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Preferential demethylation of DNA cytosine on the chromosomes restricted to germ cells in the spermatocytes but not the spermatogonia in the inshore hagfish, Eptatretus burgeri

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Abstract

Chromosome elimination and chromatin diminution occur in various species including single-cell ciliates and several multicellular animals. DNA methylcytosine (5mC) and histone modifications have been identified as markers of the eliminated DNA and chromatin in ciliate and finch. Here we examined the levels of 5mC and 5-hydroxymethylcytosine (5hmC; an intermediate of active DNA demethylation) in the male testicular cells of the inshore hagfish Eptatretus burgeri and simultaneously detected germline-restricted repetitive sequences (EEEb1) to identify the chromosomes restricted to germ cells (E-chromosomes). We detected 5mC and 5hmC signals at all chromosomes in the spermatogonia and in all of the interphase nuclei whereas 5mC signals were selectively located on the chromosomes without EEEb1 signals in the spermatocytes’ metaphase, suggesting no 5mC signal on the E-chromosomes. No significant difference in 5hmC levels between the E-chromosomes and the other chromosomes, was detected in the spermatocytes. This chromosome-specific hypomethylation has never been detected in mouse or zebrafish germ cells. These results therefore suggest that the DNA methylation pattern of the E-chromosomes, namely those presumptively eliminated in somatic differentiation, are altered just before or during meiosis. This exclusive alteration of the methylation pattern may play a key role in the chromosome elimination in hagfish species’ embryogenesis.

Keywords: DNA methylation, chromosome elimination, chromatin diminution, hagfish

Introduction

In general, genomic DNA is conserved in all cells of multicellular organisms throughout their development and differentiation. However, it is known that in some species, the germ and somatic cells show different numbers of chromosomes and DNA amounts. The first example of this exceptional phenomenon was discovered in a nematode, Parascaris equorum (Boveri 1887). In P. equorum, all somatic cells have only a chromatin fraction, whereas parentally derived large chromosomes have been completely maintained in the germline cells. Similar processes were reported in several species of flies, chironomids, copepods, hagfish, and sea lamprey (Tobler 1986; Smith et al. 2009). In Tetrahymena, a ciliated protozoan, over 90% of the micronuclear DNA (germline genome) including several thousand internal eliminated sequences (IESs) is precisely excised from their macronucleus (somatic genome) during nuclear differentiation, and thus this genome-wide DNA rearrangement is also considered an analogous event (Yao et al. 2014). These phenomena are striking events that result in both qualitative and quantitative differences between the germline and somatic genome, although the elimination process differs in various aspects among species.

Kohno et al. (1986) reported the chromosome elimination in a jawless vertebrate Eptatretus burgeri, a similar phenomenon was observed at least seven other hagfish species belonging to two families, Eptatretidae and Myxinidae (E. stoutii, E. okinoseanus, E. cirrhatus, Paramyxine atami, P. sheni, Myxine glutinosa, and M. garmani). In those species, two to 62 chromosomes and 20.9%–74.5% of the total germline genomic DNA are expelled from presumptive somatic cells (Kohno et al. 1998). The chromosomes restricted to germ cells (E-chromosomes) are mostly composed of constitutive heterochromatin, and 16 families of highly repetitive DNA sequences have been characterized as eliminated elements (Kubota et al. 1993, 1997, 2001; Goto et al. 1998; Kojima et al. 2010) to date.

An understanding of how cells recognize the chromosomes to be eliminated is likely to provide important insights into the molecular mechanisms of DNA eliminations. Studies of chromosome elimination in various organisms revealed that heterochromatinization is a common feature of the eliminated chromosomes or chromatin (Tobler 1986). In the ciliates Tetrahymena thermophila and Oxytricha trifallax, not only the methylation of histone H3 lysine 9 and 27 (H3K9me and H3K27me), but also DNA cytosine methylation (5-methylcytosine; 5mC) are observed in eliminated DNA regions, specifically during the process of programmed DNA elimination (Taverna et al. 2002; Liu et al. 2007;
Postberg et al. 2008; Bracht et al. 2012; Horrell and Chalker 2014). In Acritotus lucidus (Diptera, Chironomidae), post-translational modifications of histone H3 have a crucial role in chromatin diminution (Staiber 2012). These results suggest that epigenetic modifications may have a role in the recognition of the eliminated chromosomes (chromatin) in hagfishes.

