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RESEARCH HIGHLIGHT

PGC7, H3K9me2 and Tet3: regulators of DNA methylation in zygotes

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In zygotes, a global loss of DNA methylation occurs selectively in the paternal pronucleus before the first cell division, concomitantly with the appearance of modified forms of 5-methylcytosine. The adjacent maternal pronucleus and certain paternally-imprinted loci are protected from this process. Nakamura *et al.* recently clarified the molecular mechanism involved: PGC7/Stella/Dppa3 binds to dimethylated histone 3 lysine 9 (H3K9me2), thereby blocking the activity of the Tet3 methylcytosine oxidase in the maternal genome as well as at certain imprinted loci in the paternal genome.

DNA methylation is a crucial epigenetic modification that regulates imprinting (differential silencing of maternal or paternal alleles) and repression of retrotransposons and other parasitic DNA, as well as possibly X-chromosome inactivation and cellular differentiation. DNA methylation needs to be faithfully maintained throughout the life cycle, since loss of DNA methylation can result in gene dosage problems, dysregulation of gene expression, and genomic instability due to retrotransposon reactivation [1]. Nevertheless, genome-wide loss of DNA methylation has been observed during germ cell development [2] and in the paternal pronucleus soon after fertilization [3].

For almost a decade, the global decrease of DNA methylation observed in the paternal genome within a few hours of fertilization was ascribed to an "active", replication-independent process [3]. The maternal pronucleus is spared and instead undergoes "passive", replication-dependent demethylation during early embryogenesis, arising from inhibition of the DNA maintenance methyltransferase Dnmt1 (Dnmt1 is normally recruited to newly-replicated DNA because of the high affinity of its obligate partner, UHRF1, for hemi-methylated DNA strands, which are produced from symmetrically-methylated CpG dinucleotides as a result of DNA replication). The basis for active and passive demethylation of the paternal and maternal genomes remained a mystery until proteins of the TET family – TET1, TET2 and TET3 in humans – were discovered to be Fe(II)- and 2-oxoglutarate-dependent enzymes capable of oxidizing 5-methylcytosine (5mC) in DNA [4-6]. TET enzymes serially convert 5mC into 5-hydroxymethyl-cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) [5, 7, 8].

With the generation of specific antibodies to 5hmC, it became clear that the supposed "active demethylation" of the paternal pronucleus in mouse zygote after fertilization was due to the inability of anti-5mC antibodies to recognize 5hmC and other 5mC oxidation products [9, 10]. The enzyme responsible for 5mC oxidation was shown to be Tet3, which unlike Tet1 and Tet2 is highly expressed in mouse oocytes and zygotes. RNAi-mediated depletion of Tet3 decreased the staining of the paternal pronucleus with 5hmC, suggesting that immediately after fertilization, Tet3 in the zygote selectively oxidizes 5mC in the paternal genome to 5hmC [9, 10].

How is the maternal pronucleus protected from Tet3 activity? Nakamura *et al*. [11] previously showed that zygotes lacking PGC7/Stella/Dppa3 lose asymmetric regulation of DNA methylation, instead showing global loss of 5mC staining in both paternal and maternal pronuclei. This was correlated with hypomethylation at several maternally-imprinted loci (*Peg1, Peg3, Peg10*) in *PGC7*-deficient zygotes, as judged by bisulfite sequencing. Further, certain paternally-imprinted loci (*H19, Rasgrf1*), which are normally protected from global loss of methylation in the paternal genome, also became hypomethylated in *PGC7*-deficient zygotes. These data suggested that PGC7 protects the maternal genome, as well as certain paternally imprinted loci, from loss of 5mC.

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In their recent publication, Nakamura *et al*. [12] elegantly extended these findings to address the mechanism involved. Based on the fact that a major difference between maternal and paternal genomes is that the maternal genome contains histones, whereas the DNA of the entering sperm is tightly packaged with protamine, they asked whether PGC7 recognizes specific histone marks. Indeed, the maternal genome harbors considerable levels of the histone mark H3K9me2 [11], leading them to examine whether PGC7 distinguishes maternal and paternal genomes by recognizing H3K9me2 in the maternal genome. Using wild-type (WT) ES cells and ES cells deficient in the G9a lysine methyltransferase which generates H3K9me2 mark, they showed that PGC7 associated loosely with nucleosomes and chromatin lacking H3K9me2, but tightly if H3K9me2 was present. The binding was recapitulated using recombinant bacterially-expressed PGC7 and histone tail peptides, indicating a direct interaction of PGC7 with the H3K9me2 mark. In agreement, genomic loci enriched with H3K9me2 recruited PGC7 as judged by chromatin immunoprecipitation (ChIP), but this recruitment was abrogated in *G9a*-deficient ES cells. These data indicated that PGC7 targets genomic regions occupied by nucleosomes containing H3K9me2 (Figure 1); an interesting extension would be to ask whether loss of maternal *G9a* also results in 5hmC conversion in the maternal pronucleus in zygotes.

