On the origin of speciation

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Abstract

Charles Darwin proposed the theory of evolution that natural selection leads to the evolution of organisms in “On the Origin of Species.” However, he did not show how new species differentiate and fix. Speciation requires a system in which genes are not mixed by interspecific hybridization, and reproductive isolation, especially postmating reproductive isolation, is considered the most reliable guarantee. Haldane proposed that heterogametic sex was absent, rare, or sterile in interspecific hybrid F1. Dobzhansky and Muller predicted that postmating reproductive isolation occurs when mutations occurring at two or more interacting loci exhibit incompatibility in the hybrid. Genes that satisfy this observation and prediction are considered speciation genes. Here, the author would like to review the findings on reproductive isolation and speciation to consider the candidate conditions for the speciation genes and present the genes that fit these conditions.

Introduction

In 1859, Charles Darwin presented the theory of evolution that natural selection leads to the evolution of organisms in “On the Origin of Species”[1], which dramatically and revolutionarily advances the understanding of life. However, due to the limitations of genetics at the time, it was impossible to explain ‘the origin of speciation,’ which is how evolved populations are fixed and maintained as new species. There are many species definitions[2], but it would be simplest and clearest to make it a population where genes are not mixed by mating with other populations. Reproductive isolation, especially postmating reproductive isolation, is considered the most reliable guarantee of gene flow blockage.

It is not uncommon to form hybrids by mating between species considered morphologically heterogeneous and maintain their hybrids (i.e., syngameon)[3]. Furthermore, some species can be judged to be heterogeneous only after reproductive isolation is confirmed by crossing between those that were morphologically isogeny (i.e., cryptic species). The sympatric mix of cryptic species is believed to be far greater than currently reported[4]. Morphological differences seem to have nothing to do with speciation. Haldane proposed that heterogametic sex (XY male or ZW female, etc.) was absent, rare, or sterile in interspecific hybrid F1 (i.e., Haldane's rule)[5]. In mammals, hybrid males develop spermatogenic deficiency (i.e., hybrid male sterility, HMS) or inviability (absent or rare). As a result, hybrid F2 does not occur, and the species is conserved. Dobzhansky and Muller predicted that reproductive isolation is caused by mutations occurring at two or more interacting loci, and the gene functionally diverges in each individual and shows incompatibility only in hybrid (i.e., Dobzhansky-Muller incompatibilities, DMI)[6,7]. Haldane, Dobzhansky, and Muller's observation and prediction about postmating reproductive isolation seem very primitive and essential in considering the speciation genes. That is, in interspecific hybrid, mutations at two or more loci cause incompatibility only in the heterogametic sex (two karyotypes, XY male type and ZW female type), and gene flow is blocked by two phenotypes (sterility or inviability). DMI model genes can be said to be speciation genes. If DMI model genes are molecular evolutionarily neutral[8] and not subject to selection pressure and speciation is established solely by that mutation, it will block the gene flow of a population with gene pools of precisely the same phenotype. Therefore, the two gene pools immediately after differentiation will be exactly the same except for DMI model genes. The subsequent accumulation of gene mutations in the two populations will change the phenotype and lead to natural selection, eventually making even mating difficult (i.e., premating reproductive isolation)[9]. Therefore, even if the incompatibility genes detected between the two species are involved in fertility since a large proteome is involved in reproduction, it must be carefully considered whether they are speciation genes or mutations that occur after speciation. It is not easy to imagine that the system involved in the highly essential event of biological evolution differs for each taxon. There is a possibility that common genes and systems are working in taxa, where Haldane's rule is established in heterogametic sex and shows sterility and inviability. At the very least, the gene set is predicted to be preserved in a closely related species taxon, indicating incompatibility. Here, the author considered the conditions of the speciation genes by reviewing subsequent findings based on Haldane's rule and the DMI model. Many speciation genes have been reported so far, but none of them satisfy these conditions, so the author would like to present a hypothesis.

Conditions for speciation genes

Two karyotypes

Haldane showed that the phenomenon that causes reproductive isolation in hybrid is observed in heterogametic sex (two karyotypes, XY, XO male and ZW, ZO female)[5]. This phenomenon is not observed in homogametic sex (XX or ZZ), suggesting that the cause lies in the common system of single-copy genes on the X or Z chromosome. Moreover, the fact that the same phenomenon (hybrid sterility) is expressed in XY-type males and ZW-type females strongly suggests that the cause is not specific to spermatogenesis or oogenesis but is a mechanism common to the gametogenesis of both sexes.