In many animals and plants, the 5mC of the CpG dinucleotide is often associated with their gene expression status. Recently, 5-hydroxymethylcytosine (5hmC) was identified as an intermediate for the replication-dependent and/or replication-independent demethylation of 5mC by Ten-eleven Translocation (TET) family members (Tahiliani et al. 2009; Gu et al. 2011; Hackett et al. 2013). A study of a double knockout mouse of Tet1 and Tet3 suggested that 5hmC also acts as a key regulator for the early development and the reprogramming during gametogenesis (Kang et al. 2015). In the present study, we examined the distribution of 5mC and 5hmC among the metaphase chromosomes and interphase nuclei in testes from the inshore hagfish, Eptatretus burgeri, to test the above-mentioned possibility that 5hmC acts as a key regulator for the early development and the reprogramming during gametogenesis. In this study, immunofluorescence (IF) staining clearly revealed that 5mC signal was completely excluded from certain chromosomes in meiotic spermatocytes but not mitotic spermatocytes and spermatogonia. By the combination of fluorescence in situ hybridization (FISH) using a germline-restricted highly repetitive DNA family, EEEb1 (eliminated element of E. burgeri repetitive DNA family, EEEb1 (eliminated element of E. burgeri) and IF staining of 5mC, all of certain chromosomes in meiotic spermatocytes but not mitotic spermatocytes and spermatogonia. By the combination of fluorescence in situ hybridization (FISH) using a germline-restricted highly repetitive DNA family, EEEb1 (eliminated element of E. burgeri 1; Kubota et al. 2001) and IF staining of 5mC, all of the 5mC-negative chromosome has EEEb1 signals. This is the first report of an epigenetic approach to chromosome elimination in a hagfish species, and our results strongly suggest that DNA demethylation during meiosis may act as a hallmark of the subsequent chromosome elimination.

Materials and methods

Animals and chromosome preparations

The inshore hagfish E. burgeri was collected from Katase Bay in Kanagawa, Japan. Chromosomes from the testes were prepared as described with slight modifications (Evans et al. 1964; Kohno et al. 1986). After hypotonic treatment with 0.075M KCl for 10 min, the testis was fixed with methanol/acetic acid (1:1) fixative solution for 10 min at room temperature (RT). The fixative solution was then changed to a 60% methanol/acetic acid (1:1) fixative solution. After a 5-min incubation, lobules consisting of many follicles were gently pulled out from testes cells with the use of fine forceps and unraveled. When the lobules appeared “flat” and opaque, the supernatant fluid was transferred into a centrifuge tube and centrifuged at 1,500 rpm for 5 min at RT. The supernatant was discarded, and the collected cells were resuspended in methanol/acetic acid (3:1) fixative. After several exchanges of fixative, the cells were stored at −20°C.

Immunofluorescence staining for 5mC or 5hmC, and Immuno-FISH using EEEb1

Chromosomes slides prepared by an air-dry technique were treated for 30 min at 3°C with RNase A (Sigma Aldrich, St. Louis, MO, USA) at 100 μg/ml in 1× standard saline citrate (SSC). After three washes for 2 min each time with 2× SSC, the slides were dehydrated through a 70% and 100% ethanol series and air-dried. Chromosomal DNA was then denatured in 70% formamide/2× SSC at 70°C for exactly 2 min and then immediately dehydrated in 70% ethanol for 10 min on ice. The slides were treated with 100% ethanol for 10 min at RT, followed by air-drying. The slides were incubated in blocking buffer (3% bovine serum albumin [BSA]/Dulbecco’s modified phosphate-buffered saline) for 30 min at RT, and then with a primary mouse monoclonal anti-5mC antibody (1:500, Active Motif, Carlsbad, CA) or rabbit polyclonal anti-5hmC antibody (1:500, Active Motif) in blocking buffer overnight at 4°C.