Next, Nakamura *et al*. [12] tested by immunocytochemistry whether PGC7 in zygotes also required H3K9me2. It is known that H3K9me2 staining is concentrated in the maternal but not the paternal pronucleus [13]. Using conventional staining methods in which the cells are first fixed and then permeabilized to allow antibodies to enter the cell, the authors observed in their earlier study that PGC7 bound to both pronuclei [11]. Remarkably, by simply reversing the order of the fixation and permeabilization steps – permeabilizing first to allow the loss of loosely bound proteins by dissociation, then fixing and staining – they found that PGC7 associated much more tightly with the maternal pronucleus that bears H3K9me2 mark. Injection of mRNA encoding Jhdm2a (an H3K9me1/ me2-specific demethylase) into zygotes eliminated staining for H3K9me2 as well as PGC7 in the maternal pronucleus, and concomitantly caused loss of 5mC and acquisition of 5hmC. Taken together, these data strongly suggested that PGC7 was selectively recruited to the maternal pronucleus through binding H3K9me2, and that this binding protected zygotic maternal DNA from oxidation of 5mC to 5hmC and beyond (Figure 1).

These findings led Nakamura *et al*. to investigate how PGC7 controls Tet3 activity in zygotes. They showed (in cells that were permeabilized before fixation and immunocytochemistry) that Tet3 was tightly associated only with the paternal pronucleus in WT zygotes, but was present in both pronuclei in *PGC7*-deficient zygotes. When PGC7 was prevented from binding to the maternal pronucleus by injection of *Jhdm2a* mRNA, Tet3 became tightly associated with

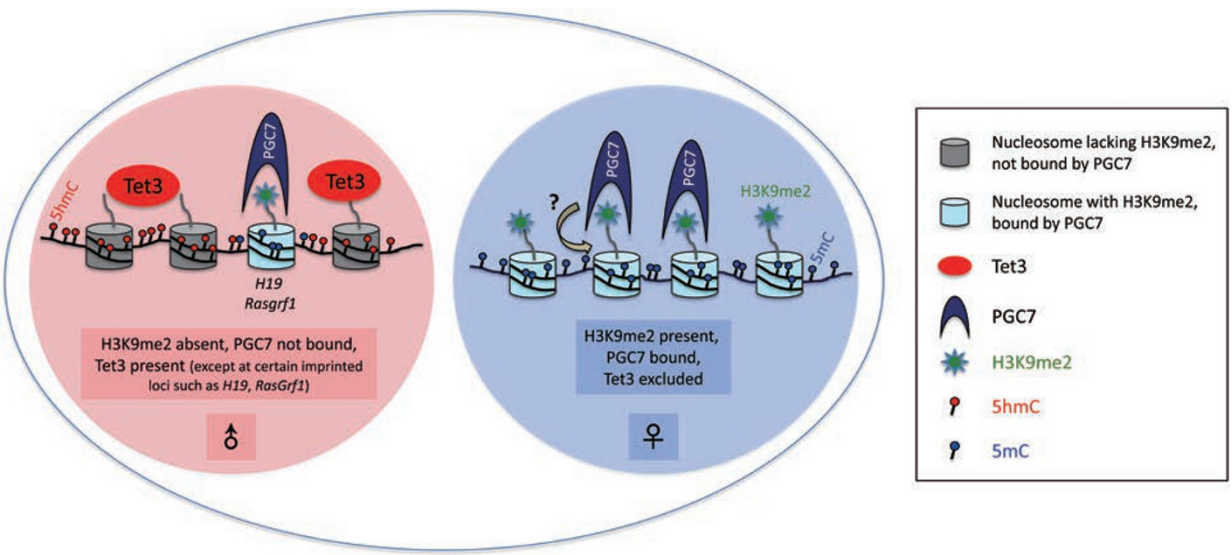

Figure 1 Schematic view of paternal (left) and maternal (right) genomes soon after fertilization. Paternal and maternal pronuclei are indicated with immunostaining results in the boxes. PGC7 binds H3K9me2 in the maternal pronucleus and at certain paternally-imprinted loci (*H19, Rasgrf1*) in the paternal pronucleus, thereby potentially regulating chromatin organization to interfere with Tet3 accessibility.

both pronuclei. In other words, loss of PGC7 or loss of H3K9me2 that recruits PGC7 had the same effect – eliminating selective association of Tet3 with the paternal genome. The implication is that PGC7 — which preferentially binds the maternal genome — somehow promotes the selective binding of Tet3 to the paternal genome, thus permitting rapid 5mC oxidation in paternal but not maternal DNA (Figure 1).