Two phenotypes

Haldane showed two phenotypes of sterility or inviability (absent or rare) in the hybrid heterogametic sex[5]. Hybrid sterility is observed in mice with a meiotic arrest in primary spermatocytes rather than mitotic arrest in spermatogonia[10]. Hybrid inviability is due to impaired embryogenesis, and Orr et al. have shown that it is caused by mitotic arrest in Drosophila[11]. The two phenotypes are meiotic or mitotic arrest, suggesting cell division disorders cause it.

Orr recognizes inviability in hybrid XXY females by crossing females with attached two X chromosomes and heterogeneous males in two species of fruit flies that usually exhibit hybrid male sterility. For this reason, Orr argues that the genetic causes of Haldane's rule differ between sterility and inviability[12]. It shows two sets (4 loci) of DMI model genes. However, when two gene sets showing two phenotypes due to incompatibility coexist in an interspecific hybrid, the sterility genes set observed after development would not be expressed because the inviability genes set is thought to be expressed at the developmental stage in normal hybrids. From Orr's observation, it cannot be denied that the DMI model genes are one set, showing two phenotypes depending on the expression time or cells. Furthermore, it is suggested that the heteromorphic chromosome contains factors that can control hybrid incompatibility.

Two genes

In the DMI model, at least two genes (loci) can be functionally diverged by mutations that do not cause incompatibility[6,7]. When two mutated genes meet in a hybrid, the incompatibility occurs in heterogametic sex, causing sterility or inviability. This fact suggests that there is a loose tolerance for the interaction between the two genes such that individual mutations do not interfere with homeostasis, including fertility.

There are two possibilities for the results due to the incompatibility of two DMI model genes: 1) Loss of original function due to disruption of protein-protein interaction or incompatibility of epistatic and effector genes. 2) Disorders by misexpression (or overexpression) of harmful functions due to incompatibility of suppressive epistatic and effector genes. In the knockout verification of these genes in mice, sterility would not be seen in the effector gene of 2), but in other cases, it is predicted that sterility will occur only in males.

Large-X effect and large-Z effect

X chromosome replacement by backcrossing between heterogeneous has a more significant disproportionate effect on hybrid fitness than autosomal chromosomes (i.e., large-X effect)[13]. Haldane’s rule predicts that even if a harmful mutation occurs in recessive X-linked allele, it is injurious in heterogametic hybrids. However, in homogametic hybrids, a harmless dominant allele masked it and no disorder appears (i.e., dominance theory)[14]. Furthermore, in the DMI model, two genes need to be expressed, that is, to be dominant, but in heterogametic sex, the genes on the X chromosome are single copies, so they are expressed regardless of dominant or recessive. For this reason, heterogametic sex is said to be affected by mutations[6]. Both have shown the importance of the X chromosome in hybrid sterility. Furthermore, a large Z effect[15] has also been confirmed in ZW-type birds and butterflies that exhibit hybrid female sterility. This finding reinforces the speculation from Haldane's rule that DM model genes are involved in events common to male and female gametes.

Hybrid sterility 1 (Hst1) on chromosome 17[16] and hybrid sterility X2 (Hstx2) on chromosome X[13,17] are mapped as hybrid sterility loci by quantitative trait locus (QTL) analysis using consomic strain based on the sperm count and testis weight of mice as indicators. There are six protein-coding genes in the Hst1 locus, and meiotic histone H3 methyltransferase, Prdm9, is further identified as a hybrid sterility gene. Prdm9 caused meiotic arrest due to chromosomal synaptic failure by its defect and was considered the causative gene of hybrid sterility[18]. However, the Prdm9 knockout mouse shows infertility not only in males but also in females[19]. Furthermore, it is shown that it is not essential for meiosis[20], so it is hard to believe that Prdm9 is the effector gene of the DMI model.

Hstx2 contains ten protein-coding genes and 22 microRNAs (miRNAs), but the hybrid sterility gene has not yet been identified. Morimoto et al. confirm that knockout mice of six protein-coding genes, except for genes not to be involved in spermatogenesis (there is disagreement about Fmr1 as

