The many roles of TET1 in the post-implantation mouse embryo.

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Abstract: The TET dioxygenases mediate DNA demethylation in pre-implantation embryos and in primordial germ cells, yet limited studies address their contribution to the global gain of DNA methylation following implantation. Here, we discuss our recent study revealing that *Tet1* is expressed and functions non-redundantly in the early post-implantation mouse embryo. Ablating TET1 affects the methylation status of primed epiblast cells; however, the majority of gene expression regulation by TET1 seems to be independent of any gain or loss in methylation/hydroxymethylation due to TET1 omission. Interestingly, we reveal a gene repressive effect of TET1. Moreover, we show that loss of TET1 leads to developmental defects resulting in embryonic lethality with different penetrance depending on the genetic background of the mice.

Keywords: TET1, epiblast, extra-embryonic ectoderm, primed pluripotency, DNA methylation, transcription repression

Introduction

The mouse embryonic development involves several key stages. After fertilization, the totipotent single cell zygote (E0.5 days post coitum) will develop to form the different lineages of the body. Around the time of implantation (E3.5), the mouse embryo is composed of three distinct cell types: the trophectoderm, the primitive endoderm, and the inner cell mass (ICM). From E3.5-E6.25, the trophectoderm gives rise to the ectoplacental cone and extra-embryonic ectoderm (ExE), the primitive endoderm forms the visceral and parietal endoderm and the ICM will organize into a pluripotent epithelial layer called epiblast. The primitive streak cells appear at E6.5, followed by a series of events leading to the formation of the three lineages: ectoderm, mesoderm and endoderm. The latter stage is called gastrulation(*1*).

Highly dynamic changes in the DNA methylome occur during early mammalian embryogenesis, first as widespread loss of 5-methylcytosine (5mC) in the fertilized zygote, subsequently as *de novo* methylation in the epiblast following implantation of the blastocyst, and again as global demethylation and reprogramming of imprints during specification of primordial germ cells (PGCs) (*2, 3*). During these transitions, cells of the embryonic lineage experience two states of pluripotency – a naive ground state of hypomethylation reflective of the ICM in early blastocysts and PGCs, and a differentiation-prone primed state at peri/post-implantation (*4-6*).

The Tet-eleven translocation family (TET1-3) is a family of 2-oxoglutarate and Fe(II)-dependent dioxygenases capable of DNA demethylation by converting 5-methylcytosine (5mC) into 5-hydroxymethycytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (*7, 8*). Because the tight regulation of DNA methylation/demethylation is developmentally crucial(*9, 10*); the precise physiological functions of TET and 5hmC in mammalian development became topics of intense investigation. Several studies suggested that global waves of DNA demethylation are mediated by TET3 in the zygote; but subsequently other studies debated that and revealed that the TET3-driven accumulation of 5hmC observed in the late zygote is not required for the initial loss of paternal 5mC signal(*11*). The other two member of the family, TET1 and TET2, are highly expressed in embryonic stem cells (ESCs), in the ICM of E3.5-E4 embryos when the *de novo* methylation occurs and in PGCs

when demethylation and imprinting reprogramming arises(*12, 13*). Pluripotent stem cells have relatively high levels of 5hmC, which decrease during cellular differentiation(*7, 14, 15*). *Tet1* and *Tet2* together act downstream of Oct4 and regulate 5hmC levels in mouse ESCs (*14, 16*). An early study suggested that *Tet1* is required for ESC self-renewal(*15*). Several reports have subsequently showed that loss of *Tet1* do not affect mouse ESCs maintenance but rather affect their differentiation potential *in vitro(14, 17-19)*. In particular, ESCs depleted of *Tet1* display skewed differentiation towards endoderm and generate trophoblastic giant cells during teratoma formation(*14, 20*). TET1 was also reported to have a role in mouse PGCs development by regulating demethylation at a subset of meiotic genes(*21*), epigenetic regulation of genomic imprinting(*22*) and in neurons and brain tissues(*23*).