PGC7 is a small protein (150 amino acids (aa) in the mouse, 159 aa in humans) whose sequence is only moderately conserved. Nakamura *et al*. [12] showed that the binding of PGC7 to H3K9me2 required the N-terminal half of PGC7, whereas its ability to exclude Tet3 from the maternal pronucleus required the C-terminal half. It is unclear how Tet3 exclusion is mediated. One possibility is that the C-terminal region of PGC7 sterically excludes Tet3 from binding, either to DNA or to a chromatin mark; another is that the C-terminal region of PGC7 is capable of altering chromatin configuration to prevent the binding of Tet3 to chromatin. In support of the latter hypothesis, the rate with which micrococcal nuclease (MNase) digested high-molecular weight chromatin was significantly slower in WT ES cells in which PGC7 was present, compared to *PGC7*$^{-/-}$ and *G9a*$^{-/-}$ ES cells in which PGC7 was either absent or not recruited to DNA because of the loss of H3K9me2 mark. In contrast, DNA methylation did not alter the chromatin association of PGC7 or its ability to protect high-molecular weight chromatin from MNase digestion, as shown by using *Dnmt1*$^{-/-}$*Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ triple knockout ES cells that completely lack DNA methylation.

How does PGC7 protect paternally-imprinted loci from Tet3-mediated 5mC oxidation? Although the haploid sperm genome is mostly packaged with protamine, a genome-wide analysis revealed that 4% of the genome of mature human sperm bears nucleosomes located at developmental and imprinted genes [14]. Nakamura *et al*. [12] found that among paternally-imprinted differentially methylated regions (DMRs), the *H19* and *Rasgrf1* DMRs contained H3K9me2 whereas the Meg3 DMR did not, consistent with their previous finding that in *PGC7*-deficient zygotes, the *H19* and *Rasgrf1* DMRs were hypomethylated but the Meg3 DMR was unaffected [11]. Therefore, PGC7 may be recruited to paternally-imprinted loci through H3K9me2-containing nucleosomes that pre-exist in the sperm haploid genome upon fertilization. Alternatively, Nakamura *et al*. point out that protamine in the sperm is replaced soon after fertilization by the histone H3.3 variant, which in somatic cells does not bear H3K9me2 mark.

In conclusion, Nakamura *et al*. [12] demonstrate unambiguously that PGC7 specifically binds to H3K9me2 in the maternal genome in zygotes, where its global occupancy excludes Tet3 and inhibits Tet3-mediated 5mC oxidation. This novel finding provides new insights into the global alterations of DNA methylation status that occur during early embryogenesis. Follow-up questions abound. First, can PGC7 protect other methylated loci such as transposable elements and the X-chromosome? It would be interesting to assess H3K9me2 at these loci. Second, how does the N-terminal half of PGC7 recognize H3K9me2? Structural characterization of this interaction may elucidate a novel epigenetic "reader" domain specific for H3K9me2. Third, PGC7 is a marker for cells of the inner cell mass, and is co-expressed with Tet1 and Tet2 rather than Tet3 in ESCs [15]. Does PGC7 also antagonize Tet1 and Tet2 and protect imprinted loci in ESCs? Fourth, how does PGC7 inhibit the access of Tet3 to chromatin? Considering that PGC7 is small and is not equipped with known enzymatic domains, it is likely that PGC-interacting proteins, rather than PGC7 itself, function to regulate chromatin status. Fifth, how is Tet3 recruited to paternal chromatin – are there specific histone or other epigenetic marks that facilitate Tet3 recruitment? Finally, while technically challenging, it seems imperative to identify the target genes of PGC7 and Tet3, by profiling the genomic location of 5hmC and other 5mC oxidation products in the paternal and maternal genomes of zygotes from WT, *Tet3*-deficient and *PGC7*-deficient mice.

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