The slides were then washed three times for 10 min in Tris-buffered saline containing 0.05% Tween 20 (TBST) at RT and then incubated with an Alexa488-conjugated rabbit anti-mouse IgG antibody (1:5000, Cell Signaling Technology, Beverly, MA) for 5mC or an Alexa555-conjugated mouse anti-rabbit IgG antibody (1:5000, Cell Signaling Technology) for 5hmC at RT. Two hours later, the slides were washed three times for 5 min each time in phosphate-buffered saline with Tween 20 (PBST), and chromosomal DNA was counterstained for 5 min in TBST containing 0.4 μg/ml Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA). Finally, the slides were mounted with the Prolong Gold (Life Technologies).

For the immuno-FISH analysis, after final washing, slides were postfixed for 10 min in 2% paraformaldehyde/TBST at RT and rinsed in TBST three times for 10 min each time at RT prior to FISH. The FISH was performed using the plasmid inserted EEEb1 as the probe. The DNA probe was labeled with digoxigenin-11-dUTP by DIG-Nick Translation Mix (Roche, Mannheim, Germany) in accord with the manufacturer’s instructions.

After precipitation with 25 μg of yeast tRNA, the labeled probe DNA was thoroughly resuspended in 50 μl of formamide, and denaturation was then performed at 70°C for 10 min. The denaturation of chromosomal DNA hybridization, washing and detection were performed as described (Kubota et al. 1993). Again, chromosomal DNA was denatured with 70% formamide/2× SSC at 70°C for 2 min and then immediately dipped in ice-cold TBST. The mixture of the probe DNA (10 μl; 200 ng) and the hybridization solution (10 μl) containing 2 mg/μl BSA (Roche), 10% dextran sulfate (Pharmacia Biotech, Little Chalfont, UK), and 2× SSC was deposited onto the denatured chromosomes on each slide, and then each slide was covered with Parafilm and incubated overnight at 37°C in a dark humid chamber.

The slides were then extensively washed through a wash buffer series: 2× SSC/0.05% Tween 20 for 10 min, 50% formamide/0.5× SSC for 20 min, 2× SSC/0.05% Tween 20 for 20 min at 42°C, and TBST for 5 min at RT. For the detection of hybridized probe DNA, slides were incubated in TNB buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.5% blocking solution [Roche]) for 30 min at 37°C and anti-DIG solution (TNB buffer containing anti-DIG fluorescence Fab fragments [4 ng/μl; Roche]) for 45 min at 37°C in a dark humid chamber. After the slides underwent three washes in TBST, chromosomal DNA was counterstained for 5 min in TBST containing 0.4 μg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA), followed by a finally wash in TBST.

Slides were then mounted using the Prolong Gold (Thermo Fisher Scientific). All images of immunofluorescence and FISH were collected using a light microscope (Axio Imager
Results

Hypomethylation of the E-chromosomes in spermatocytes but not spermatogonia

To investigate the chromosomal distribution of both 5mC and 5hmC in hagfish germ (testis) cells, we performed immunofluorescence staining using antibodies against 5mC and 5hmC raised in mouse and rabbit, respectively. The histological analysis of the hagfish testes was well described by Patzner (1977): the testes are composed of lobules that consist of many follicles of different sizes, and spermatogenesis occurs synchronously in all follicles. We observed not only the interstitial cells which are morphologically equivalent to Leydig cells but also sertoli cells in these follicles, similar to higher vertebrates.

The immunostaining of 5mC and 5hmC showed various signal patterns in interphase cells (Fig. 1) in good agreement with the above observation. In a total of more than 400 nuclei of the interphase cells from 15 testes, most of the nuclei (70.3%) showed 5mC and 5hmC signals uniformly distributed as small pinpoint signals in the entire nuclei (Fig. 1A). Our colocalization analysis using ImageJ revealed that approx. 12% of the 5hmC signal coincided with 5mC signal (Fig. 1B). In contrast, 11.3% of the cells with small nuclei had intensive signals of both 5mC and 5hmC (Fig. 1A,C). Another 12.5% of the nuclei also showed strong 5mC signals, whereas the 5hmC signals were mostly similar to those in the majority of cells (Fig. 1A,C).

