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RNA-directed epigenetic regulations of DNA rearrangements

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Abstract

Ciliated protozoa undergo extensive DNA rearrangements, including DNA elimination, chromosome breakage and DNA descrambling, when the germline micronucleus produces the new macronucleus during sexual reproduction. It has long been known that many of these events are epigenetically controlled by DNA sequences of the parental macronuclear genome. Recent studies in some model ciliates have revealed that these epigenetic regulations are mediated by non-coding RNAs. DNA elimination in *Paramecium* and *Tetrahymena* is regulated by small RNAs that are produced and operated by an RNAi-related mechanism. It has been proposed that the small RNAs from the micronuclear genome can be used to identify eliminated DNAs by whole-genome comparison of the parental macronucleus and the micronucleus. In contrast, DNA descrambling in *Oxytricha* is guided by long non-coding RNAs that are produced from the somatic parental macronuclear genome. These RNAs are proposed to act as templates for the direct descrambling events that occur in the developing macronucleus. The possible evolutionary benefits of these RNA-directed epigenetic regulations of DNA rearrangement in ciliates are discussed here.

Keywords

RNAi; non-coding RNA; heterochromatin; DNA rearrangement; DNA elimination; ciliate; *Tetrahymena*; *Paramecium*; *Oxytricha*; Argonaute; Dicer

Introduction

Recent advances in technology have enabled us to convert our somatic cells into pluripotent stem (iPS) cells, which can produce all types of cells in our body, by introducing a handful of transcription factors [1]. This is possible because cells in our skin, muscle and brain are all derived from a single zygotic cell and thus have the same DNA. However, we can find an exception for this rule in the B- and T-cells in our blood; to recognize numerous different invaders such as viruses and bacteria, they have the ability to express over one million different receptors. Because humans have only ~25,000 genes, it is impossible to express such varieties of receptors even if all genes are allocated to their production. Instead, immune cells produce multiple different receptor-coding genes by making DNA rearrangements. For example, the human immunoglobulin (Ig) heavy chain locus in the germline has ~50 V_H, 27 D_H and 6 J_H encoding genes; one of each regional gene is recombined by DNA rearrangement during B cell maturation to generate ~8,000 different Ig heavy chains. Similar DNA rearrangements produce ~200 Ig light chains from a germline light chain locus. Because Ig consists of a heavy chain and a light chain, a repertoire of ~1.6 million (= 8000 × 200) antibodies can be produced [2].

Similarly, many different eukaryotes undergo a variety of DNA rearrangements during certain developmental processes, such as mating-type switching in yeast and chromatin diminution in the somatic cell lineages of parasitic nematodes and crustaceans [3]. Additionally, elimination of entire chromosomes has been observed in somatic cells of early embryos of hagfish and sciarid flies [4]. Therefore, developmentally regulated DNA rearrangements are widespread among eukaryotes. One of the most extreme examples of DNA rearrangement has been observed in the ciliated protozoa. Recent studies have revealed that some types of DNA rearrangements in ciliates are epigenetically regulated by small and/or long non-coding RNAs. In this review, I focus on these epigenetic regulations of DNA rearrangements in ciliates. For recent advancements on the molecular mechanisms controlling DNA rearrangements, please see other reviews [5, 6].

DNA rearrangement in ciliated protozoa

Ciliates are a group of primarily single-celled eukaryotes common almost everywhere there is water. They are similarly evolutionarily distant from Unikonts (including animals, fungi and amoebas) and Plantae (including algae and land plants), and belong to another supergroup, Chromalveolata. Most ciliates show nuclear dimorphism by the presence of a germline micronucleus and a somatic macronucleus (Fig. 1A). Only the micronucleus has the ability to undergo meiosis, which, followed by fertilization, forms the zygotic nucleus. The zygotic nucleus produces new micro- and macronuclei for the next generation. DNA rearrangements occur during the differentiation from the zygotic nucleus to the new macronucleus (Fig. 1A). In this process, approximately 20% of the micronuclear DNA is deleted in the oligohymenophorean ciliates *Paramecium* and *Tetrahymena*, while more than 95% of the genome is eliminated in some spirotrich ciliates, such as *Euplotes*, *Stylonychia* and *Oxytricha* [7]. Endoreplication of macronuclear chromosomes also occurs during macronuclear development. The degree of endoreplication is greater in spirotrichs than oligohymenophoreans. For example, *Tetrahymena* has ~60 copies of macronuclear chromosomes while *Oxytricha* has ~2,000 copies [7].

