Short Communication

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Effects of Black Raspberry Supplementation on Methylation Pathways in *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ Double Knockout Mice with Early-stage Myelodysplastic Syndrome

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Myelodysplastic syndromes (MDS) are a subset of myeloid malignancies defined by clonality of immature hematopoietic stem cells that leads to faulty blood cell development. These syndromes can lead to an increased risk of infection and may transform into acute myeloid leukemia, making it critical to determine effective treatments for the condition. While hypomethylating agents such as azacitidine and decitabine, as well as stem cell transplants, have been delineated as favored treatments for MDS, not all patients are physiologically receptive to these treatments. However, black raspberries (BRBs) have been shown to exert hypomethylating effects in various malignancies, with minimal adverse effects and thus a broader range of potential candidacies. This study aimed to investigate the potential of BRBs to exert such effects on MDS using *Addition of Sex Combs Like*/*Tet Methylcytosine Dioxygenase 2* (*Asxl1*/*Tet2*) double knockout mice (*Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$), which typically manifest symptoms around 25 weeks of age, mirroring genetic mutations found in humans with MDS. Following a 12-week dietary supplementation of *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice with 5% BRBs, we observed both hyper- and hypomethylation at multiple transcription start sites and intragenic locations linked to critical pathways, including hematopoiesis. This methylation profile may have implications for delaying the onset of MDS, prompting a need for in-depth investigation. Our results emphasize the importance of exploring whether an extended BRB intervention can effectively alter MDS risk and elucidate the relationship between BRB-induced methylation changes, thus further unlocking the potential benefits of BRBs for MDS patients.

Key Words Myelodysplastic syndromes, Methylation, Black raspberries, *Asxl1*/*Tet2* double knockout

INTRODUCTION

Myeloid malignancies such as leukemias, myelodysplastic syndromes (MDS), and myeloproliferative disorders afflict patients across a myriad of demographics. These disorders frequently result from dysregulation of pathways that, under normal circumstances, control the proliferation or development of hematopoietic cells [1]. MDS, a subtype of myeloid malignancies, are characterized by cytopenia, dysplastic morphology of hematopoietic cells, and genetic indication of

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clonal hematopoiesis [2].

While significant strides have been made in better discerning pathways that may be targeted for therapeutic purposes in MDS, hypomethylating agents (HMAs) remain the first-line therapy for this condition. HMAs such as azacitidine and decitabine have shown efficacy in both lower- and higher-risk MDS, but they may have significant adverse effects and a critical proportion of high-risk MDS patients fail to meaningfully benefit from HMA therapy due to comorbidities and demographic factors [3]. Thus, exploration has begun regarding alternative therapies that might provide a broader range of efficacy and reduced adverse effects. Our previous research has focused on the chemopreventive potential of a diet supplemented with black raspberries (BRBs), but this research has also shown that BRBs exert hypomethylating effects in conditions such as ulcerative colitis and colorectal cancer [4,5]. It is thus feasible to explore whether the hypomethylation caused by BRBs may be of benefit in MDS.

The *Vav-Cre*, with *Addition of Sex Combs Like* (*Asxl1*) knockout (KO) and *Tet Methylcytosine Dioxygenase 2* (*Tet2*) KO, was used in this study. *Asxl1* mutations in humans are associated with myeloid malignancies such as MPN, MDS, and AML- those with such mutations exhibit epigenetic dysregulation leading to alteration of histone markers. *Asxl1* mutations are also associated with worsened prognosis and reduced survival rates [6]. The combination of the *Asxl1* and *Tet2* KOs in mice is known to replicate MDS; *Tet2* is often mutated with *Asxl1* in human patients with MDS, and the addition of the *Tet2* KO leads to a more rapid fatality in mice [7].

The *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice were thus utilized for the purposes of a mechanistic evaluation regarding how BRBs might modulate the early-stage progression of myeloid malignancies such as MDS. However, prior to determining mechanisms, it is important to determine whether an effect is present. Therefore, this study was intended to explore the potential of BRBs to affect the regulation of hematopoietic pathways and to determine which of these pathways are most significantly modified.

