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LETTER TO THE EDITOR

TET-catalyzed 5-methylcytosine hydroxylation is dynamically regulated by metabolites

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Dear Editor,

DNA methylation suppresses gene expression and plays a broad and important role in diverse biological processes such as development and tumor suppression [1, 2]. DNA methylation is generally thought to be relatively stable and heritable. This view is recently changed by the discovery of TET (ten-eleven translocation) family of dioxygenases which catalyze three sequential oxidation reactions: converting 5mC first to 5hmC, and then 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC) [3-7]. A subsequent decarboxylation of 5caC could then lead to DNA demethylation [3]. While the biological function and catalytic mechanism of TET enzymes are being extensively investigated, much less is currently known about their regulation. TET proteins are the members of Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases, which require Fe(II) as a metal cofactor and α-KG as a co-substrate [8, 9]. In various types of tumors, pathological accumulation of three metabolites, 2-HG, succinate and fumurate, that share structural similarity with α-KG, results in a competitive inhibition of α-KG-dependent TET activity, leading to a hypermethylation phenotype [10, 11]. α-KG is produced and consumed mainly by four different metabolic pathways: as a key intermediate in the TCA cycle for energy metabolism, as an entry point for several 5-carbon amino acids to enter the TCA cycle (anaplerosis) after being converted into glutamate, being reduced back to isocitrate and then citrate for the eventual synthesis of acetyl-CoA, and being used as a co-substrate for multiple α-KG-dependent dioxygenases. These features of α-KG led us to investigate how a change in energy metabolism may influence TET activity *in vivo*.

We administered glucose in mice via an intraperitoneal injection protocol, which resulted in a rapid increase in blood glucose levels, reaching peak values at $t = 30$ min after injection, followed by a recovery at $t = 60$ min post injection (Figure 1A and Supplementary information, Figure S1A). GC-MS analysis revealed a rapid increase in hepatic α-KG levels that paralleled with the dynamic pattern of blood glucose, reaching peak values at $t = 30$ min and returning to normal level at $t = 60$ min post injection (Figure 1B , Supplementary information, Figure S1B). Strikingly, genome-wide 5hmC levels were rapidly increased in various mouse tissues (the liver, kidney, and muscle) within 30 min of glucose injection as determined by dot-blot analysis (Supplementary information, Figures S1C). LC-MS/MS analysis confirmed that global 5hmC level was rapidly increased by 1.4-fold ($P < 0.01$) in mouse livers within 30 min after glucose injection (Figure 1C and Supplementary information, Figures S1D). Notably, global 5hmC level rapidly declined/recovered at $t = 60$ min post glucose injection (Figure 1C), which may at least in part be due to the decrease of α-KG after glucose withdrawl. Global 5mC levels were not changed by glucose injection (Supplementary information, Figure S1E). Global 5fC levels in the mouse liver were elevated slightly at $t = 30$ min after glucose injection, but unlike those of 5hmC, continuously increased and reached a near-significant increase by 26.7% at $t = 60$ min after glucose injection (Supplementary information, Figure S1E). 5caC levels were below the threshold of reliable detection (data not shown).

Moreover, we found that high levels of blood glucose and hepatic α-KG were maintained for an additional 30 min ($t = 30+30$ min) after repeated injection (Figure 1A, 1B and Supplementary information, Figure S1A). Global 5hmC level was maintained at a high level in various mouse tissues at $t = 30+30$ min after repeated glucose injections as determined by both dot-blot and LC-MS/MS analyses (Figures 1C and Supplementary information, Figure S1C). Global 5mC levels were not substantially changed (Supplementary information, Figure S1E), while 5fC levels were significantly ($P < 0.05$) increased at $t = 30+30$ min after repeated glucose injections as compared to $t = 0$ min (Supplementary information, Figure S1E). The mRNA and protein levels of Tet2 and Tet3 were not altered in mouse livers after glucose shock (Supplementary information, Figure S1F). Moreover, Tet3 protein was predominantly in the nuclei of mouse hepatocytes at $t = 60$ min after single or repeated glucose injection, in-