A variety of DNA rearrangements has been reported in several different ciliates and I classify them here into four types. A Type I rearrangement is defined by the elimination of internal DNA segments followed by ligation of the two flanking macronuclear-destined sequences (Fig. 1B, I). The eliminated DNAs vary in length (~30 bp to ~20 kbp) and in their sequence nature (single copy sequences, transposons and other repeated sequences). Some of the internal DNA segments are precisely excised at the nucleotide level while others are imprecisely eliminated with variable boundaries. These are called internal eliminated sequences (IESs; in *Paramecium*, only the precisely excised single-copy sequences are called IESs). A Type II rearrangement is similar to a Type I rearrangement but leads to chromosome fragmentation followed by the addition of telomeres (Fig. 1B, II). A Type III rearrangement involves chromosome breakage at the Cbs (chromosome breakage sequence) site accompanied by short DNA trimming and *de novo* telomere formation (Fig. 1B, III). Finally, Type IV rearrangements involve “descrambling” of protein-encoding DNA segments (Fig. 1B, IV). In this process, “scrambled” protein-encoding sequences in the micronucleus are joined and assembled (scrambled) into the proper order in the macronucleus. Not all types of DNA rearrangements can be observed in a single ciliate species. For example, *Tetrahymena thermophila* show only Type I and III rearrangements, and Type IV rearrangements have been detected only in some spirotrich ciliates. The types of DNA rearrangements observed in different ciliate species are summarized in Fig. 1C.

As a result of these DNA eliminations and fragmentations, *Paramecium* and *Tetrahymena* produce macronuclear chromosomes with an average size of ~300 kbp and ~800 kbp, respectively [7]. On the other hand, spirotrich ciliates have ~20,000 extensively fragmented

macronuclear chromosomes with an average size of ~2 kb. They often contain only single genes and are referred to as “gene-sized” chromosomes [7].

Epigenetic effects of parental macronuclear sequences on DNA rearrangement in the new macronucleus

Several lines of evidence suggest that there are epigenetic mechanisms that use the primary sequence of the parental macronucleus to direct DNA rearrangements in the developing macronucleus.

The first case of such an epigenetic effect on DNA rearrangement was uncovered through the analysis of the d48 mutant of *Paramecium tetraurelia*. In wild type cells, Type II DNA rearrangements occur proximal to the A-type surface antigen gene (A-gene), which is retained in the macronucleus (Fig. 2A). The d48 mutant has a deletion of the A-gene in the macronucleus, while the micronuclear A-gene remains intact. In the sexual progeny of d48 mutants, the A-gene is eliminated from the new macronucleus, and thus the d48 mutation is epigenetically inherited in the next generation (Fig. 2B) [8]. Moreover, microinjection of DNA fragments containing the A-gene into the parental macronuclei of d48 mutants can induce retention of the A-gene in the new macronuclei (Fig. 2C) [9, 10]. Epigenetic effects in DNA rearrangements are also seen when IESs are introduced into the parental macronuclei of wild type cells. In *Paramecium*, injection of a plasmid containing an IES in the G surface antigen gene into the macronucleus causes retention of this IES in the newly formed macronucleus (Fig. 2D) [11]. Similar epigenetic effects in IES eliminations were observed in *Tetrahymena* [12]. These phenomena indicate that both Type I and II rearrangements (DNA elimination) can be influenced by the DNA sequence of the parental macronucleus, such that the new macronucleus copies the sequence pattern of the parental macronucleus. To achieve these maternal regulations, some sequence-specific information must be transferred from the parental macronucleus to the new macronucleus.