MATERIALS AND METHODS

All protocols were carried out in accordance with institutional guidelines for animal care dictated by the Medical College of Wisconsin Animal Care and Use Committee (protocol approval number AUA2430). *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice were bred as described [7] to knock out both *Asxl1* and *Tet2* genes using *Vav-Cre*, a hematopoietic-specific *Cre*. 4 to 5 week-

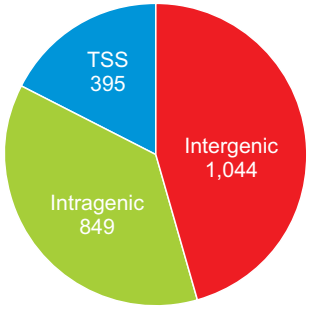

Figure 1. Differentially methylated regions (DMRs) de novo before and after black raspberry intervention. Among 2288 identified DMRs reaching false discovery rates < 0.05, 395, 849, and 1,044 were located at transcription start site (TSS), intragenic and intergenic regions, respectively.

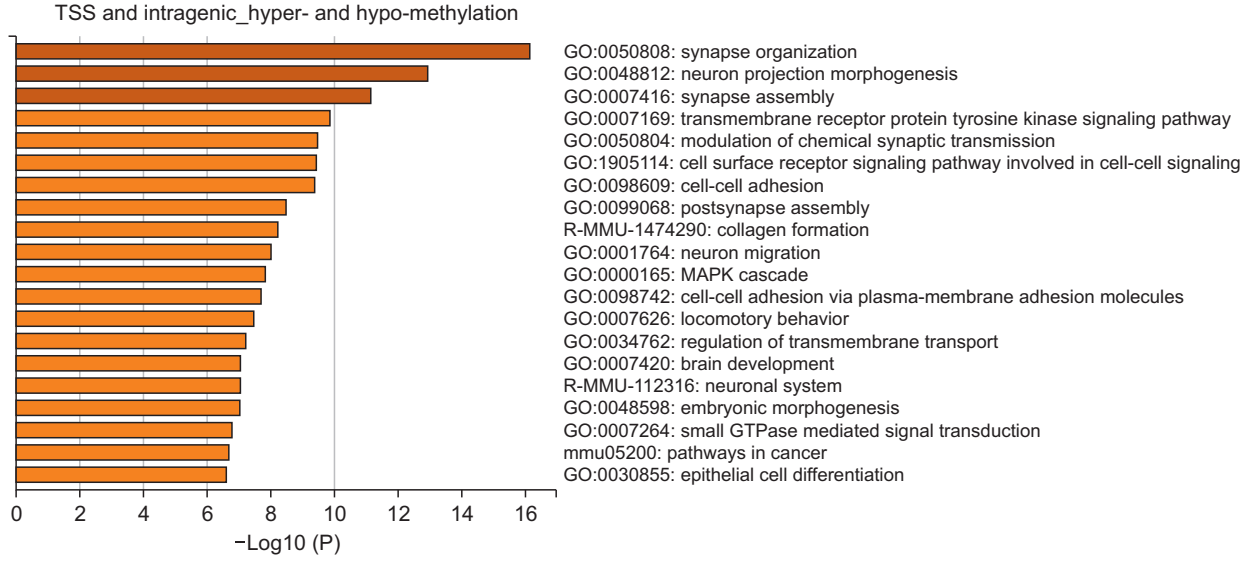

Figure 2. Metascape pathway analysis of differentially methylated regions at combined hypermethylated and hypomethylated transcription start site (TSS) and intragenic regions. Synapse organization (GO:0050808) and neuron projection morphogenesis (GO:0048812) pathways were most significantly affected by black raspberry supplementation.

old wild-type (n = 6) and *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ (n = 10) mice were fed 5% BRBs for 12 weeks. The circulating blood was used for the Wright–Giemsa stain, and CD45^{+} cells from circulating blood were used for the Reduced Representation Bisulfite Sequencing (RRBS) assay; data analysis was performed as described [8]. A modified software based on Metilene and Wilcoxon Signed Rank Test was applied to identify the differentially methylated region (DMR) de novo among samples. A DMR is required to contain at least five CpG sites and exhibit a 5mC difference of greater than 10% among samples. The methylation rate of each region was calculated as the average methylation rate of each site across the region. Benjamin-Hochberg procedure was used to control false discovery rates (FDR). DMR with FDR < 0.05 from Wilcoxon signed-rank test was considered significant. We divided genomic regions of interest into three genomic features: Transcription start site (TSS), intragenic regions, and intergenic regions. TSS regions were defined as 1,000 bp upstream and downstream of a TSS. Intragenic regions were defined as regions between the start and end of a transcript. The remaining regions not covered by TSS and intragenic regions were defined as intergenic regions. Then, the identified significant DMRs were assigned to one of the above three regions.

