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5-hydroxymethyl-cytosine enrichment of non-committed cells is not a universal feature of vertebrate development

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5-hydroxymethyl-cytosine (5-hmC) is a cytosine modification that is relatively abundant in mammalian pre-implantation embryos and embryonic stem cells (ESC) derived from mammalian blastocysts. Recent observations imply that both 5-hmC and Tet1/2/3 proteins, catalyzing the conversion of 5-methyl-cytosine to 5-hmC, may play an important role in self renewal and differentiation of ESCs. Here we assessed the distribution of 5-hmC in zebrafish and chick embryos and found that, unlike in mammals, 5-hmC is immunochemically undetectable in these systems before the onset of organogenesis. In addition, Tet1/2/3 transcripts are either low or undetectable at corresponding stages of zebrafish development. However, 5-hmC is enriched in later zebrafish and chick embryos and exhibits tissue-specific distribution in adult zebrafish. Our findings show that 5-hmC enrichment of non-committed cells is not a universal feature of vertebrate development and give insights both into evolution of embryonic pluripotency and the potential role of 5-hmC in its regulation.

Introduction

DNA methylation (5-methyl-cytosine, 5-mC) is a major epigenetic modification of vertebrate genomes contributing to the regulation of gene expression during development.‡,§ Another form of modified cytosine, 5-hydroxymethyl-cytosine (5-hmC), has recently attracted considerable attention due to its possible involvement in ESC maintenance and differentiation.‡,§ 5-hmC is produced by enzymatic oxidation of 5-mC, catalyzed by Tet (Ten-11 translocation) proteins (Tet1/2/3),‡,§ which are, according to several reports, important for mouse ESC (mESCs) self-renewal and/or lineage specification.‡,§,8 Unlike 5-mC, 5-hmC is enriched in both mouse and human ESCs, compared with most differentiated cells.‡,8,10-13 The elevated levels of 5-hmC are lost upon ESC differentiation and reappear during the generation of induced pluripotent stem cells (iPSC); thus, the enrichment of this DNA modification correlates with a pluripotent state.‡,8,12 In addition, we have previously shown that 5-hmC signal is present throughout mouse pre- and post-implantation development, being highly enriched in embryos at morula and blastocyst stages.‡,8 Moreover, according to several reports 5-hmC is immunochemically detectable in mouse zygotes, where the paternal genome is subjected to a genome-wide 5-mC oxidation.‡,12,14,15 Genome-wide mapping studies of 5-hmC in mouse and human ESCs (hESCs) demonstrated that it is predominantly localized in enhancers and gene bodies, as well as in binding sites of pluripotency regulators Oct4 and Nanog‡,5,16,17 and it has been recently proposed that the balance between pluripotency and lineage commitment during development may be closely linked to the balance between hydroxymethylation and methylation in the genome‡ and, moreover, that Tet1 may play a key role in orchestrating the balance between pluripotent and lineage committed states.§

Since 5-hmC has been studied to date exclusively in mammalian systems, the data obtained using other vertebrate models may yield important insights into its function. Here we assessed the 5-hmC distribution during embryonic development of two non-mammalian model systems, zebrafish (Danio rerio) and chick (Gallus gallus).

Results

The dynamics of genomic 5-hydroxymethyl-cytosine distribution in early zebrafish development. Since the zebrafish
genome contains the homologs of all 3 mammalian Tet genes (see Materials and Methods), we looked at the dynamics of genomic 5-hmC content during zebrafish embryonic development. Previously we characterized the 5-hmC distribution in mammalian development and detected 5-hmC signal in a range of mouse embryonic and adult tissues. Notably, both mouse blastocysts and hESCs exhibited intense 5-hmC staining (Fig. 1A and B). However, we could not detect any 5-hmC signal in sections of paraffin-embedded zebrafish embryos at cleavage, blastula and gastrula stages under the same experimental conditions (Fig. 1C, 1,000 cells, dome, tailbud views and Fig. S1A). A detectable 5-hmC signal was first evident only in 10 somite stage embryos, and was then present throughout all later stages of zebrafish development (Fig. 1C).