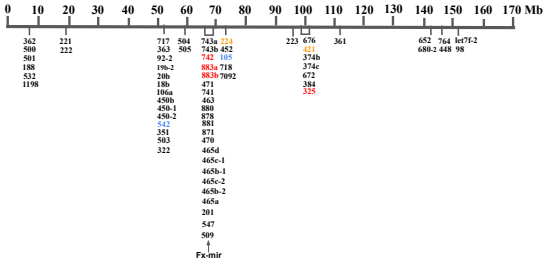
described later.), do not cause infertility[21]. Therefore, the hybrid sterility gene of the Hstx2 locus is more likely to be microRNAs rather than the protein-coding genes. Hstx2 locus almost coincides with the human Xq27.3 region called the fragile-X region. The fragile-X region is composed of the protein-coding genes SLITRK2 and FMR1 and 22 microRNAs sandwiched between them and is located only on the X chromosome, and this composition is conserved in mammals[22]. This region is easily physically cleaved and was initially noted in studies of fragile sites abundant on chromosomes, demonstrating that the fragility lies in the FMR1 mutation[23]. Recently, X-linked miRNA has been attracting attention for promoting evolution because it shows a fast evolution speed. The miRNAs (Fx-mir) in this fragile-X region (Hstx2) are composed of the hsa-miRNA888 cluster (miR888~892c, 7 genes, 10 mature miRNA) and the hsa-miR506 cluster (miR506~514b, 15 genes, 20 mature miRNA) in humans[23] (Table S1). In mice, 22 genes (44 mature miRNA) are present as Fx-mir[24] (Table S2). Some mouse Fx-mir are weakly expressed in various organs, including the ovary, but most are strongly expressed only in the testis[24]. MiRNAs are non-coding RNAs with a length of 20~25 nucleotides that mainly bind to mRNA 3'UTR and suppress gene expression. MiRNAs exist widely from fungi to plants and animals[25]. In the hybrid male sterility of Nematoda, the interaction between the X chromosome and the autosomal loci was shown to be essential[26], suggesting that the autosomal genes controlled by Fx-mir are potential effector genes in hybrid sterility. Furthermore, if the miRNA is an epistatic gene of DMI model genes, the effector gene is not expressed in normal gametogenesis, and its misexpression is presumed to cause disorders. Since miRNA and mRNA do not have a 1:1 relationship and a single miRNA typically downregulates target mRNA by only about 20~40%, multiple miRNAs are required to regulate more strongly[24,27]. This fuzzy relationship between miRNA and mRNA seems to guarantee that individual mutations in DMI model genes can occur without functional impairment. More than half of the miRNA clusters usually have a paralogous cluster at different loci, but it has not been found in Fx-mir[22]. In the DMI model, it was expected that a single copy of the gene on the X (Z) chromosome of the heterogametic sex would guarantee incompatibility, and Fx-mir satisfies this condition.

Faster-X effect and faster-Z effect

In general, X-linked genes, especially male-specific genes, are said to evolve faster than autosomal genes (i.e., faster-X effect and faster male effect)[28,29,30]. Since the genes on the male X chromosome are single copies, mutations are easily fixed, and intense selection pressure is applied to highly competitive sperm, it is suggested that their rapid evolution contributes to the speciation of mammals (i.e., sexual selection)[29]. However, this speculation breaks down since the faster-Z effect has been confirmed in birds (ZW female type)[31]. As mentioned above, the genes for which mutations are always confirmed between species immediately after speciation may be only the DMI model genes. In addition, mutations in DMI model genes after species differentiation lead to the following differentiation, so regardless of the neutrality of molecular evolution[8], DMI model genes will appear to evolve relatively faster than other genes. Evolution is not driven by fast evolution due to the selection pressure of X-linked genes, but they appear to be fast because it is directly involved in evolution. Because of the existence of the sympatric cryptic species, the sterility and inviability as a phenotype of DMI model genes are neither the result of natural selection nor undergoing selection pressure. In that sense, speciation may be considered an intrinsic, autonomous event, unlike the extrinsic, heteronomous evolution by natural selection.

The nucleotide substitution rate of miRNAs expressed mainly in the testis was 25 times higher on the X than on the autosomal chromosome, and there was no significant difference in the substitution rate of miRNAs not expressed in the testis between the X chromosome and the autosomal chromosome[32]. That is, X-linked miRNAs expressed in the testis (X-linked testis miRNA, Xt-mir hereafter) evolves rapidly. In mice, 77 Xt-mirs, including Fx-mir, were detected and distributed over the entire X chromosome centering on Fx-mir (Fig.1). Xt-mir in the mouse testis was expressed partially in spermatogonia and mostly in round spermatids from spermatocytes, escaping meiotic sex chromosome inactivation (MSCI)[33]. Xt-mir, expressed in spermatocytes showing meiotic arrest in hybrid testis, is a likely candidate for the DMI model genes. Furthermore, since miRNAs and mRNAs are in a co-evolutionary relationship[24], among the many target genes of Xt-mir, those corresponding to DMI model genes are predicted to evolve particularly rapidly. Among the DMI model genes, the evolution of the intermolecular action part, which is mainly related to incompatibility, seems faster than other parts. Of the nucleotide substitutions in primate random primary miRNAs (not including Fx-mir), 2.5% were found in mature miRNAs, compared with 19.5% in mature miRNAs of hsa-miR506 cluster[34]. DMI model genes should be the most fixed in the species because if mutated, they will be lost due to sterility or inviability or become new species. Hsa-miR509 has three copies and all three work and the number of copies was the same among races and firmly fixed within the species[29]. Fx-mir (or Xt-mir) clusters are very different from other miRNAs in evolution, and their involvement in speciation is strongly suspected, further strengthening the possibility of DMI model genes.