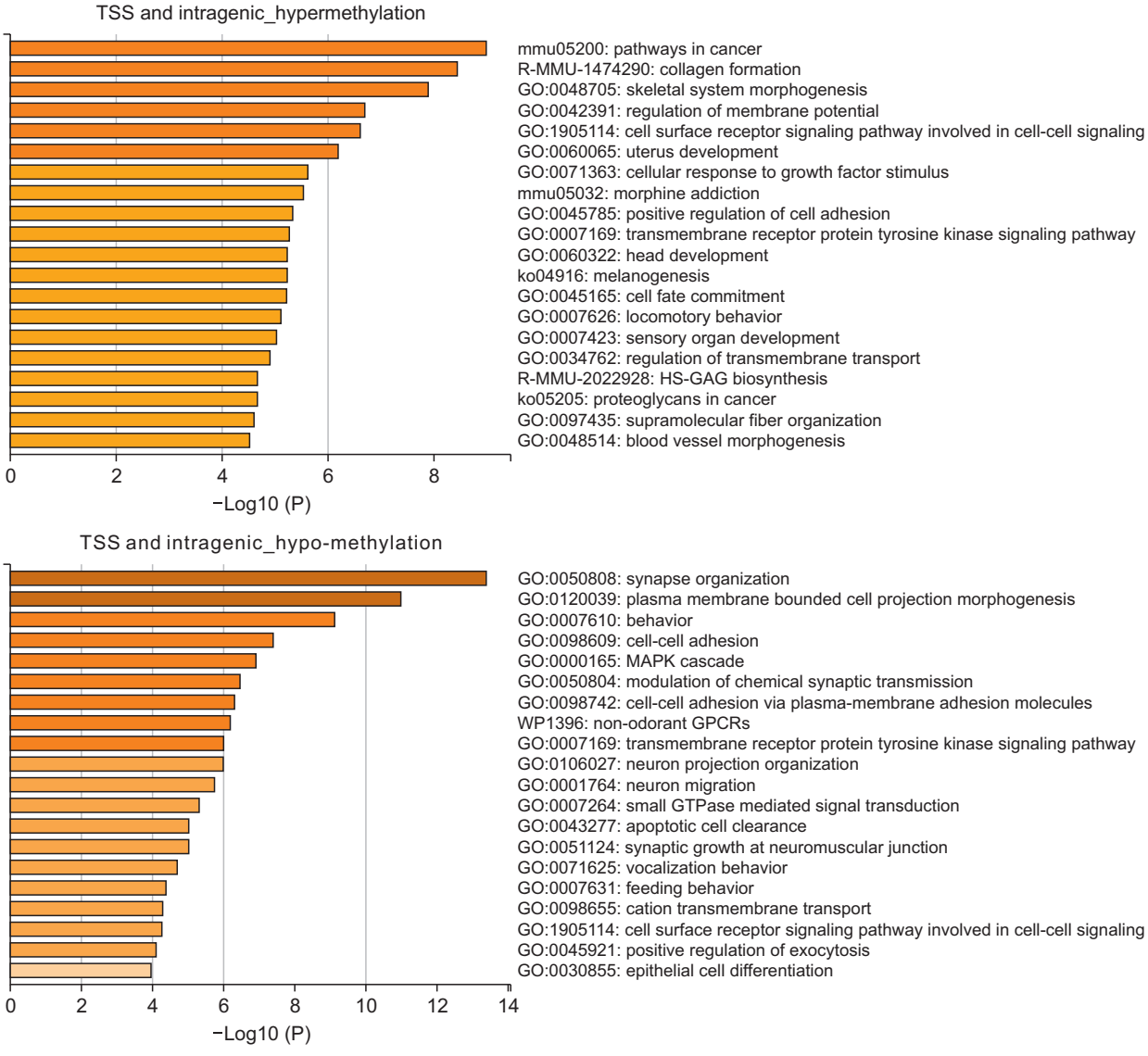

Figure 3. Metascape pathway analysis of differentially methylated regions (DMRs) at contrasting hyper/hypomethylated transcription start site (TSS) and intragenic regions. Hypomethylation at these DMRs was associated mainly with synapse organization (GO:0050808) and morphogenesis of plasma membrane-bounded cell projections (GO:0120039). Hypermethylation was associated with cancer pathways (mmu05200) and collagen formation (R-MMU-1474290). HS-GAG, heparan sulfate-glycosaminoglycan; MAPK, mitogen-activated protein kinase; GPCR, G protein coupled receptors.

