B, C). Consistent with a previous study [30], GZ161 treatment led to significantly decreased Gb3 relative to sham control.

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**Table 1.** Summary of treatment and cell models used in this study.

| Genetic model                  | Effect of the treatments               |
|-------------------------------|----------------------------------------|
| IMFE1(mock)                   | Overexpresses α-gal A                 |
| IMFE1(α-gal+)                 |                                        |

| Chemical model                |                                        |
|-------------------------------|----------------------------------------|
| IMFE1-sham                    |                                         |
| IMFE1-DGJ treatment           | Inhibits residual activity             |
| IMFE1-GZ161 treatment         | Inhibits GSLs synthesis                |

**Fig. 1.** Cell models and the effect of α-gal A deficiency in methylation of specific CpG sites in AR gene promoter. (A) Summary of ‘genetic’ and ‘chemical’ cellular models used in this study. (B) Gb3 immunofluorescence staining demonstrating markedly increased lysosomal Gb3 storage in DGJ-treated IMFE1 cells compared to sham-treated control. Arrows indicate some of the Gb3 positive signals (red color). Scale bar, 25 μm. (C) Gb3 levels in sham-, DGJ-, or GZ161-treated IMFE1 cells measured by LC-MS/MS (n = 3). (D) Top: Map of 5’ region of human AR gene containing two ‘hot spot’ CpG regions. Each CpG is indicated by a vertical line. CpG nucleotide positions relative to transcription start site (indicated by right angle arrow), and the core-promoter region with a length of 150 bp that contains the elements essential for promoter activity (indicated by grey bar) are shown. Bottom: Methylation level (%) of individual CpG sites.

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(F, G) Average methylation level of 3 CpG sites in AR hot spot 2 (n = 3–4). (H) Average methylation level of 3 CpG sites in LINE-1 (n = 2).
As the first step to test whether α-gal A deficiency and GSL accumulation affect DNA methylation, we analyzed methylation status of specific CpG sites in the androgen receptor (AR) gene as a marker. The AR gene was chosen because we had previously found that AR transcription is upregulated in Fabry disease [6]. Furthermore, the correlation between AR expression and DNA methylation has been well studied [31–33]. The 5′ region of human AR gene contains a ~3-kb CpG island with >80 CpG sites; among these, there are two specific CpG regions (‘hot spots’), whose methylation is closely associated with AR gene silencing in prostate cancer cell lines [33]. These two hot spots (constituted by 4 and 3 CpG sites, respectively) are located near the transcription start site (Fig. 1D). We analyzed these CpG sites using bisulfite pyrosequencing, a sensitive and reliable method for quantitative determination of methylation [34]. In hot spot 1, the CpG site at nucleotide position –121 had a higher methylation level in IMFE1 (mock) than in IMFE1(α-gal+) cells, while the other three CpG sites (–123, –125, and –131) remained unchanged (Fig. 1D). The average

Fig. 2. DNA methylation array and RNA sequencing. (A) Principal component analysis plot. Samples in each group (n = 3 per group) were clustered together, except a sham control from chemical model (arrow) that was excluded from the subsequent analysis. (B) Summary of numbers of DMPs and DMRs in each treatment group. (C) Venn diagram showing high proportion of common DMPs between different treatment groups. Numbers of DMPs were shown. (D) Majority of common DMPs between DGJ- and GZ161-treatment groups had the same direction of changes (hyper- or hypo-methylation) in both treatments. (E) RNA-seq results for collagen genes and profibrotic genes. Data were presented as fold-change [IMFE1(mock): IMFE1(α-gal+)]. *FDR < 0.05. (F) Map of DMPs (vertical lines) in COL4A1 and COL4A2 genes. Two arrows indicate transcription from a shared bi-directional promoter (shown as a triangle). Data were presented as methylation difference (delta beta values, IMFE1(mock) vs. IMFE1(α-gal+)). Most DMPs in these two genes (13/17 and 14/20 probes, respectively) exhibited hypomethylation in IMFE1(mock).
methylated level of the 4 CpG sites in hot spot 1 was not substantially different between IMFE1(α-gal−) and IMFE1(mock) cells (Fig. 1E). In contrast, all three CpG sites in hot spot 2 (+44, +49, and −54) had decreased methylation levels in IMFE1(mock) compared to IMFE1(α-gal+) cells (Fig. 1D). The average methylation level of the 3 CpG sites in hot spot 2 was significantly lower in IMFE1(mock) compared to IMFE1(α-gal+) cells (Fig. 1F).