Figure 1. Xt-mir mapping on the mouse X chromosome
Numbers: mouse X-linked miRNAs number expressed in the testis (Xt-mir). Colored numbers: Xt-mir targeting Spag1-2 mRNA 3'UTR with 7-8mer matching. Red numbers: Xt-mir expressed mainly in spermatocytes~spermatids. Orange numbers: Xt-mir expressed in spermatocytes and organs other than the testis. Blue numbers: Xt-mir expressed in spermatogonia, spermatocytes, postnatal testis, and organs other than testis[33].



Infertility

Azoospermia and oligospermia may result if reproductive dysfunction is caused by significant mutations or deletions in the DMI model genes that exceed the permissible range in germ cells. Furthermore, as a process of speciation, the son will be infertile if both partners are carriers of the incompatible gene. Investigating the causes of infertility in humans may provide clues to the speciation genes. 5% of males have infertility, of which 75% have idiopathic sperm dysfunction of unknown cause[35]. The miR888 cluster was downregulated in the testis of non-obstructive azoospermia (NOA) patients compared to obstructive azoospermia patients[36]. Misexpression of target genes due to Fx-mir downregulation is thought to impair spermatogenesis, and Fx-mir and its target genes may be candidates for DMI model genes.

Apoptosis or not?

Most reports refer to cell death due to the meiotic arrest of hybrid sterility as apoptosis. However, there are no characteristic histological findings of apoptosis, such as nuclear rupture or apoptotic body in hybrid testis[37,38]. Usually, in the histological proof of apoptosis, cells stained in response to ruptured nuclear DNA by TdT-mediated dUTP nick-end labeling (TUNEL) assay are judged as apoptotic cells, but they do not react to mitochondrial DNA (mtDNA). Therefore, apoptotic cells seem to be unconditionally determined only by positive staining. However, the cytosol of positive cells also appears to be stained in all reported micrographs. The spermatocyte has a minimal cytosol/nucleus ratio, so even if cytosol is stained, it is difficult to recognize it overlapping with counterstain in a standard light microscopic image. Hayashida et al. showed by TUNEL assay using confocal fluorescence microscopic image that spermatocytes of mouse hybrid sterility testis have mtDNA disruption but nuclei[38]. Recently, Yu et al. showed that the direct cause of hybrid sterility in scallops is cell cycle arrest due to ATP depletion in interspecific hybrid F1 gonads. Mutations, rearrangements, depletions, etc. of mtDNA are thought to cause mitochondrial dysfunction, but it is unclear why hybrid gonads cause structural changes (or disruption?) of mtDNA[39]. The TUNEL assay of the Xenopus (frog) embryo, which exhibits hybrid inviability, shows a different image without nuclear staining and nuclear condensation compared to staining with apoptosis-inducing agents, suggesting cell death that is not apoptosis (it appears to be stained around the nucleus)[40]. Hybrid sterility and inviability may be caused by mtDNA depletion rather than apoptosis. The fact that the terminal image of hybrid sterility in very distant taxa, such as mammals and bivalves, was shown to be an unusual cell death that is not apoptosis suggests the possibility that the principle of speciation has a mechanism common to all organisms.

Summary of speciation genes conditions

From the above, speciation genes conditions can be summarized as follows: 1) Speciation genes may be one set of Fx-mir (or Xt-mir) and effector genes on autosomes controlled by this. 2) Meiotic and mitotic arrest show sterility and inviability, respectively, due to the difference in expression time or cells of two genes incompatibility. 3) Speciation genes may involve cell division mechanisms common to meiosis and mitosis. 4) Hybrid incompatibility leads to cell death that is not apoptosis due to mtDNA destruction. 5) Effector genes impair gametogenesis or embryonic development due to their misexpression in the phase where their expression is usually suppressed in gonads and embryos of the heterogametic sex (both XY and ZW organisms). 6) Speciation genes are associated with mechanisms common to male and female gametogenesis. 7) There is a possibility that speciation genes exist among the causative genes of human infertility of unknown

etiology. 8) Not only the Xt-mir but also the target gene should evolve rapidly. 9) The genes and mechanisms of speciation may be conserved across taxa.