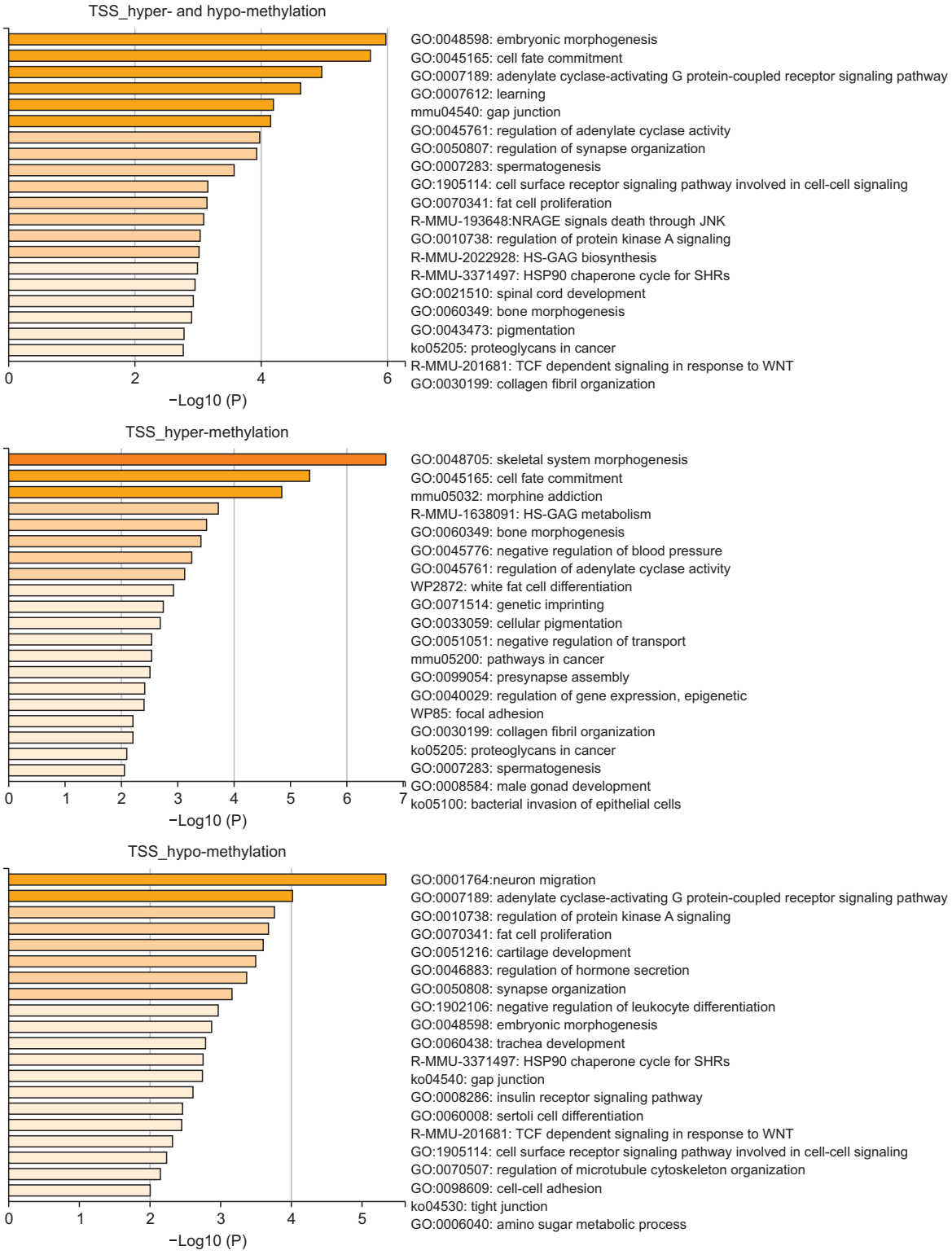

Figure 4. Metascape pathway analysis of differentially methylated regions (DMRs) at combined and contrasting hyper/hypomethylated transcription start site (TSS) regions. Embryonic morphogenesis (GO:0048598) and cell fate commitment (GO:0045165) pathways were most significantly affected by black raspberry supplementation. When this evaluation was specified regarding methylation status to better discern the effects of each type of methylation, it was found that hypomethylation at these DMRs was associated mainly with neuron migration (GO:0001764) and adenylate cyclase-activating G-protein-coupled receptor signaling (GO:0007189), whereas hypermethylation was associated with skeletal system morphogenesis (GO:0048705) and cell fate commitment. NRAGE, p75 neurotrophin receptor-interacting protein; HS-GAG, heparan sulfate-glycosaminoglycan; SHR, steroid hormone receptor; TCF, T-cell factor; WNT, Wingless-related integration site.