Because the methylation status was changed more significantly in hot spot 2 than hot spot 1, we only studied hot spot 2 in subsequent studies. Consistent with findings in the ‘genetic’ model, short-term treatment with DGJ led to decreased methylation in all three CpG sites, with the greatest change at +44, compared to sham control (Fig. 1D). The average methylation level of hot spot 2 was significantly decreased in DGJ-treated cells (Fig. 1G). The methylation levels of hot spot 2 were unchanged in GZ161-treated cells relative to sham control (Fig. 1D, G).

We analyzed long interspersed element-1 (LINE-1), the repetitive DNA elements that are randomly inserted throughout the human genome and thus are used as a marker for global methylation [35]. Methylation levels of three individual CpG sites in LINE-1 were not different between IMFE1(mock) and IMFE1(α-gal+) (Fig. 1H).

Collectively, these data suggested that changes in α-gal A activity and GSL levels in Fabry endothelial cells lead to altered DNA methylation in a highly CpG site-specific manner.

3.2. Genome-wide methylation changes in Fabry disease cellular models

We further tested the link between Fabry disease and DNA methylation in a genome-wide analysis. DNA methylation of a total 865,859
sites was analyzed in the ‘genetic’ and ‘chemical’ models using Infinium MethylationEPIC. After quality control filtering, 793,793 sites with reliable methylation data were used for further analysis. Principal component analysis showed that samples in each group clustered together (except one sample), and that treated cells were well separated from controls (Fig. 2A). We identified >230,000 differentially methylated probes (DMPs; P < 0.05) between IMFE1(α-gal−) and IMFE1 (mock), and > 50,000 DMPs in DGJ- or GZ161-treated cells versus sham-treated cells (Fig. 2B). Large numbers of differentially methylated regions (DMRs), which are composed of multiple DMPs, were also identified (Fig. 2B).

Of note, there was substantial overlapping in DMPs between different treatments. >23,000 DMPs were common between DGJ- and GZ161-treatments, which are ~40% of total numbers of DMPs in each group (Fig. 2C). Similarly, ~35% of DMPs in DGJ- or GZ161-treated groups overlapped with DMPs in the ‘genetic’ model (Fig. 2C).

Because DGJ-treatment increases whereas GZ161 decreases substrate accumulation, one might expect that the direction of methylation changes (i.e., hyper- or hypomethylation relative to sham control) of the common DMPs in these two treatments should have an inverse relationship. On the contrary, the directions of methylation changes in these two treatments were highly consistent; 64.4% of common DMPs were hypermethylated in both treatments, and 35.4% were hypomethylated (Fig. 2D). Although detailed mechanisms of each treatment used to modulate DNA methylation can vary and will require further investigations, the high proportion of overlap in DMPs between different treatments suggests that these common DMPs might represent CpG sites whose methylation status has higher susceptibility to the changes in α-gal A activity and GSL levels. A total of 1044 genes were deduced from