Many speciation genes have been identified in yeast, thale cress, fruit fly, mouse, etc., and many are involved in transcriptional or translational regulation[41], but the underlying common mechanism remains unclear. Many hybrid sterility or inviability genes have been identified in fruit flies. Among them, the lethal hybrid rescue (Lhr) and the hybrid male rescue (Hmr) are reported as two distinct interacting genes[42]. However, both recognize few orthologs except Brachycera (fly) (ORTHOSCOPE, http://yurai.aori.u-tokyo.ac.jp/orthoscope/Actinopterygii.html). Below, the author would like to present a hypothesis that almost matches these conditions.

Proposing a candidate for speciation genes set

Maternal mitochondrial DNA inheritance and hybrid male sterility

Maternal mitochondrial DNA inheritance (MMI) system In most sexually reproducing eukaryotes, the mtDNA of one gamete is eliminated after mating between gametes (i.e., uniparental mtDNA inheritance, UMI)[43]. In mammals, sperm mitochondria enter the egg together with the nucleus during fertilization, but sperm mtDNA is selectively eliminated from the egg, and mtDNA is inherited maternally (i.e., maternal mitochondrial DNA inheritance, MMI)[44]. The MMI system is strict and is completely eliminated among allogeneic species[43]. Based on speculation that this system is for the processing of sperm mtDNA damaged by reactive oxygen species (ROS), there are theories that the ubiquitin-proteasome system or autophagy of the fertilized egg selectively processes male sperm mitochondria[45,46]. However, there is no guarantee that all male sperm mitochondria have deteriorated by the time of fertilization. Hepatocyte mitochondria were not eliminated by microinjection into an embryo[47], but sperm mitochondria were eliminated by microinjection into somatic cells[48]. These facts mean that sperm mitochondria have a factor to be eliminated before being damaged, unlike somatic cell mitochondria, and somatic cells have a system that recognizes this factor and eliminates sperm mitochondria like eggs. Most of the mtDNA has been transferred to the nucleus in most eukaryotes. However, only a few genes remain in mitochondria, including genes essential for maintaining the function of mitochondria (ATP synthase, etc.)[49]. In mice, a phenomenon that sperm mtDNA disappears before the mitochondrial membrane potential is lost was observed in the fertilized egg[50]. Hayashida et al. considered that MMI is a purposeful programmed mitophagy by a system that controls mitochondria due to eliminating mtDNA essential for maintaining the function, and presented the following theory. The molecular chaperone Spag1-isoform2 (Spag1-2, cytoplasm type) protein transports endogenous retroviral integrase 15kDa (Eri15) (new accession No. LC627956.1), which has endonuclease activity in the egg cytoplasm, to mitochondria. Spag1-isoform1 (Spag1-1, mitochondria type) incorporated into the outer membrane of sperm mitochondria as a member of the translocase of the outer mitochondrial membrane (TOMM) 40 complex during spermatogenesis selectively taken in Eri15 into the matrix and destroys mtDNA. As a result, mitochondria that have lost their membrane potential are treated by the autophagy system (i.e., mitophagy)[51,52].

hybrid male sterility (HMS) system The MMI system is said to avoid competition with heterosexual mtDNA and parasites brought in by sperm mitochondria[43], but it is easily disrupted by intersubspecific and interspecific hybrid and paternal mtDNA is detected in the somatic cells of F1 individuals in mice[50]. The intermolecular reactions involved in MMI can be said to be species-specific. For this reason, Hayashida et al. thought that this system may have evolved rapidly at the forefront of speciation[38]. Spermatocytes of mutant mice lacking part of mtDNA have been shown to cause meiotic arrest[53]. Since Eri15 is also expressed in the spermatocytes[52], it is considered that Spag1-2/Spag1-3 is suppressed by the epistatic gene so that the MMI system does not operate during spermatogenesis. Hayashida et al. showed that not only Spag1-1 but also Spag1-2 was expressed in the intersubspecific and interspecific hybrid testis in mice. Compared to the fact that nuclear and nuclear DNA ruptures were observed at all stages of the artificial cryptorchidism testis, the hybrid testis showed swelling of the spermatocyte mitochondria and cleavage of only mtDNA, causing meiotic arrest due to mitophagy-induced cell death (i.e., mitoptosis) which is not apoptosis[38]. The evolutionary preservation of the MMI system is due to the need for speciation, and the residual mtDNA essential for maintaining function in mitochondria may be due to the functioning of the MMI and HMS systems.