RESULTS

Absence of phenotypic changes in 16-week-old *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice

Asxl1/Tet2 double-KO mice typically exhibit symptoms around 25 weeks of age [7]. However, *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice were 16-week-old in our study. In our preliminary assessment, we conducted a Wright–Giemsa stain analysis of the circulating blood, and no abnormal cells were observed (data not shown). In addition, these mice exhibited no discernible phenotypic alterations compared to their wild-type littermates (data not shown). Therefore, we focused on exploring the impact of BRBs on methylation at this early stage of disease development.

DMRs

A total of 2,288 DMRs were found that reached a FDR < 0.05 after the administration of BRBs (Fig. 1). Of these regions, 395 were located in TSSs, 849 were in intragenic sites, and 1,044 were in intergenic sites.

Metascape pathway analysis

Evaluation of all DMRs in TSS and intragenic sites (regardless of hyper- or hypomethylation status) demonstrated that the synapse organization (GO:0050808) and neuron projection morphogenesis (GO:0048812) pathways were most significantly affected by BRB supplementation (Fig. 2). When this evaluation was specified regarding methylation status to better discern the effects of each type of methylation, it was found that hypomethylation at these DMRs was associated mainly with synapse organization (GO:0050808) and morphogenesis of plasma membrane-bounded cell projections (GO:0120039), whereas hypermethylation was associated with cancer pathways (mmu05200) and collagen formation (R-MMU-1474290) (Fig. 3).

Analysis of all DMRs in TSS (regardless of hyper- or hypomethylation status) demonstrated that the embryonic morphogenesis (GO:0048598) and cell fate commitment (GO:0045165) pathways were most significantly affected by BRB supplementation (Fig. 4). When this evaluation was specified regarding methylation status to better discern the effects of each type of methylation, it was found that hypomethylation at these DMRs was associated mainly with neuron migration (GO:0001764) and adenylate cyclase-activating G-protein-coupled receptor signaling (GO:0007189), whereas hypermethylation was associated with skeletal system morphogenesis (GO:0048705) and cell fate commitment (GO:0045165) (Fig. 4).

Lastly, analysis of all DMRs in intragenic sites (regardless of hyper- or hypomethylation status) demonstrated that the morphogenesis of plasma membrane-bounded cell projections (GO:0120039) and synapse organization (GO:0050808) pathways were most significantly affected by BRB supplementation (Fig. 5). When this evaluation was specified regarding methylation status to better discern the effects of each type of methylation, it was found that hypomethylation at these DMRs was associated mainly with postsynaptic organization (GO:0099173) and morphogenesis of plasma membrane-bounded cell projections (GO:0120039), whereas hypermethylation was associated with extracellular matrix organization (R-MMU-1474244) and regulation of membrane potential (GO:0042391) (Fig. 6).