Subsequently, high levels of 5-hmC were detected in both epithelial and mesenchymal cells in 24 h stage embryos, with particularly high levels in skeletal muscle cells (Fig. 1C, 24 h). In 36 h embryos, 5-hmC was detected in a wide range of embryonic tissues including the neural retina and the embryonic brain (Fig. 1C, 36 h; Fig. S1B). In addition we performed an immunostaining of adult zebrafish sections and found that, similar to mammalian organs, various fish tissues possess different levels of genomic 5-hmC. While neuronal, liver and some skeletal muscle cells exhibited pronounced 5-hmC signal, 5-hmC was undetectable in smooth muscle and the endodermal gut epithelium (Fig. S2). As expected, 5-methyl-cytosine was present in cells at all the stages of zebrafish development analyzed, and the global level of DNA methylation did not vary significantly between embryonic stages and adult tissues (Fig. 1C; Figs. S1 and S2).

5-hmC is not detectable by immunochemistry in pre-streak chick embryos. To check whether the developmental pattern of 5-hmC distribution observed in zebrafish is specific for bony fish species, or more widespread among non-mammalian vertebrates, we assessed the dynamics of 5-hmC content during chick (Gallus gallus) early development. Immunostaining experiments revealed that, similar to zebrafish, 5-hmC is immunochromically undetectable in the epiblast of pre-primitive streak chick embryos at 1 h and 3 h incubation stages (Fig. 2A). By contrast, a strong 5-hmC signal was observed in chick embryos at 6-somite stage of development (28 h incubation) (Fig. 2B; Fig. S3). Staining was not uniform, with some 5-hmC enriched cells localized in the head fold and endoderm, with less pronounced signal in somites and very weak staining in the epiblast (Fig. 2B; Fig. S3). Similar to zebrafish, the 5-hmC was detected throughout the stages of later chick development. Thus most embryonic tissues in 7 d stage embryos were strongly enriched in 5-hmC (Fig. 2C–G), with highest levels of this mark in the somites and neural tube (Fig. 2C, F and G). In addition 5-hmC was detected in a range of tissues of pre-hatching chick embryos, wherein most cells in the brain, heart and skeletal muscle as well as some cells of skin, intestine and liver, exhibited pronounced 5-hmC staining (Fig. S4). As in zebrafish, 5-mC signal did not vary between chick stages of development or embryonic tissues (Fig. 2; Figs. S3 and S4).

5-hmC is immunochromically undetectable in zebrafish pre-gastrulation embryos. As our immunoassaying procedure employs peroxidase-conjugated secondary antibody, we could
compare the kinetics of peroxidase reactions by quantifying fluorescence on serial adjacent embryonic sections (or identical cell culture slides) after different incubation times with fluorescent substrate (tyramide), which would allow us to semi-quantitatively assess the levels of 5-hmC in different specimens. Based on that, we compared the 5-hydroxymethylcytosine levels in zebrafish blastula embryos with the 5-hmC content of hESCs derived from an equivalent developmental stage (blasto-cyst). Quantification of our results revealed that the intensity of hESCs staining increased with time in a linear manner (Fig. 3A and B). 5-hmC staining of 24 h zebrafish embryos exhibited similar linear dynamics (Fig. 3B). By contrast, immunostaining of dome stage zebrafish embryos did not produce detectable 5-hmC signal at any time of incubation with tyramide (Fig. 3A and B).

To ensure that our staining results were not affected by tissue-embedding procedures we assessed the 5-hmC and 5-mC contents of total DNA extracted from hESCs or zebrafish dome stage embryos using a dot-blot assay. While 5-mC was present in both hESCs and zebrafish blastula embryos at similar levels, and 5-hmC signal was indeed observed in hESCs DNA, 5-hmC was undetectable in zebrafish dome stage embryos even at the highest concentrations of genomic DNA tested (Fig. 3C). These results agree with our immunohistochemical data, and we conclude that 5-hmC levels in blastula stage zebrafish embryos are, at best, several orders of magnitude lower than in hESCs.