In the metaphase spreads, we were able to easily distinguish the germ cells (spermatogonia and spermatocytes) from the supporting somatic cells, because the chromosome number was different between the germ and somatic cells (Kohno et al. 1986). In the meiotic metaphases of the first spermatocytes, most of the chromosomes had the apparent signals of 5mC, whereas several small associated E-chromosomes had less signals (Fig. 2A and C). In contrast to 5mC, 5hmC signals were detected on all of the chromosomes in more than 80% of the metaphases. In the mitotic metaphases of the spermatogonia, however, 5mC and 5hmC signals were clearly detected on all of the chromosomes in >90% of the metaphases (Fig. 2B and D).

Hypomethylated chromosomes in the spermatocytes were restricted to germ cells, namely E- chromosomes

To explore whether the chromosomes lacking 5mC signals are the germline-restricted chromosomes, we next performed the simultaneous detection of 5mC and EEEb1. EEEb1 is one of the germline-restricted, highly repetitive DNAs, and it is located on all E-chromosomes (Kubota et al. 2001). As shown in Figure 3A, 5mC signals were uniformly detected throughout the entire nucleus of most of the interphase cells, whereas EEEb1 signals tend to make

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Figure 1. Multicolor immunofluorescence with anti-5mC antibody (green) and anti-5hmC antibody (red) in E. burgeri germ cells. Low magnification (A) and high magnification (B) images in testicular cells. Arrows and arrowheads in panel A show the cells with high 5mC signal and the cells with both high 5mC and 5hmC signals, respectively. Arrowheads in panel B show the colocalization of 5mC and 5hmC signals. Nuclei were counterstained with Hoechst (blue). C: The relative frequency of the type of cell showing the four types of 5mC and 5hmC signal patterns. Interphase nuclei of testicular cells were classified by signal intensities of 5mC or 5hmC as follows: ++: fluorescence signal (integrated brightness/signal area) was more than four times as high as the mean fluorescence signal of total 400 nuclei of testicular cells (mean signal intensity). -: fluorescence signal was less than one quarter of the mean signal intensity. Remaining nuclei were classified as “+”. Scale bar=10 μm.
DNA cytosine methylation in *Eptatretus burgeri*

We also detected 5mC signals on all of the chromosomes of the spermatogonia, although the fluorescent intensity varied slightly among the chromosomes (Fig. 3B). In the spermatocytes however, 5mC signals were hardly detected on the chromosomes harboring EEEb1 signals, although 5mC signals could be easily detected on the other chromosomes (Fig. 3C). These results indicated that all of the chromosomes in the mitotic cells were methylated, but E-chromosomes were unmethylated or hypomethylated in meiotic cells.

**The demethylation activity was uniform in all chromosomes in the germ cells**

The most likely explanation for the above results is the preferential demethylation of the E-chromosomes during meiosis. 5mC is enzymatically demethylated by the Tet methylcytosine dioxygenase Tet1-3 through three consecutive oxidation reactions (Tahiliani *et al.* 2009). 5hmC is one of the intermediate products of these oxidation processes; thus, DNA demethylation activity can be estimated from the level of 5hmC. As shown in Figure 4A, 5hmC signals were mostly found in the whole interphase nucleus containing the region corresponding to EEEb1 signals. If the E-chromosomes are exclusively demethylated before meiosis, 5hmC appear to be preferentially detected on the nucleoplasm harboring EEEb1 signals. However, we could not detect those cells.

In the mitotic chromosome spreads of the spermatogonia, 5hmC signals were also detected on all chromosomes. There was no difference between the chromosomes harboring and not harboring EEEb1 (Fig. 4B). In the meiotic chromosome spreads of spermatocytes, 5hmC signals were frequently detected on the small, dumbbell-shaped E-chromosomes as the other chromosomes (Fig. 4C).