Because both *Tet1* and *Tet2* are expressed in the pluripotency state, the physiological importance of *Tet1* is likely subdued by *Tet2* in naive pluripotency. *In vivo*, *Tet1* and *Tet2* are relatively enriched in the ICM of the mouse blastocysts compared to the trophectoderm (*15*). Since *Tet1* and *Tet2* expression decreases to low levels in epiblast stem cells (EpiSCs), it was presumed that both genes are likely silenced soon after implantation(*24, 25*). However, even though EpiSCs are derived from epiblast of E6.0-E6.5 post-implantation embryos, *in vitro* they resemble the E7.5 anterior primitive streak (PS) of the early mouse gastrula. Recently, it was shown that ESCs adapted to 2iL (ground-state pluripotency) can be differentiated *in vitro* in presence FGF2 and Activin into epiblast-like cells (EpiLCs) that resemble molecularly the E5.5-6.0 “pre-streak” epiblast(*26*). In addition, Sohni et al(*27*) recently showed a dynamic reorganization of active cis-regulatory domains that sustains *Tet1* but rapidly silences *Tet2* during this naive-to-prime transitions of the epiblast, raising the question of TET1 involvement in the implanted epiblast when *de novo* methylome re-patterning occurs.

To understand the role of TET1 during embryonic development, several groups produced *Tet1* Knock-out (KO) mice but the phenotypes and the embryonic lethality of *Tet1*-deficient mice has been a subject of controversy. In a *Tet1* gene trap (GT) line (RRG140, donor strain 129P2/OlaHsd), embryonic lethality was observed in mixed strain background; after backcrossing for three to six generations to the C57BL/6J (B6) strain viable $Tet1^{GT/GT}$ mice were obtained(*22*). In another *Tet1* Knock-out B6 strain in which exons 9-12 encoding the catalytic domain are removed, partial embryonic lethality was observed (30% of KO embryos born viable)(*28*). However other studies, based on targeted mutant models with deletions of exon 5 or exons 11-13, reported that *Tet1* KO mice are born without any apparent loss *in utero*(*29, 30*) and suggested that TET1 is dispensable for embryonic development. Alternatively, recent studies have examined embryonic defects in combined *Tet* double and triple KOs (DKOs and TKOs)(*28, 31*). In particular, a recent study demonstrated severe gastrulation defects in *Tet* TKO embryos caused by the perturbation of Nodal-Lefty signaling(*31*). However, neither double KOs of *Tet1* and *Tet2,* nor of *Tet1* and *Tet3*, showed such defects(*20, 32*). The lack of phenotype in the latter two cases may be due to the hypormorphic deletions generated by *Tet1* KO strategies targeting 3’ exons. As a result of all those discrepancies, the contribution of TET1 to mouse embryonic development remained unclear. Recently, we aimed to clarify the importance of TET1 and identify its role in early post-implantation stage of the mouse embryo(*33*).

Authors’ results

In our research group, we defined kinetics of expression of *Tet1* and 5hmC profile in early pre- and post-implantation stages. We showed that *Tet1* is the only *Tet* paralog expressed, mainly in the epiblast (Epi) around E6.5 of post-implantation development. Further, we showed that *Tet1* expression diminished rapidly at E7.5 and persisted only faintly in the head folds and neural tube by E8.5. *Tet2* and *Tet3* were undetectable or lowly expressed at E6.5-8.5, consistent with previous reports(*31, 34*). After axial rotation of the embryo, expression of the three *Tets*

progressively increased in the developing brain of E9.5-10.5. In line with transcript detection of *Tet1*, we demonstrated by immunohistochemistry 5hmC enrichment in the E6.5 epiblast compared to the extra-embryonic ectoderm confirming that TET1 have a unique and non-redundant functions in the E6.5 embryo (Figure 1).

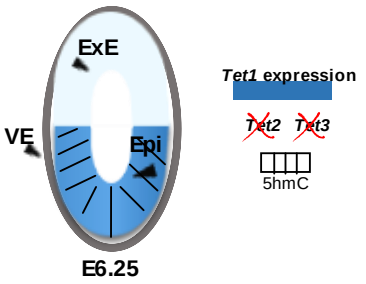

Figure 1: Schematic representation of different levels of *Tet1* expression in epiblast and extra-embryonic ectoderm of E6.25 embryos, where *Tet2* and *Tet3* are not detectable. 5hmC is enriched in the epiblast cells. VE, visceral endoderm; ExE, extra-embryonic ectoderm; Epi, epiblast.