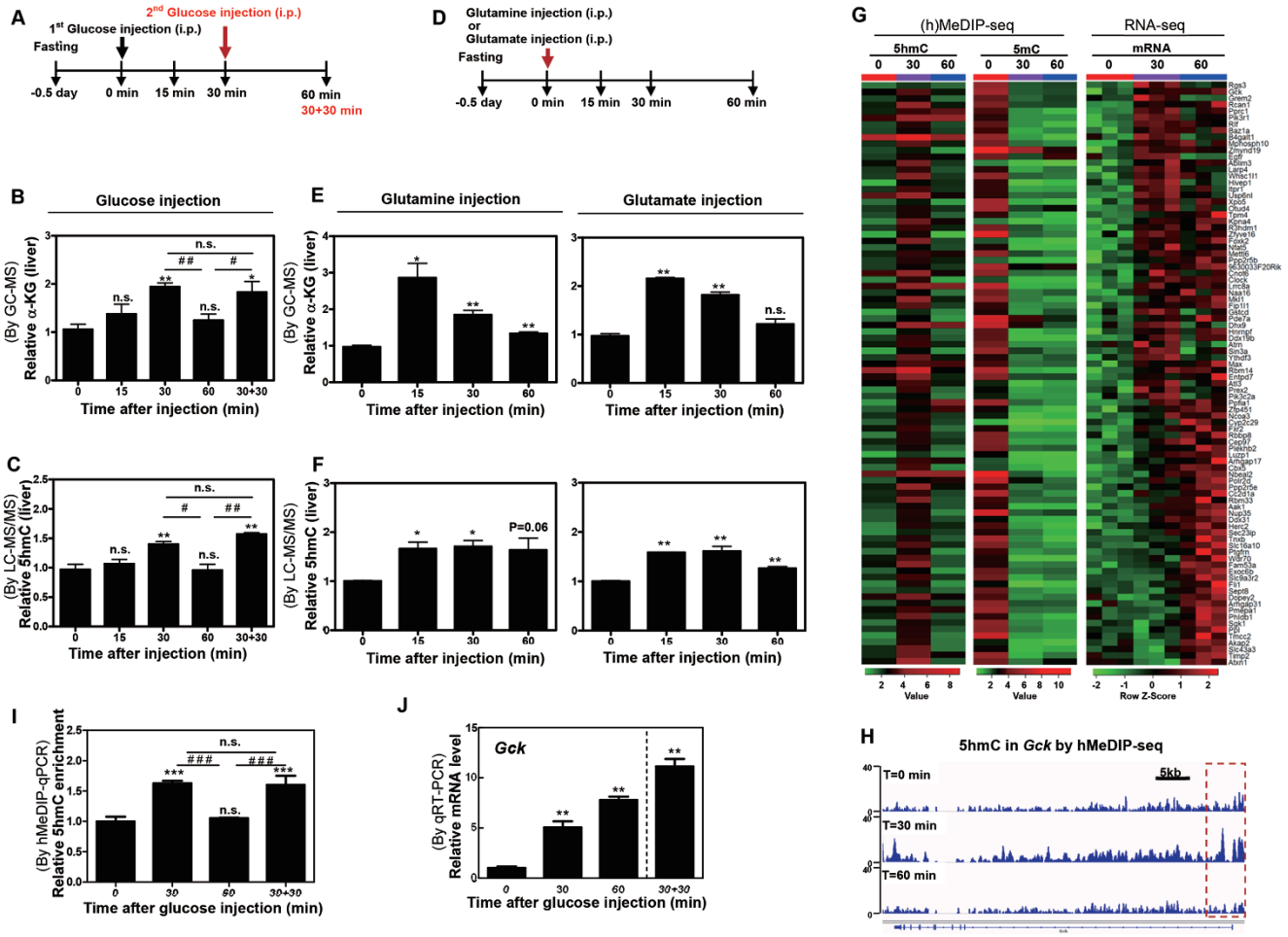

Figure 1 TET-catalyzed 5hmC production is dynamically regulated by metabolites. **(A)** The strategy for glucose injection in mice. **(B)** Relative α-KG levels in mouse livers at the indicated time points after glucose injection, as determined by GC-MS analysis. **(C)** Relative genome-wide levels of 5hmC in mouse livers at the indicated time points after glucose injection, as determined by LC-MS/MS analysis. **(D)** The strategy for glutamine or glutamate injection in mice. **(E)** Relative α-KG levels in mouse livers at the indicated time points after glutamine or glutamate injection, as determined by GC-MS analysis. **(F)** Relative genome-wide levels of 5hmC in mouse livers at the indicated time points after glutamine or glutamate injection, as determined by LC-MS/MS analysis. (G) Heatmaps of hMeDIP-seq, MeDIP-deq, and RNA-seq analyses in mouse livers at the indicated time points after glucose injection. Genes were listed, which displayed ≥ 1.3-fold increase in 5hmC and ≥ 2-fold decrease in 5mC at t = 30 min post glucose injection, and exhibited significant up-regulation in mRNA expression ($P < 0.05$ and fold change > 1.2 at either t = 30 or t = 60 min post glucose injection). A red to green color scale indicates values from high to low, and row-scaling is used to facilitate visualization. **(H)** hMeDIP-seq peaks in the *Gck* gene visualized in Integrative Genomics Viewer (IGV). One region labeled by a red-dotted box was shown as an enlarged drawing in Supplementary information, Figure S1H. (I) Relative 5hmC levels in one selected region of the *Gck* gene (chr11:5948151bp-5948348bp) in mouse livers at the indicated time points after glucose injection, as determined by hMeDIP-qPCR analysis. **(J)** mRNA expression of the *Gck* gene in mouse livers at the indicated time points after glucose injection, as determined by qRT-PCR analysis. Data shown represented the mean ± SD. **(B, C)** n = 2-5 per group; **(D-G)** n = 3 per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for comparison with t = 0 min; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ for the indicated comparison. n.s. = not significant.

dicating that glucose shock did not affect the subcellular localization of Tet3 (Supplementary information, Figure S1F). Therefore, the observed dynamic changes in global 5hmC levels in mouse tissues after glucose injection are apparently not caused by changes in Tet expression, protein stability, and subcellular localization. Collectively, our results support a notion that *in vivo* α-KG is a rate-limiting factor and that an increase in the intracellular concentration of α-KG resulting from glucose injection can rapidly, within 30 min, raise the levels of TET reaction product, 5hmC.