Tet1/2/3 transcripts are either low or undetectable in zebrafish embryos before the onset of organogenesis. To investigate the expression of Tet genes in zebrafish development, we analyzed a whole-transcriptome gene expression time course data set (Zebrafish Genome Sequencing Group, Wellcome Trust Sanger Institute), which was queried for Tet1/2/3 genes, along with a range of reference markers (EF1α, actin α a1, Dnmt3, Ndr1 and Ndr2). In agreement with our immunostaining results, neither Tet2 nor Tet3 was expressed and only 25 reads mapping to Tet1 were identified in zebrafish embryos at the 2 cell stage (Fig. 4). Moreover, in gastrulating zebrafish embryos at 6 hpf, no expression was detected from any Tet gene (Fig. 4). Expression of the Tet1/2/3 starts between 6 and 24 hpf in zebrafish development, achieving the highest levels at 48 hpf. By contrast, the content of DNA methyltransferase Dnmt3 transcripts was higher during early development decreasing after 24 hpf and the orthologs of mammalian pluripotency regulators Oct4 (pou5f1) and Nanog were expressed exclusively in 2 cell stage and 6 hpf embryos (Fig. 4). As expected, the nodal related signaling molecules were only significantly expressed during early development, the expression of EF1α was relatively high at all the developmental stages and skeletal muscle actin α a1 exhibited high levels of expression only after the muscle formation at 24 hpf (Fig. S5).

Discussion

Our results show that whereas mammalian blastocysts and ESCs derived from them, are highly enriched for 5-hmC, this modification is immunohistochemically undetectable in relevant cells of zebrafish and chick embryos (Fig. 5). Since the conversion of 5-hmC into 5-formylcytosine (5-FC) and 5-carboxylcytosine (5-caC) is also catalyzed by Tet1/2/3 proteins,19,20 which are virtually not expressed during early zebrafish development according to our expression analysis (Fig. 4), it is extremely unlikely that these newly found forms of oxidized 5-methylcytosine are present at significant levels in pre-organogenesis zebrafish embryos. It is
All vertebrate embryos have multipotent non-committed cells before gastrulation, and a number of studies argue for evolutionary conservation of the core factors that regulate pluripotency in development. Nevertheless, whether the architecture of the pluripotency gene regulatory network is conserved between mammals and non-mammalian vertebrates is debatable. Thus, embryonic stem cell-like (ESC-like) cells derived from chick and fish embryos are able to contribute to somatic chimera, but have limited ability to contribute to the germ line, and do not self-renew indefinitely like bona fide ESC. A recent report suggests that multiple components of the pluripotency network are novel to mammals or have acquired new expression domains in mammalian development. Since an emerging consensus on a role for 5-hmC in mammalian ESCs implies its participation in maintaining the pluripotency gene regulatory network, it is therefore possible that 5-hmC enrichment in the DNA of early embryos is also a mammalian innovation and may account for such specific features of the mammalian pluripotency network as the ability to maintain long-term self-renewal, which has been unachievable for non-mammalian ES-like cultures to date. Alternatively, as a recent report brings the perceived role of Tet1, and therefore potentially, 5-hmC during development of mammals into question, our observation that 5-hmC enrichment in non-committed cells is not a universal feature of vertebrate development may contribute to elucidating the role of this modification in mammalian ESCs.

Materials and Methods

ES cell culture. HUES-7 hESCs were maintained on Matrigel in feeder-free conditions in conditioned medium (CM) and trypsin passaged. To prepare CM, mouse embryonic fibroblasts (MEFs) were mitotically inactivated with mitomycin C (MMC) (10 μg/ml, 2.5 h) and seeded at 4.8 × 10^6 cells per T75 flask. After 24 h the inactivated MEFs were incubated with DMEM-F12 supplemented with 15% KnockOut Serum Replacement, 100 μM β-mercaptoethanol (β-ME), 2 mM GlutaMAX, 1% nonessential amino acids (NEAA), and 4 ng/ml βFGF for 24 h. CM was then harvested and supplemented with an additional 4 ng/ml βFGF.