Small RNA-directed chromatin rearrangement

The finding that the micronuclear genome is bi-directionally transcribed during conjugation in *Tetrahymena* [13] provided the first indication that micronuclear RNAs could play a role in the maternal regulation of DNA rearrangements. Although the micronucleus is transcriptionally inert in most life stages, an exception to this has been observed during prophase meiosis in *Tetrahymena* [14]. It was determined that these micronuclear RNAs are processed to ~28-29 nucleotide small RNAs, called scan (scn) RNAs, by the Dicer-like protein Dcl1p [15, 16] and then form complex with the Argonaute protein Twi1p [17, 18]. Dcl1p and Twi1p are essential for the production and stable accumulation of scnRNAs, respectively, and for Type I rearrangements (IES elimination) [15, 16, 17]. Dicer and Argonaute family proteins are conserved core components of the RNAi-machinery and are involved in post-transcriptional as well as transcriptional gene silencing in many eukaryotes [19]. Dicer-dependent accumulation of scnRNAs and its requirement in DNA rearrangements (Type I and II) has also been reported in *Paramecium* [20]. Therefore, scnRNAs derived from micronuclear transcripts play a pivotal role in DNA elimination in oligohymenophoreans in a manner similar to RNAi-directed transcriptional gene silencing in other eukaryotes.

The results above suggest that scnRNAs act as messengers that transmit maternal information from the parental macronucleus to the new macronucleus. How can they direct precise DNA rearrangements? In *Tetrahymena*, although scnRNAs complementary to both the macronuclear-destined sequences and the micronuclear-specific sequences (mostly IESs in *Tetrahymena*) are produced in the early conjugation stage, only the latter scnRNAs

become gradually enriched in the mid conjugation stages [18, 21]. In the mid conjugation stages, the scnRNA-Twi1p complex is localized to the parental macronucleus. Later, the complex, including scnRNAs enriched in IES sequences, is transported into the new macronucleus. Therefore, it is reasonable to speculate that some mechanism specifically degrades scnRNAs complementary to the genomic DNA in the parental macronucleus, and this could be the basis of the maternal regulation of DNA rearrangements (Fig. 3) [18].

The molecular mechanism that induces selective degradation of scnRNAs complementary to the macronuclear genome is still unclear. Our study in *Tetrahymena* [21] suggested that nascent non-coding transcripts mediate the interaction between chromatin and scnRNA-Twi1p complexes in the parental macronucleus and that this interaction is dependent on the putative RNA helicase Ema1p. Moreover, *EMA1* knockout strains have defects in the selective degradation of scnRNAs and in DNA eliminations. Therefore, we have proposed that scnRNA degradation is induced by a base-pairing interaction between scnRNAs and nascent non-coding transcripts from the parental macronucleus. The importance of the parental macronuclear transcripts for proper IES eliminations has also been demonstrated in studies of another ciliate, *Paramecium tetraurelia* [22]. Down-regulation of non-coding transcripts from the parental macronucleus by an RNAi technique blocked the scanning process in the targeted regions and induced ectopic DNA elimination.

Methylation of histone H3 at lysines 9 (H3K9me) and 27 (H3K27me) and the chromodomain protein Pdd1p, which binds to these modifications, are specifically accumulate on IESs and are required for DNA elimination [23]. Therefore, IESs are wrapped in heterochromatin structure before they are eliminated. Since disruption of *TWII* inhibits H3K9me accumulation [24], the RNAi-related pathway is upstream of heterochromatin formation.