**Discussion**

We here newly observed the possibility of a relationship between DNA cytosine methylation and chromosome elimination in *E. burgeri*, a lower vertebrate. The chromosomes to be eliminated were hypomethylated, whereas other chromosomes were rather methylated in the spermatocytes. Because those differential methylation statuses have never been detected in mitotic spermatogonia, the demethylation of specific chromosomes appears to occur in meiosis. Programmed DNA rearrangement such as chromosome elimination has been described in single-cell ciliates (Protozoa) and a multitude of multicellular animals including over 100 species. A common feature in these organisms that exhibit DNA elimination is the discarding of the heterochromatin including large amounts of repetitive sequences, although some genes are also eliminated in several species (reviewed by Wang and Davis 2014). Recent studies on *Oxytricha* and *Stylonychia* indicated that eliminated DNA-specific de novo methylation was observed on repetitive elements during...
Macronuclear development (Bracht et al. 2012; Juranek et al. 2003). Histone 3 lysine 9 and lysine 27 trimethylation (H3K9me3 and H3K27me3) was also deposited on DNA destined for elimination in ciliates and finch (Taverna et al. 2002; Lui et al. 2007; Schoenmakers et al., 2010; del Priore and Pigozzi 2014). These results revealed that a number of epigenetic modifications are associated with chromatin diminution/chromosome elimination in hagfish species, although the methylation/demethylation distribution is opposite in *E. burgeri*.

It is generally believed that DNA methylation correlates with the genome stability. James et al. (1999) suggested that DNA hypomethylation might increase the chromosome nondisjunction. Therefore, segregation defects caused by the hypomethylation of specific chromosomes might be involved in the chromosome elimination in hagfish species.

We also observed various patterns of 5mC and 5hmC signals in the interphase nuclei of the hagfish testes. The localization of 5mC and 5hmC in the germ cells has been well investigated in mice. In the interphase nuclei of undifferentiated germ cells at embryonic day 13.5, the 5mC colocalized with the 5hmC, whereas the nuclei...
of prospermatogonia showed a rather uniform 5mC staining, consistent with the occurrence of global de novo methylation. In these nuclei, the 5hmC appeared mainly in the nuclear periphery (Li et al. 2013). In the case of adult male germ cells, however, 5mC and 5hmC were distributed in the whole nuclei, and 80%–90% of all CpG dinucleotides in sperm was fully methylated (Mayer et al. 2000; Oswald et al. 2000). In good agreement with these reports, we obtained similar results in our experiments. These results suggested that variations of 5mC and 5hmC localization patterns in hagfish germ cells are attributed to the developmental stages of the germ cells during germ cell development.

The mechanisms of DNA methylation and demethylation in fish are generally conserved in mammals, because zebrafish possess most of the basic enzymes for DNA methylation and demethylation (DNA methyltransferase, Dnmt 1, 3a/b, TET family and MBD/MECP families: Goll and Halpern 2011; Vastenhouw et al. 2010; Wu et al. 2011). In addition, the global DNA demethylation and re-establishment during early embryogenesis seen in mammals has also been observed in zebrafish (McGowan and Martin

Figure 4. Distributions of 5hmC and EEEb1 in the testicular cells from E. burgeri. An interphase nucleus (A), metaphase chromosomes in a spermatogonium (B) and a spermatocyte (C) were immunostained by anti-5hmC antibody (red) and hybridized with labeled EEEb1 probes (green). Nuclei and chromosomes were counterstained with Hoechst (blue). Scale bar=10 µm.
2002; Mhanni and McGowan 2004; MacKay et al. 2007). In mice and zebrafish, chromosome-specific demethylation has never been detected during meiotic germ cell development, suggesting that the hypomethylation of the chromosomes to be eliminated might have a special role in hagfish species. Further examinations of the distribution of methylation/demethylation machinery (DNMT and TET families) and histone modifications will contribute to our understanding of the molecular mechanisms and biological significance of the chromosome elimination in hagfish species.

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