| Pathways | Number of genes | P value | Genes | Pop Hits | Pop Total | Fold Enrichment | Bonferroni | Benjamini | FDR |
|----------|-----------------|---------|-------|----------|-----------|-----------------|------------|-----------|-----|
| Adherens junction | 9 | 0.0043 | EGFR, PTPRM, PTPRF, ACTN4, SMAD4, LM07, WASL, CTNN1A, CTNN3 | 71 | 6879 | 3.419 | 0.6387 | 0.6387 | 5.497 |
| cGMP-PKG signaling pathway | 14 | 0.0054 | SLC8A3, MYLK3, MRV1, ATP1A4, NPR1, ATP2B2, MF2D, PLCB3, ATP6B, GATA4, CACNA1F, CALM2, ADRAD1, OPRI1 | 158 | 6879 | 2.390 | 0.7185 | 0.4694 | 6.798 |
| Calcium signaling pathway | 14 | 0.0148 | EGFR, SLC8A3, NOS1, PTGER3, MYLK3, GRIN2A, ATP2B2, PLCB3, PDE1A, RYR1, CACNA1F, ADRAD1, CALM2, CACNA1B | 179 | 6879 | 2.109 | 0.9686 | 0.6724 | 17.499 |
| Nicotine addiction | 6 | 0.0151 | PLCB3, MYLK3, ATP1A4, SLCA4, CTRF2, CA2, KCNQ1, CALM2 | 40 | 6879 | 4.046 | 0.9702 | 0.5846 | 17.734 |
| Gastric acid secretion | 8 | 0.0176 | PLCB3, MYLK3, ATP1A4, SLCA4, CTRF2, CA2, KCNQ1, CALM2 | 73 | 6879 | 2.956 | 0.9835 | 0.5603 | 20.405 |
| Glutamatemic synapse | 10 | 0.0246 | DLGAP1, PLCB3, SLC1A3, GRIA2, GRIK1, GRM7, GRIN2A, HOMER1, SHAN2, GNG7 | 114 | 6879 | 2.364 | 0.9968 | 0.6173 | 27.395 |
| Central carbon metabolism in cancer | 7 | 0.0299 | FGFR2, PIK3, EGFR, NTRK1, FGFR3, NTRK1, SLC2A1 | 64 | 6879 | 2.950 | 0.9991 | 0.6337 | 32.329 |
| Adrenergic signaling in cardiomocytes | 11 | 0.0311 | ATP2B2, PLCB3, BCL2, ATP6B, ATP1A4, CACNA1F, CACNA2D3, KCNQ1, PPP2R2C, ADRAD1, CALM2 | 138 | 6879 | 2.150 | 0.9993 | 0.5987 | 33.349 |
| Inositol phosphate metabolism | 7 | 0.0463 | INPP1, PLCB3, PKC3CG2, PLC1, IPMK, MTPM7, INPP5A | 71 | 6879 | 2.659 | 0.9999 | 0.7045 | 45.636 |
| Regulation of actin cytoskeleton | 14 | 0.0466 | FGFR2, EGFR, FGFR3, ACTN4, DEPH2, MYLK3, ITGAI, ITGAV, ITGAX, CFL2, SCIN, MOS, WASL, FG3 | 210 | 6879 | 1.798 | 0.9999 | 0.6682 | 45.815 |
| Phosphatidylinositol signaling system | 8 | 0.0696 | INPP1, PLCB3, PKC3CG2, DKG, IPMK, MTPM7, INPP5A, CALM2 | 98 | 6879 | 2.202 | 0.9999 | 0.7804 | 60.394 |
| Retrograde endocannabinoid signaling | 8 | 0.0790 | PLCB3, GABBR3, GRIA2, GABBR6, GABBR6, CACNA1F, GNG7, CACNA1B | 101 | 6879 | 2.136 | 0.9999 | 0.7961 | 65.243 |
| Neuroactive ligand-receptor interaction | 16 | 0.0863 | AVPR2, GABBR3, PTGER3, GRIK1, GZMA, GABBR2, GABBR6, GRIN2A, ORH1, ORH2, GMR7, GALR2, CNR2, ADRAD1, OPRI1, GRD1 | 277 | 6879 | 1.558 | 0.9999 | 0.7990 | 68.602 |
| Insulin secretion | 7 | 0.0933 | PLCB3, GIP, SLC2A1, ATP6B, ATP1A4, CACNA1F, RIMS2 | 85 | 6879 | 2.221 | 1 | 0.8015 | 71.563 |
| Salivary secretion | 7 | 0.0974 | ATP2B2, PLCB3, NOS1, ATP1A4, SLC2A4, ADRAD1, CALM2 | 86 | 6879 | 2.195 | 1 | 0.7937 | 73.161 |
We identified models Fabry disease cellular models were relatively evenly distributed in cancers, where the methylation changes preferentially occur in specific regions (Cpg island shores) [38], the differentially methylated Cpgs in Fabry disease cellular models were relatively evenly distributed in various gene regions and chromosomes, and did not cause global changes in methylation level.