SPAG1 SPAG1 was discovered as one of the target proteins of anti-sperm antibodies in unexplained infertile males[54]. In mice, Spag1-1 (114 kDa) is expressed only on the outer mitochondrial membrane of the testis. However, in epididymal sperm, it is post-translationally modified and detected at 166 kDa[51], suggesting that it has an important function even after maturation. The ortholog of SPAG1 is widely recognized from fungi to plants and animals (ORTHOSCOPE). It has been shown that SPAG1 has a high synonymous substitution rate among sperm proteins that are said to have fast evolution[28]. SPAG1 may be target gene that have a molecular co-evolutionary relationship with Xt-mir[24] as a DMI model gene.

Spag1-2 (64kDa) or Spag1-3 (75kDa) are widely expressed in the cytoplasm of organs other than

the testis, and only one or both are expressed depending on the tissue, and it seems that they complement each other. Spag1-1 has three TPR domains, and Spag1-2 has two TPR domains [51,52]. In humans, although three isoforms of 60 (or 50), 92 to 95, and 104 to 106 kDa have been detected, the intracellular localization, etc. have not been sufficiently investigated for each isoform. It is estimated that 92 to 95 and 104 to 106 kDa isoforms are cytoplasm type (Spag1-3, Spag1-2) and 60 (or 50) kDa isoform is mitochondria type (Spag1-1), and the molecular sizes are reversed in humans and mice[55,56,57]. SPAG1 is expressed in both types in cancer cells and undifferentiated respiratory epithelial cells[55,57], and only 50 kDa isoform in sperm[56] and is considered to be a cancer-testis antigen (CTA)[58]. SPAG1-2 provides a platform for quaternary protein folding of proteins via the TPR domain[59,60] that is involved in protein-protein interactions as a member of the R2SP complex (SPAG1, PIH1D2, RUVB1/2), which is a co-chaperone complex, and is involved in the assembly of protein complexes such as dynein arms[57,61] (its mutation causes primary ciliary dyskinesia due to dysplasia of the axoneme dynein arm). The R2SP complex is identified as a ubiquitous R2TP (RPAP3, PIH1D1, RUVB1/2)-like chaperone, where RPAP3 is located at SPAG1-2 of R2SP and is particularly strongly expressed in the testis. Its assembly function is most robust at the proper temperature of the testis, 32°C, and is optimized for the testis environment[61]. This fact means that R2TP malfunctions in high-temperature environments. It has been observed that hybrid males of flower beetles exhibit malformations, and hybrid females also exhibit malformations in a high-temperature environment of 34°C[62]. The SPAG1/Eri15 axis may also be related to hybrid inviability.

Translocase of outer mitochondrial membrane 34 (TOMM34) is a protein with the highest homology to SPAG1[51]. TOMM34 also has 2 TPR domains, is localized in the cytoplasm and outer mitochondrial membrane, and is mainly expressed in the testis[63]. TOMM34 not only forms a large chaperone complex with Hsp70/Hsp90, etc., and provides a platform for protein folding but also shuttles mitochondrial precursor proteins to mitochondria and imports them into the matrix via TOMM34 on the outer mitochondrial membrane[64]. However, Tomm34 knockout mouse shows no obstacles[65]. Some compensation functions probably worked. Tomm34 has a high similarity to Spag1 in terms of localization and functionality.

Eri15 In mice, Eri15 is present in the cytoplasm as a multimer with the Spag1-2 protein in most somatic cells as well as in the ovary[52]. This fact can explain the abovementioned observation that sperm mitochondria were eliminated by microinjection into somatic cells[48]. In the mitochondria matrix, the pH is alkaline, the Mn^{2+} concentration is high[52], and the Zn^{2+} concentration is three orders of magnitude lower than that of the cytoplasm[66]. The activity of recombinant Eri15 is most vital at pH 8.5 and is enhanced by Mn^{2+}. Normal retroviral integrase has a Zn^{2+} binding site at its N-terminus and requires Zn^{2+} for its activity[67], whereas Eri15 is truncated in this portion and endonuclease activity is preserved, but on the contrary, the activity is suppressed by Zn^{2+}[52]. Eri15 is highly optimized for the environment in the mitochondria matrix. The ortholog of Eri15 is widely conserved in plants and animals (ORTHOSCOPE).