In addition to glycolysis, glutaminolysis can also contribute to α-KG production via converting glutamine to glutamate and then to α-KG. To further investigate

whether a change in α-KG levels may influence TET-mediated 5mC oxidation *in vivo*, mice were administered with either glutamine or glutamate via an intraperitoneal injection protocol (Figure 1D). GC-MS analysis demonstrated a rapid increase in α-KG levels in mouse livers, which reached peak values at t = 15 min after glutamine or glutamate injection (Figure 1E). Consistent with dot-blot results (Supplementary information, Figure S1G), LC-MS/MS analysis demonstrated that global 5hmC level was rapidly increased by 1.7-fold ($P < 0.05$) and 1.6-fold ($P < 0.01$) in mouse livers within 15 min of glutamine and glutamate injection, respectively (Figure 1F). Moreover, we found that a global decrease of 5mC level paralleled with the increase in 5hmC level after glutamine or glutamate injection (Supplementary information, Figure S1G). Together, these data further support the idea that α-KG is a critical determinant of the dynamic nature of 5hmC *in vivo*.

Emerging evidence indicates that 5hmC may directly contribute to gene regulation in addition to being an intermediate in the 5mC demethylation process. We thus carried out combined RNA-seq and hydroxymethylated DNA immunoprecipitation (hMeDIP)-seq analyses to identify genes whose transcriptional activation in mouse livers after glucose injection was associated with increased 5hmC and decreased 5mC (Figure 1G and supplementary information, Table S1A and S1B). Supporting a functional role of 5hmC in gene activation, we observed that the *Gck* gene, which encodes glucokinase (Hexokinase 4), was activated rapidly at t = 30 min after glucose injection, and this transcriptional activation was associated with remarkably increased 5hmC and decreased 5mC levels in the *Gck* gene (Figure 1G-1H and Supplementary information, Figure S1H). Glucokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P) and plays an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor. Interestingly, 5hmC at the same regions of *Gck* was rapidly decreased to an almost basal level at t = 60 min after glucose injection, while 5mC was less changed during glucose withdrawal (Figure 1G). In addition to *Gck*, other 89 genes were also identified whose transcriptional activation after glucose shock was found to be related with increased 5hmC and decreased 5mC levels (Figure 1G). Moreover, we have identified 732 genes whose mRNA expression was down- or up-regulated without significant changes in 5hmC levels at t = 30 and 60 min after glucose injection (Supplementary information, Table S1C and S1D).