We further validate *Tet1* expression profile using the TET1-Gt mouse reporter model, in which a β-galactosidase LacZ cassette is inserted in the intron 2-3 of *Tet1* reporting the expression and disrupting the downstream transcription(*21*). In addition, the X-gal staining revealed positive cells in the extra-embryonic amnion and chorion of E7.5-8.5 embryos. The specific expression of *Tet1* observed in the epiblast of early gastrulating embryos is consistent with a potential role in regulating germ-layer specification *in vivo*. Afterwards, we analyzed TET1 KO phenotypes in the *Tet1* gene-trap (GT) strain. Interestingly, $Tet1^{GT/GT}$ embryos obtained from backcross generations N≤3 appeared developmentally abnormal at E8.0-8.5 and none survived beyond E9.5, demonstrating fully penetrant lethality. Further backcrossing (N>5) viable *Tet1*-deficient offspring to escape lethality but those KO mice were born below the mendalian ratio as previously reported(*21*). Background-dependent variability in penetrance was previously reported in few mouse KO models (i.e. TGFβ 1 KO model)(*35*); moreover, background-dependent severity of certain diseases was described (i.e. Neural tube defects)(*36*). Interestingly, the TET1 KO mouse model shows a surprising contrast to the more commonly observed strain-dependent lethality in inbred KO mice that are relieved in outbred stocks(*35*). To validate the observed phenotypes, we generated a new B6 line containing a *Tet1*-targeted mutation ($Tet1^{tm1Koh}$), in which the LacZ reporter cassette is inserted immediately at the ATG start codon ablating the full coding sequence of *Tet1*. In this independent B6 strain, we again observed embryonic defects in KO embryos and by E9.5, several *Tet1*-null embryos showed deformities. Our phenotypic analyses of the TET1 KO in both strains revealed the importance of TET1 in the early mouse embryonic development and that a complete deletion of *Tet1* is enough to result in an abnormal embryogenesis.

In addition, the X-Gal staining performed suggested a low expression of *Tet1* in the ExE of E6.0-E6.5 embryos. Intrigued by the observation that *Tet1* is expressed at differential levels in both Epi and ExE lineages, we performed RNAseq on Epi and ExE samples separately collected from wt, Het and *Tet1* KO E6.25 B6 embryos. Analysis for differentially expressed (DE) genes, in the epiblast, suggested a precocious differentiation and entry into mesendodermal fate upon loss of TET1. In the ExE, an increase in genes involved in the oxidative phosphorylation was detected. This is in line with the notion that TET1 is preventing precocious differentiation, because a shift in metabolic dependence towards oxidative pathways is a hallmark of stem cell differentiation(*37*). Our results showing low expression and a role of TET1 in the ExE are without a doubt very interesting and provocative. This might reveal the basis of many epigenetic defects affecting the future placental development.

To analyse the effect of TET1 loss on the methylome at the primed state, we performed loss-of-function analysis using the *in vitro* counter-part of the primed epiblast, the Epiblast-Like Cells (EpiLCs). First, we analysed methylation and hydroxymethylation state in wt, *Het* and TET1 KO ESCs in serum and 2iL (ground state) cultures and in EpiLCs using dot

blot, mass spectrometry and DIP analysis. Although loss of TET1 in serum and 2iL-cultured *Tet*$^{Gt/Gt}$ ESCs had minimal effects on global 5hmC/5mC levels, we have observed loss of hydroxymethylation in TET1 KO EpiLCs compared to *Het* and wt cells. The maintenance of 5hmC levels in the absence of TET1 in ESCs correlates with recent study showing that *Tet1* depletion by *si*RNA shows only ~ 15% decrease in genomic 5hmC levels than Tet1 depletion(*38*). This could be due to a compensation effect on 5hmC levels by TET2 that is expressed as well in ESCs. In view of very low levels of *Tet2* and *Tet3* expression in the E6.5 mouse epiblast and EpiLCs, our results shows that TET1 is the predominant DNA oxygenase enzyme functioning in the primed epiblast and may have a key role in this cellular state.