RNA-directed DNA descrambling in *Oxytricha*

Type IV DNA rearrangements (DNA descrambling) in the spirotrich ciliate *Oxytricha* are also regulated by non-coding RNAs. *Oxytricha* predominantly utilizes long RNAs to guide the descrambling event (Fig. 4A), although some role of small RNAs in this rearrangement has been suggested [25]. The long RNA-guided DNA descrambling has first been proposed as a theoretical model [26] and recently demonstrated experimentally [27]. Bi-directional transcription of the parental macronuclear genome occurs in early conjugation in *Oxytricha*. Spirotrich ciliates have very short “gene-sized” macronuclear chromosomes, and the macronuclear long RNAs are likely produced by “telomere to telomere” transcription (Fig. 4A). Disruption of specific parental macronuclear long RNAs by RNAi inhibits descrambling of the corresponding loci in the new macronucleus (Fig. 4B), and injection of artificial RNAs reprograms the descrambling orders (Fig. 4C). Therefore, Type IV DNA rearrangements in *Oxytricha* are epigenetically regulated by the DNA sequence of the parental macronuclear genome, and long macronuclear RNAs act as templates in these events (Fig. 4A). Moreover, the occasional transfer of point mutations from RNA templates to the new macronuclear DNAs has been observed [27]. This indicates that there is an RNA-directed proofreading mechanism. Although the molecular mechanisms involved in RNA-guided DNA chromosome rearrangements and RNA-directed proofreading are currently unclear, future studies on these phenomena would provide us clear examples about how acquired somatic mutations can be transmitted to the next generation without altering the germline genome.

Evolutionary advantages of epigenetically-regulated DNA rearrangement

Epigenetic regulations of DNA rearrangements by macronuclear DNA sequences must have some evolutionary advantage for the survival of ciliates. Three potential benefits of these events can be proposed.

The first advantage is sequence-independent recognition of molecular parasites such as viruses and transposons. Because many of the eliminated sequences during Type I and II DNA rearrangements are related to transposons, it is thought that one of the major roles of DNA rearrangements in ciliates is the elimination of transposons from the transcriptionally active macronucleus. Indeed, exogenous sequences experimentally introduced into the micronucleus are eliminated by Type I rearrangement during macronuclear development [28, 29]. This epigenetic mechanism could target any type of DNA inserted at any locus of the micronucleus for elimination.

The second advantage is the inheritance of acquired somatic mutations in the next sexual generations. Because the macronucleus is polyploid and its chromosomes are amitotically segregated during vegetative growth, mutations in the macronuclear chromosomes can be assorted positively or negatively. If a mutation is beneficial to cells, it can be fixed in a population during vegetative growth. The epigenetic regulations of the DNA rearrangements described above could act as a mechanism that transmits beneficial somatic mutations to the next generation. Although only large deletions in the macronucleus can be inherited in the next sexual generations by the small RNA-directed DNA rearrangement system in the oligohymenophorean ciliates *Paramecium* and *Tetrahymena*, RNA-guided DNA rearrangements in *Oxytricha* can result in the transmission of translocations and even point mutations to the next generations.

The third advantage is the inheritance of beneficial variants among alternatively rearranged forms of a gene. DNA rearrangements can produce variants of macronuclear chromosomes from a single micronuclear genome by alternative rearrangements. If some specific variant (or combinations of variants) is advantageous to the growth and/or survival of cells, it would dominate over other variants as in the mechanism described above. Once some variant is fixed in the macronucleus, the pattern of DNA rearrangement in the next sexual generation can be biased toward this variant form by the epigenetic mechanism. Also, because the micronucleus retains the original genome, cells have a chance to quickly return to an unbiased rearrangement when the variant loses its advantage for survival by some environmental change.

Conclusion

Although the epigenetic regulations of DNA rearrangements in the ciliated protozoa have been known for a long time, their molecular bases have only recently begun to be revealed. Small RNA-directed DNA elimination in the oligohymenophorean ciliates *Paramecium* and *Tetrahymena* and long RNA-guided DNA descrambling in the spirotrich ciliate *Oxytricha* were discussed in this review. How these seemingly distinct mechanisms have evolved in different classes of ciliates is unclear. Future studies on other ciliate classes may help to solve this issue. Additionally, the detailed molecular mechanisms regulating these events, such as the biogenesis and selection of scnRNAs in *Tetrahymena* and *Paramecium*, the transcription of long template RNAs in *Oxytricha* and the induction of DNA rearrangements by these non-coding RNAs, are still mostly unknown. I expect established genetic tools in these model ciliates, such as gene knockout and RNAi techniques, and fully sequenced genomes will help to answer these unsolved questions in the near future.