Hosts infected with the virus use apoptosis to remove infected cells and prevent the spread of the infection, but the virus avoids apoptosis via mitophagy in various ways[68]. Herpes simplex uses amino terminally truncated isoform UL12.5 (which gives mitochondria directivity by truncation) of alkali endonuclease UL12 involved in replication to eliminate host mtDNA and prevent apoptosis[69]. Xia et al. have shown that oncolytic measles virus-infected lung cancer cells induce mitophagy, suppress apoptosis through decreased cytochrome c release, and thus favor virus replication, and ultimately, cancer cells cause necrosis due to ATP depletion[70]. This fact indicates that the final form of mitoptosis due to excessive mitophagy is necrosis rather than apoptosis. Spermatocytes of mutant mice with partial deletion of mtDNA exhibit meiotic arrest[53]. As mentioned above, interspecific hybrid F1 gonads of scallops indicate that cell cycle arrest due to ATP depletion is the direct cause of hybrid sterility[39]. Dmc1 that causes meiotic arrest due to synaptic failure by the defect is ATP-dependent[71]. Therefore, the synaptic failure and subsequent meiotic arrest observed in hybrid sterility testis may be caused by ATP depletion due to excessive mitophagy resulting from disruption of mtDNA by the SPAG1/Eri15 axis. The meiotic arrest of hybrid spermatocytes does not lead to apoptosis, probably because the apoptosis pathway is interrupted by programmed mitophagy for active quantity control, not passive quality control.

It is said that 8~10% of mammalian genomes are occupied by the endogenous retrovirus genes[72], and some are used by the host, such as syncytin, which is involved in human placental formation[73]. It is possible that Eri15, a protein derived from retrovirus, is used to eliminate mitochondria in the host.

Mitophagy and Warburg effect

Recently, the association between many diseases and mitophagy has been pointed out[74]. However, most the research subjects are quality control of mitochondria, and little is understood about programmed mitophagy for quantity control. In oocytes, it was said that a temporary number reduction called the bottleneck effect was performed to homogenize mtDNA[75]. It was suggested that Spag1 is involved in mitochondria synthesis and dynamic regulation during meiosis in mouse oocytes[76]. The copy number of mtDNA is actively reduced during spermatogenesis[77]. In

erythroblasts differentiated from hematopoietic stem cells, mtDNA degradation by ANKLE1 protein with endonuclease activity causes mitophagy to become mature erythrocytes. ANKLE1 is usually expressed only in erythroblasts but also in cancer and is associated with the risk of developing ovarian and breast cancers. Ectopic expression in mammary gland cells was shown to induce mitophagy through mtDNA degradation, shifting metabolism from oxidative phosphorylation to glycolysis (i.e., Warburg effect)[78] and avoiding apoptosis[79].

Most stem cells, including cancer stem cells, have few mitochondria and exhibit the Warburg effect[80]. Aurrière et al. have identified and examined 70 mitochondria-related CTA out of 276 CTA since cancer cells in the hypoxic state in the center of the tumor and spermatogonia, primary spermatocytes outside the blood-testis barrier at the outer edge of the seminiferous tubule have a commonality of the metabolic shift to glycolysis. Only two, SPATA19 and COX6B2, were associated with mitochondria metabolism, and neither was found to have the effect of shifting metabolism to glycolysis[81]. They included SPAG1 in the list of CTA searched but did not pick it up as a mitochondria-related CTA. Chromatin-remodeling complexes, which play an essential role in mitosis, are ATP-dependent[82]. The observation above by Xia et al. that ATP depletion by the measles virus in lung cancer cells led to necrosis[70] indicates that rapidly proliferating cells cannot survive by glycolysis alone. If the stem cells are depleted of ATP by mitophagy at the stage of development, the mitotic arrest will occur, and the development will stop. The reason why the mitotic arrest is not seen in hybrid sterility testis[37,83] is that when the mitotic arrest is caused in spermatogonia, it becomes mitotic arrest in the whole stem cells at the same time. Its phenotype is not detected due to developmental failure. Not only meiosis arrest but also mitosis arrest in hybrids may be caused by ATP depletion due to excessive mitophagy.

As described above, SPAG1-1 and SPAG1-2 are expressed in cancer cells[55]. In lung adenocarcinoma cells, the overexpression of SPAG1-2 increased autophagy and decreased cell proliferation and colony formation[84]. 94, 106 kDa SPAG1 isoform expression increases with the differentiation of human respiratory epithelial cells, whereas only 60 kDa isoform shows a constitutive expression[57]. This fact may indicate expression in stem cells, which exist in a certain number of cultured cells. Multiple SPAG1 proteins of ~50 and ~100 kDa are expressed in iPS cells during differentiation induction[85]. SPAG1 may be the CTA involved in the Warburg effect of stem cells. Cell division and proliferation may be associated with the risk of apoptosis. Stem cells may cause programmed mitophagy by SPAG1/Eri15 to avoid apoptosis and, as a result, eventually shift from oxidative phosphorylation to inefficient glycolysis[17]. From the above, it is possible to explain that mitotic or meiotic arrest is caused by cells expressing one set of DMI model genes and showing two phenotypes, inviability and sterility, respectively.