We next confirmed a role of TET1 in shaping the methylome in the primed state by performing whole genome bisulfite sequencing (WGBS) in parallel bisulfite (BS) and oxidative bisulfite (oxBS), in wt and KO EpiLCs. This base resolution analyses allowed us to identify significant numbers of differentially methylated regions (DMRs) with predominantly higher levels of DNA methylation in the TET1 KO compared to wt. These DMRs overlapped predominantly gene body and promoter regions. In addition, we detected depressed levels of 5hmC in KO EpiLCs at CGIs. Subsequently, we performed TET1 antibody chromatin immunoprecipitation-sequencing (TET1 ChIP-seq) and showed that TET1-bound regions were globally centered at unmethylated sites but exhibited elevated 5mC and reduced 5hmC levels in KO compared to wt EpiLCs. Collectively, our results strongly suggest that TET1 regulates DNA methylation via hydroxymethylation at gene proximal promoters in the primed epiblast.

Surprisingly, the majority (70%) of DE genes were upregulated in the KO Epi, EpiLCs and ExE, suggesting a direct or indirect role of TET1 in gene repression and that the role of *Tet1* in regulating gene transcription may be as well independent of its catalytic demethylating activity. Indeed, it was shown that most TET1-target genes are still similarly affected by Tet1KD in *Dnmt* triple KO ES cells deficient in both 5mC and 5hmC(*17*) and multiple studies have implied a dual role of *Tet1* in both activation and repression of its target genes(*17, 19, 39-41*) but without any detailed clarification. To confirm this, we re-expressed TET1, either as a full-length wt transcript or a catalytic mutant that is unable to convert 5mC to 5hmC and identified several genes regulated by TET1 independently of its catalytic activity in EpiLCs. In addition, we identified a relatively unknown Jumonji-family member, JMJD8, which is downregulated upon loss of TET1. We showed by methylation and ChIP-qPCR that *Jmjd8* is a direct TET1 target. Then, we confirmed that JMJD8 is a transcriptional repressor by using a dual reporter assay and that a truncated form (N-JMJD8) is present in the nucleus and co-localizes with TET1-bound sites in EpiLCs. Others have suggested that TET1 recruits co-repressors including Sin3A/HDAC (*42*) and the Polycomb-repressor complex (PRC2) (Wu et al 2011). Here, we show another possibility of an indirect transcriptional repression by TET1 through *Jmjd8* down-regulation.

In addition, we attended to understand the basis of the difference in the penetrance between KO mice of mixed and inbred background. For that we derived ESCs from both B6 congenic and CD1 out-crossed TET1 GT mice and converted them further to EpiLCs. RNA-seq and qPCR analyses, performed on samples from both backgrounds, showed a considerable enhancement in the differential expression in the CD1 KOs relative to the KOs with B6 background. A short list of possible strain-specific modifiers was determined but further experiments are needed for validation.

In our study, we provide provocative fresh insights into several aspects of epigenetic regulation in early mouse embryonic development. Among other things, we addressed a major unresolved question in the field - the extent of functional redundancy among TET enzymes. Previously, all studies depicting the expression levels of *Tet1-3* focused on pre-implantation and post-gastrulation stages, and showed that two or all three TET enzymes are co-expressed which

has clouded efforts to identify the roles of each individual. Our analyses clarify their dynamic expression in the early mouse embryonic development and demonstrate that their spatial and temporal patterns of expression are largely divergent in the early post-implantation pre-streak stage. Importantly, we define a stage during mouse embryonic development, prior to gastrulation, where TET1 is solitary expressed and play a crucial non-redundant role.

We provide an explanation why previously studied *Tet1*-mutant mice, which ablated the TET1 catalytic domain, had little or no defects *in utero*(*28, 30, 43*), by analyzing two independent *Tet1* null mouse strains. In these alternative strains that ablate also the 5' coding sequence, severe post-gastrulation defects resulted, suggesting that while the catalytic domain of TET1 can be dispensable, further regulation by the N-terminal domain is critical for post-gastrulation events. We also demonstrated a hitherto unknown role of *Tet1* in extra-embryonic development. In addition, our studies highlight the highly complex and pleiotropic mechanisms used by TET1 to regulate its target genes in the primed state and that a deregulation in those mechanisms can lead to an abnormal embryonic development and might be the basis of post-natal epigenetic disorders.

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