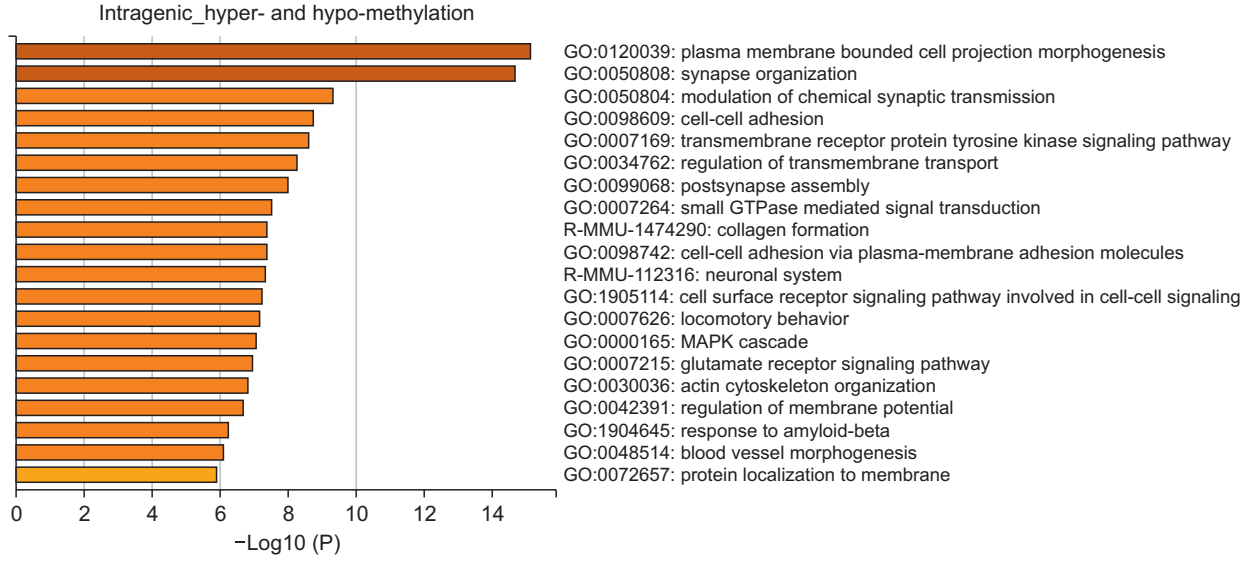

Figure 5. Metascape pathway analysis of differentially methylated regions at combined hypermethylated and hypomethylated intragenic regions. Morphogenesis of plasma membrane-bounded cell projections (GO:0120039) and synapse organization (GO:0050808) pathways were most significantly affected by black raspberry supplementation. MAPK, mitogen-activated protein kinase.