3.3. Differential mRNA expression and methylation in Fabry cellular models

We analyzed the transcriptome in the genetic model using RNA-seq. We identified >3,000 differentially expressed genes between IMFE1 (α-gal-) and IMFE1/mock (cells for FDR < 0.05; Supplementary Table 1) and 85 relevant signaling pathways (Supplementary Table 2). By integrating the methylation array and RNA-seq data (details, see Materials and Methods), we identified 21 genes that exhibited both differential DNA methylated regions and gene expression (Supplementary Table 3). Among the signaling pathways identified from RNA-seq (Supplementary Table 2), we took specific interest in extracellular matrix-related pathways, such as those involved in focal adhesion, and ECM-receptor interaction. Fibrosis is a prominent pathological feature in Fabry disease [39]. We found that 6 out of 13 collagen genes in the RNA-seq results were differentially expressed (FDR < 0.05), and they were exclusively upregulated in IMFE1/mock relative to IMFE1 (α-gal-) cells (Fig. 2E). SERPINE1, which encodes profibrotic protein plasminogen activator inhibitor-1 (PAI-1) [40], was also upregulated (~43%) in IMFE1/mock cells (Fig. 2E). TGF-β1, which is a key mediator of fibrotic process [41] and induces expression of several collagens and PAI-1 [42,43], was significantly upregulated in IMFE1/mock cells (Fig. 2E). These gene changes are consistent with the enhanced fibrosis in Fabry disease and also with previous laboratory findings, e.g., upregulated SERPINE1 in Fabry mouse penis [7], and enhanced TGF-β1 immunostaining in Fabry patient’s kidney biopsies [44].

Among these genes, COL4A1 and COL4A2 exhibited significant changes in methylation status. Most DMPs in these two genes (13/17 and 14/20 probes, respectively) exhibited hypomethylation in IMFE1 (mock) cells (Fig. 2F). COL4A1 and COL4A2 genes are arranged head-to-head on opposite strands of chromosome 13, and are separated by a shared, bidirectional promoter [45] (Fig. 2F). This short promoter (127 base pairs) does not show significant transcriptional activity, and requires regulatory elements present on distant portions of both genes [45,46]. The coordinated expression of both genes is necessary for function of the protein. In agreement with this, both genes were upregulated in IMFE1 (mock) cells to the same extent (25%) (Fig. 2E). Earlier studies suggested that DNA demethylation correlated with induction of COL4A1/2 expression during differentiation of F9 teratocarcinoma cells [47]. Our data suggests that Fabry disease is associated with decreased methylation of both COL4A1 and COL4A2 genes, and this may contribute to upregulated transcription of these genes. Peptides encoded by COL4A1 and COL4A2 form collagen type IV α1(4)β12 heterotrimer, which is the major component of basement membranes. This protein is involved in a number of physiological and pathophysiological conditions, including angiogenesis and brain small vessel diseases [48]. It is possible that increased expression of COL4A1/2 contributes to the development of vasculopathy in Fabry disease.

3.4. Potential basis of altered DNA methylation in Fabry disease

To study the potential basis of the altered DNA methylation, we tested whether levels of SAM, the methyl donor for DNA methylation, and other methionine cycle metabolites are changed in Fabry disease. Relative to IMFE1 (α-gal-) cells, the level of methionine was significantly increased and cystathionine was decreased in IMFE1 (mock) cells (Fig. 3A). We also analyzed kidney tissues from Fabry and WT mice. Consistent with the findings in the cellular models, the methionine level was significantly increased in Fabry mouse kidneys compared to WT mice (Fig. 3B). SAH in Fabry mice trended towards a higher level (P = 0.053). There was a strong positive correlation between levels of methionine and cystathionine in both WT and Fabry mouse kidney tissues (Fig. 3C, left). Methionine also positively correlated with SAM/SAH ratio in WT mice kidneys, but not in Fabry mouse kidneys (Fig. 3C, right).

Methionine cycle metabolism plays pivotal roles in the methylation process [49]. A number of animal studies using dietary approaches - either restricting or supplementing methionine or its metabolites betaine and choline - demonstrated the role of these compounds in modifying DNA methylation [50,51]. However, an increased methionine level was not always associated with increased SAM. For instance, 6 weeks of methionine supplementation in rats caused increased renal concentration of SAH, but no change in SAM and SAM/SAH [52]. Moreover, it was suggested that the effect of methionine supplementation on DNA methylation might be gene-specific, rather than global [53]. Collectively, our findings suggest that the disturbed methionine cycle could be part of the basis for the altered DNA methylation patterns in Fabry disease.