Pre-implantation mouse embryo culture and immunostaining. Mouse embryos were produced by mating super-ovulated F1 females with CD1 males and cultured according to standard procedures. The blastocyst stage embryos were immunostained as described in reference 12, using anti-5-hmC (Active Motif, 1:5,000 dilution) and anti-5-mC (Eurogentec, 1:200 dilution) antibodies and tyramide signal enhancement system (Perkin Elmer).

Zebrafish and chick embryo culture. Wild-type zebrafish embryos, obtained from in house breeding, were maintained at 28.5°C as described previously in reference 30. White Leghorn fertile eggs from Henry Stewart and Co., were incubated in a humidified incubator as previously described in reference 31. At the indicated time points, embryos were dissected, washed in PBS and stored in cold 4% paraformaldehyde until embedding.

Immunocytochemistry, immunohistochemistry and imaging. For immunocytochemistry cells were fixed in 4% formaldehyde for 15 min. Paraffin embedded formaldehyde fixed sections of zebrafish and chick embryos and adult tissues were used for immunohistochemistry. Tissue sections were de-waxed according to standard procedures. Cells and tissue sections were permeabilized for 15 min with PBS containing 0.5% Triton X-100, incubated in 4 N HCl for 1 h at 37°C and then neutralized in 100 mM TRIS-HCl (pH 8.5) for 10 min, followed by a standard
immunostaining protocol. Anti-5-hmC (Active Motif, 1:5,000 dilution) and anti-5-mC (Eurogentec, 1:200 dilution) primary antibodies were used. Peroxidase-conjugated anti-rabbit secondary antibody and the tyramide signal enhancement system (Perkin Elmer) were employed for 5-hmC detection. Control staining without primary antibody produced no detectable signal. Images were acquired using a Nikon ECLIPSE 90i immunofluorescence microscope and Volocity software.

**Image quantification.** Image quantification was performed using Fiji software. Slides with serial adjacent sections were processed in identical conditions with varying times of incubation with tyramide and were imaged at the same exposure settings. Mean intensities were measured for 10–20 random cell nuclei on each region of interest for each sample. Mean values of the mean intensities were plotted onto graphs. Experimental error is expressed as SEM.

**Dot blot assays.** Dot blot assays were performed as reported previously in reference 32, using anti-5-hmC (Active Motif, 1:5,000 dilution) and anti-5-mC (Eurogentec, 1:1,000 dilution) primary antibodies. Equal dilutions of hESCs and zebrafish embryonic DNA were loaded onto membrane. The dilution rate between two neighboring experimental points equaled 10x.

**Deep-sequencing based gene expression analysis.** Three Tet orthologs, Tet-1 (ENSDARG00000075230), Tet-2 (ENSDARG00000076928) and Tet-3 (ENSDARG00000062646) were identified in the zebrafish genome (version Zv9) by homology. Each gene has two identified transcripts, although only Tet1 has transcripts, which differ significantly from one another, with one not producing a protein. Zebrafish time course data set is deposited in the NCBI short read archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/?study=ERP000400). These data were provided by the zebrafish genome sequencing group at the Wellcome Trust Sanger Institute and cover 7 time points during development, 2 cell stage, 6 h post fertilization (hpf), 24 hpf, 48 hpf, 72 hpf, 120 hpf and adult head with an average of 56 x 10^6 mappable reads per sample. The reads were mapped to the Zebrafish genome (version Zv9.64) using Tophat version 1.2.33 The resulting bam files were processed using Htasq-count to generate a count file for each transcript.34 These data were further processed to calculate RPKM (Reads Per Kilobase of exon model per Million mapped reads) values for each gene according to.35 This data set was queried for each of the Tet genes, along with a range of suitable reference markers.

**Figure 4.** The expression of Tet1/2/3, Dnmt3 and the pluripotency regulators’ orthologs pou5f1 and Nanog during indicated zebrafish developmental stages. The left y-axis and bars show RPKM (Reads Per Kilobase of exon model per Million mapped reads) values, while the right y-axis and crosses identify the absolute transcript count.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 5. A schematic representation of 5-hmC distribution throughout the developmental stages of mouse, zebrafish and chick. The cells in non-committed state are indicated.