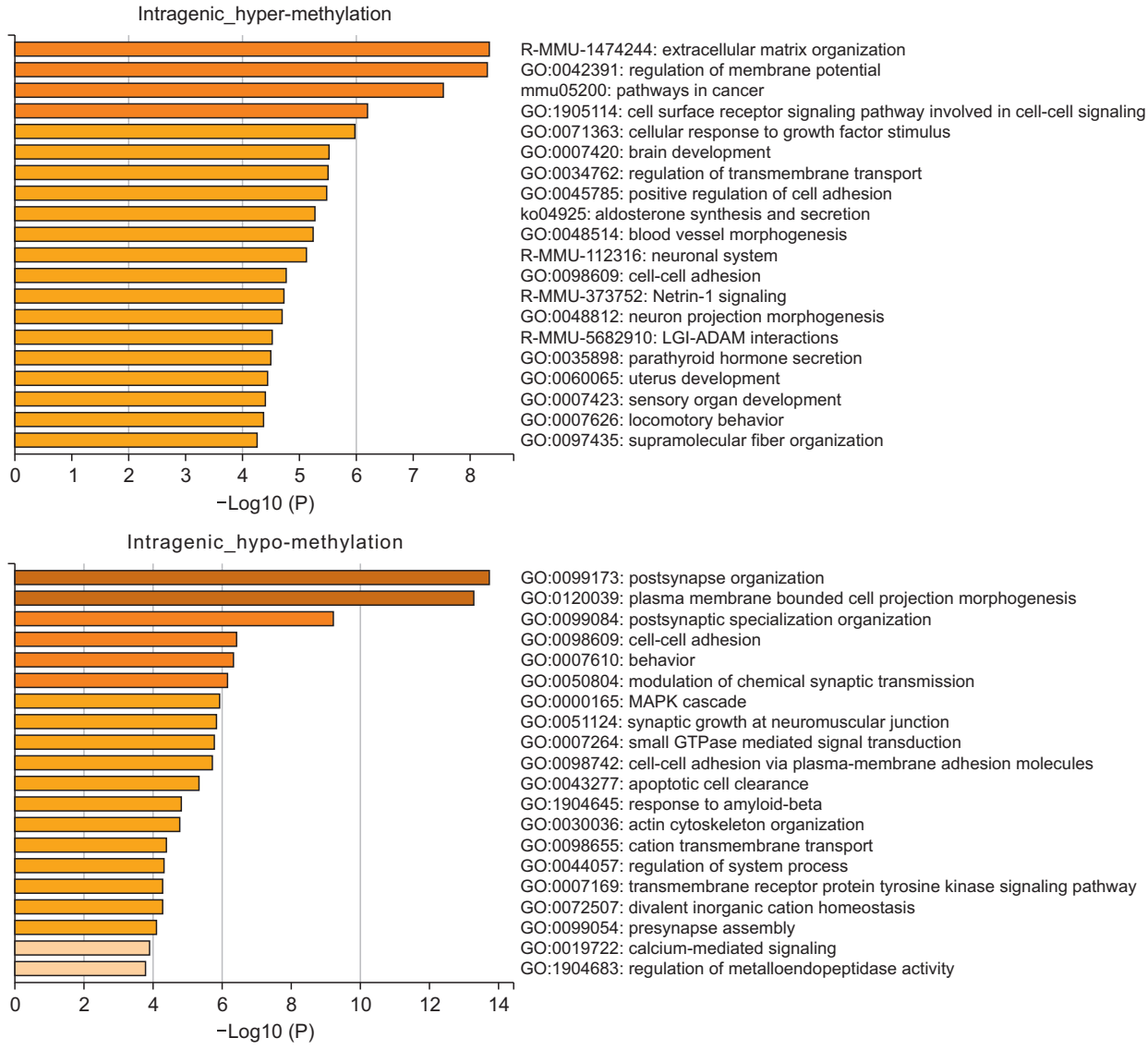

Figure 6. Metascape pathway analysis of differentially methylated regions (DMRs) at contrasting hyper/hypomethylated intragenic regions. Hypomethylation at these DMRs was associated mainly with postsynaptic organization (GO:0099173) and morphogenesis of plasma membrane-bounded cell projections (GO:0120039). Hypermethylation was associated with extracellular matrix organization (R-MMU-1474244) and regulation of membrane potential (GO:0042391). LGI-ADAM, leucine-rich, glioma-inactivated 1-a disintegrin and metalloprotease domain.

DISCUSSION

We determined in previous studies that a diet supplemented with BRBs has the potential to exert hypomethylating effects in both murine models and human patients. With *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice as a model, we found in the current study that BRBs also induce hypomethylation in an MDS-like phenotype. This hypomethylation mainly affects receptor signaling pathways, neuronal development, and cellular morphogenesis. At both TSS and intragenic sites, hypomethylation activates the maturation of neurons and synapses. In mice, a neurovascular bundle supports the marrow and regulates the proliferation of hematopoietic stem cells, which suggests that the effects on neuron-related pathways seen after the administration of BRBs may in turn affect the development of MDS [9]. However, Flores-Figueroa and Gratzinger [9] also note that this neurovascular bundle does not exist in humans, so this phenomenon may be limited to mice. The morphogenesis of plasma membrane-bounded cell projections was also modulated by hypomethylation, especially at intragenic sites. As previously discussed, dysplasia of hematopoietic cells is a key feature of MDS, and thus it is possible that the hypomethylation caused by BRBs promotes a more favorable morphology, though further research is required [2].

Hypermethylation of DMRs was also seen, mainly affecting the development and performance of the extracellular

matrix, cancer pathways, and development of the skeletal system. All of these pathways may be implicated in the development of MDS; for instance, issues with bone remodeling and osteoblast numbers have been linked with MDS [10]. The mmu05200 pathway implicated in cancer development has wide-ranging effects on a number of signaling pathways and carcinomas, making it difficult to discern exactly how its hypermethylation affects MDS; however, it is notably involved with lymphoid enhancer-binding factor 1 (associated with acute myeloid leukemia) and Bcl2 (associated with B cell leukemias and lymphomas).

It is nonetheless important to acknowledge that the pathways associated with these DMRs are multifaceted in their connections and effects. Therefore, further examination is necessary to better glean precisely which aspects of the pathways are affected by BRBs. However, this study suggests directions in which to target future research.

This study demonstrated that the supplementation of diet with 5% BRBs in *Vav-cre Asxl1*$^{fl/fl}$ *Tet2*$^{fl/fl}$ mice has distinct effects on hematopoietic pathways potentially related to the development of MDS. Our pilot clinical study illustrated the hypomethylating effects of BRBs in individuals diagnosed with myelodysplastic syndrome or myeloproliferative neoplasm [8]. Pathway analysis indicates that the intragenic hypomethylation induced by BRBs plays a role in prompting leukocyte differentiation. Discrepancies observed between the outcomes of mouse and human studies may stem from variations in disease stages—earlier stages in mice and later stages in humans. The viability of BRBs as an addition to the existing MDS treatment regimen will be dependent on additional research that continues defining the precise outcomes of the biochemical and physiological changes that result from BRB supplementation.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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