From the RNA-seq results, we summarized the expression of major enzymes that regulate methionine metabolism and the methylation/demethylation processes [49,54] (Fig. 3D, E). Methionine adenosyltransferase (MAT) 2B was downregulated in IMFE1 (mock) compared to IMFE1 (α-gal-) cells (Fig. 3E). MAT catalyzes the synthesis of SAM from methionine (Fig. 3D). In MATII isoenzyme, the regulatory subunit MATβ (encoded by MAT2B) interacts with catalytic subunit MATα2 (encoded by MAT2A) to form a heterotetramer [55]. MATβ increases the activity of MATII complex by lowering its Kₘ for the substrate, methionine [56]. Therefore, downregulation of MAT2B may contribute to the increased methionine in Fabry disease cells and tissues. However, it is not clear whether MAT2B expression change is a reason for increased methionine, or is a secondary change resulting from altered methylation. Other potential explanations for the imbalanced methionine metabolism may relate to oxidative stress and dysregulated nitric oxide (NO) pathway in Fabry disease [57–59]. NO inhibits the activity of methionine synthase, the enzyme responsible for regeneration of methionine from homocysteine [60]. It is possible that decreased NO production in Fabry disease due to NO synthase uncoupling [58,59] may lead to increased methionine synthase activity.

In addition, both RNA-seq and quantitative RT-PCR revealed that ten-eleven translocation 1 (TET1), the enzyme involved in DNA demethylation [54,61], was significantly downregulated (~40%) in IMFE1 (mock) (Fig. 3E, F), suggesting that TET1 may also be involved in the altered DNA methylation homeostasis in Fabry disease.

4. Conclusions

By combining unique cellular models based on Fabry patient-derived endothelial cells that are disease-relevant, and loci-specific pyrosequencing and genome-wide arrays, the present study demonstrated that abnormal glycolipid metabolism in Fabry disease may affect DNA methylation homeostasis. The dysregulated methylation was associated with altered methionine metabolism. These findings provide new insights into the pathogenic mechanism of this complex disease. DNA methylation, together with other epigenetic mechanisms, may also play a role in the phenotypic heterogeneity noted in Fabry and other LSD.
In addition, from the perspective of therapeutics, data from α-gal A overexpression and GZ161 treatment in the cells may provide useful information for gene therapy and substrate reduction therapy, respectively. Both approaches are under active investigation currently as new therapies for Fabry patients [62–64]. As shown from the RNA-seq and methylation array results, these treatments change the expression of numerous genes as well as methylation status throughout the genome. Our results may serve as a database for investigating potential clinical biomarkers for these therapies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2022.100919.

Fig. 3. Methionine cycle metabolism and DNA methylation.
(A, B) Methionine cycle metabolites in IMFE1 cells (A) and 6-month-old male mouse kidneys (B). *P < 0.05. Data were presented as % of levels in normal controls. Met, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Cysta, cystathionine; Bet, betaine; Cho, choline; Hcy, homocysteine (total); Cys, cysteine (total). (C) Correlations between methionine levels and cystathionine (left), and SAM/SAH ratio (right) in mouse kidneys. (D) Schema of methionine cycle and DNA methylation/demethylation reactions. The major enzymes involved in these reactions were shown. MAT, methionine adenosyltransferase; GAMT, guanidinoacetate methyltransferase; GNMT, glycine N-methyltransferase; AHCY, s-adenosylhomocysteine hydrolase; BHMT, betaine-homocysteine methyltransferase; MTR, methionine synthase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine ß-synthase; CTH, cystathionine γ-lyase; GATM, L-arginine/glycine amidinotransferase; DNMT, DNA methyltransferase; TET, ten-eleven translocation; TDG, thymine-DNA glycosylase; DMG, dimethylglycine; 5mC, 5-methylcytosine. (E) Transcription levels (RNA-Seq data) of major enzymes that are involved in methionine cycle and DNA methylation/demethylation reactions. Data were presented as fold-change [IMFE1(mock): IMFE1(α-gal+)]. *FDR < 0.05. (F) mRNA levels of DNA methylation/demethylation related genes measured by quantitative RT-PCR. *P < 0.05.

Declaration of Competing Interest
None.

Data availability
Data will be made available on request.

Acknowledgements
This work was supported by Baylor Scott & White Research Institute Foundation.
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