Biography

Kazufumi Mochizuki obtained his Ph. D. from the Graduate University for Advanced Studies (Hayama, Japan) in 2000. He joined the laboratory of Professor Martin A. Gorovsky at the University of Rochester (Rochester, USA) as a post-doctoral research fellow in 2001. Since 2006, he has been a group leader at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA, Vienna, Austria). His research interests are in the regulation of chromatin structure by non-coding RNAs.

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Summary

- Several different types of DNA rearrangements are observed in ciliated protozoa.
- DNA elimination (Type I and II DNA rearrangement) and DNA descrambling (Type IV DNA rearrangement) occur in the newly developed macronucleus and are epigenetically regulated by the primary DNA sequences of the parental macronucleus.
- DNA eliminations in the oligohymenophorean ciliates *Paramecium* and *Tetrahymena* are directed by small non-coding RNAs produced by an RNAi-related mechanism.
- DNA descrambling in the spirotrich ciliate *Oxytricha* is guided by long macronuclear non-coding RNAs.

Figure 1. Life cycle and DNA rearrangements in ciliates
(A) Nuclear dimorphism and conjugation of *Tetrahymena*. Like most ciliates, *Tetrahymena thermophila* (picture) has two different types of nuclei (highlighted in red), a macronucleus (Mac) and a micronucleus (Mic), in a single cell. When enough nutrients are available, vegetative *Tetrahymena* grow by binary fission, and the macro- and micronucleus are divided independently. After prolonged starvation, two cells of complementary mating type fuse to start the sexual reproduction process, conjugation. Their micronuclei undergo meiosis, and one of the meiotic products survives and divides mitotically, giving rise to two gametic nuclei, one stationary and one migratory. Fertilization occurs after the migratory gametic nucleus crosses the conjugation bridge. The zygotic nucleus divides twice, and two products differentiate as macronuclei while the other two differentiate as micronuclei. The parental macronucleus becomes pyknotic and is resolved. (B) There are four types of DNA rearrangements in ciliates (see text for details). Green lines: macronuclear-destined sequences, red boxes: micronuclear-limited (eliminated) sequences, dashed green lines: telomeres. (C) Distribution of the four types of DNA rearrangements in ciliates. +: present, -: absent or not observed.

Figure 2. Parental macronuclear DNA sequences epigenetically regulate DNA rearrangement in the new macronucleus

Cells before conjugation (parent) are on the left and cells post-conjugation (progeny) are on the right. (A) DNA rearrangement at the A-gene locus in the wild type *Paramecium* strain. A Type II DNA rearrangement occurs, and a DNA segment (red) adjacent to the A-gene is eliminated during new macronuclear (Mac) development, while the A-gene is retained. (B) In the d48 mutant, the A-gene is absent in the parental Mac. While the micronuclear (Mic) A-gene locus is intact, the A-gene is eliminated from the new Mac. (C) Microinjection of DNA containing A-gene sequence into the d48 mutant results in the retention of the A-gene in the new Mac. (D) Type I DNA rearrangement in a wild type *Paramecium* strain. IESs are

eliminated during new Mac development. (E) Microinjection of DNA containing the red IES results in the retention of this IES in the new Mac.

Figure 3. A model for small RNA-directed DNA rearrangement in *Tetrahymena*

In the early conjugation stages, the genome of the micronucleus (Mic), including the IESs, is transcribed bi-directionally, and these transcripts form dsRNA (a). The dsRNAs are processed into small RNAs (scnRNAs) by a Dicer-like RNase III (b), and the scnRNAs are transferred to the parental macronucleus (Mac) (c). In the mid conjugation stages, any scnRNAs homologous to DNAs in the parental Mac are degraded (d). In the late conjugation stages, the scnRNAs that are not degraded in the parental Mac (those homologous to IESs) are transferred to the developing new Mac (e), where they target IESs to be eliminated by base pairing (f).

Figure 4. A model for long RNA-guided DNA rearrangement in *Oxytricha*

(A) Telomere to telomere transcription of parental macronuclear “gene-sized” chromosomes produces guide RNAs (wavy line with circles), which are then transported to the newly developed macronucleus where they act as scaffolds to guide DNA rearrangements. This model explains the effect of disruption of guide RNAs by RNAi (B) and microinjection of artificial templates (C).