To verify these sequencing data, we then performed hMeDIP-qPCR and MeDIP-qPCR analyses to track 5hmC and 5mC changes in the selected regions of *Gck*, respectively. Consistent with the hMeDIP-seq results, hMeDIP-qPCR analysis demonstrated that 5hmC was rapidly and significantly ($P < 0.001$) increased in one of the CpG-rich regions of *Gck* in mouse livers at t = 30 min after glucose injection (Figure 1I). Such a rapid gain of 5hmC was in parallel with a rapid decrease in 5mC at the same locus of *Gck* (Supplementary information, Figure S1H). Furthermore, 5hmC at the same region of *Gck* was maintained in mouse livers at t = 30+30 min after the first glucose injection if they received a second injection (Figure 1I). In contrast, 5mC levels remained at a low level at t = 60 min after single or repeated glucose injection (Supplementary information, Figure S1H). There was no change in 5hmC and 5mC levels in a control upstream region of *Gck* with comparable GC content in mouse livers after glucose injection (Supplementary information, Figure S1I). Finally, qRT-PCR analysis confirmed that the *Gck* gene became activated in mouse livers as rapidly as 30 min after glucose injection, and its expression levels were further upregulated by repeated glucose injection (Figure 1J). The expression of other glycolytic or gluconeogenic genes was, however, not significantly changed after glucose shock (Supplementary information, Figure S1J). Taken together, these results suggest that 5hmC can undergo dynamic changes, which is associated with the regulation of specific gene expression in response to altered cellular metabolic conditions.

How stable 5hmC modifications are in the genome is currently not known, but could have broad implications to the understanding of epigenetic control and gene regulation. One view holds that while the production of 5hmC is enzymatically catalyzed, its decline or loss in pre-implantation embryos is passively accomplished through a replication-coupled dilution mechanism [12]. Our results presented here reveal two novel features of 5hmC regulation. First, TET-catalyzed genome-wide 5hmC production can be dynamically regulated, and 5hmC decline can occur independently of DNA replication. Second, we provide evidence that α-KG, the co-substrate of TET enzymes and a key TCA metabolite, appears to be a critical factor in the regulation of 5hmC dynamics *in vivo*. Following the injection of glucose, glutamine or glutamate, the concentration of α-KG, which is dynamically and tightly regulated in response to cellular metabolic conditions, exhibits a pattern of increase/decrease that is closely correlated with that of 5hmC. The physiological significance of 5hmC dynamics is supported by the finding that 5hmC and 5mC levels in the promoter regions of *Gck* gene, a glycogen synthesis pathway gene, were rapidly increased and decreased, respectively, following high glucose shock. An important implication of this study is that besides developmental controls where

long-term stable gene silencing or derepression is more desirably achieved by the regulation of 5mC and 5hmC, additional cellular cues, such as metabolic conditions, could also influence the TET enzymes to regulate the status of 5mC/5hmC at specific loci and thus the expression of responsible genes. Moreover, we cannot exclude the possibility that glucose may affect DNA epigenetic modification by other mechanisms. A recent study reported that the nuclear localization and O-GlcNAcylation of TET3 was regulated by glucose metabolism [13], which was, however, not observed in our current study. It is also possible that glucose may affect the DNA repair pathway and thus demethylation. We show here that the increase of 5hmC can be maintained by repeated glucose injection, indicating that a hyper-5hmC level may be sustained by high glucose. How 5hmC is affected under hyperglycemia conditions and plays a role in gene regulation is another interesting question that warrants future investigations.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)