Eukaryotic cells are thought to have developed in the symbiosis of heterotrophic eubacteria in autotrophic archaea[49]. Before the symbiosis, there seemed to be an attack and defense system for each other, and the system changed its shape and remained after the symbiosis. It is speculated that apoptosis is the control of archaea (later eukaryotic cells) by cytochrome c of eubacteria (later mitochondria), and preventing the spread of infected cells by apoptosis of virus-infected cells may be a protective reaction of mitochondria rather than a host. Furthermore, spermatogonia and cancer stem cells may use the SPAG1/Eri15 axis to escape from the control by apoptosis of eubacteria (mitochondria) just as the virus avoided apoptosis by its own endonuclease and ancestor return to archaea with infinite proliferating ability. Since most of mtDNA has migrated to nuclear DNA[49], it cannot self-proliferate outside the cell, but it can do so inside the cell. Oncocytic tumors are benign, non-aggressive, and hypoproliferative lesions characterized by marked hyperplasia of mitochondria. As mtDNA mutations and mitochondrial structural changes are observed in oncocytic cells, there is a hypothesis that retrograde signals such as ROS due to respiratory chain disorder are transmitted from mitochondria to the nucleus, and signals that increase mtDNA copy number and mitochondrial proliferation are upregulated[86]. Viewing oncocytic tumors from the perspective of a mitochondrial ancestor return to eubacteria may help elucidate their pathogenesis.

Xt-mir and SPAG1

A single miRNA binds to mRNA in a 6 to 8 nucleotide (nt) match centered on a continuous seed sequence of 6 bases of 2 to 7nt on the 5'end (6nt match has a lower inhibitory effect than 7 to 8nt)[27]. The relationship between miRNA and mRNA is complex and uncertain, and it seems complicated to discuss the suppressive effect with only the match of the seed sequence, so experimental verification by gene knockout, etc., is required[27]. Since miRNA and miRNA cluster have evolved through genomic duplication events[87], and miRNA and mRNA are in a co-evolutionary relationship[24], the number of target sites of miRNA for one mRNA 3'UTR and the resulting elongation of mRNA should be considered evolutionarily significant. In this paper, when discussing target gene candidates for miRNA, the author will basically use the number of miRNAs showing 6 to 8nt seed match (canonical sites)[27] and the number of target sites in the 3'UTR as an index.

SPAG1 was estimated as the target of hsa-miR888 that is expressed in the human epididymis and is involved in forming the epididymis and sperm maturation[88]. According to TargetScan online software (https://www.targetscan.org/), five types (6 target sites on mRNA 3'UTR) for SPAG1-2 and

only 1 type (1 site) for SPAG1-1 of hsa-Fx-mir is targeted (Table S1). In mice, five Xt-mirs other than the three Fx-mir were predicted as targets of Spag1-2 mRNA (Table S2). Its distribution on the X chromosome (Fig. 1) is in the range of 52Mb around the Fx-mir, and it matches well with the QTL mapping according to the sperm count in the mouse hybrid shown by Bhattacharyya et al., including parts of other than the Hstx2 (Fx-mir) locus[13].

Of the Fx-mir among mouse subspecies, the KO mouse of the mmu-miR743 with SNP and the mmu-miR465 cluster with copy number polymorphism did not become infertile[71]. Of these two miRNAs, miR743 has a 6nt seed match for Spag1-2 3'UTR (Table S2). Ota et al. showed no histological abnormalities in the testes with individual KO mice of mmu-miR741, mmu-miR871, and mmu-miR880. However, with miR871+miR880 or all three KO mice, spermatogenesis is stopped in a part of the seminiferous tubule[89]. Of these three miRNAs, only miR880 has a 6nt seed match to Spag1-2 3'UTR (Table S2). However, Wang et al. reported that when 18 of the 21 Fx-mirs of the mice were knocked out simultaneously, the mice developed normally, and the testes were not histologically affected[90]. If the results of Ota et al. and Wang et al. on the KO mouse of Fx-mir are correct, it is inferred that miRNA involved in the factor that suppresses the expression of Spag1-2 is present in Fx-mir. Ramaiah et al. identified 11 Fx-mirs in mice (6 in humans) targeting Fmr1 (Table S2), which are always present downstream of Fx-mir in the fragile X region in mammals, and confirmed that the induction of each of the four miRNAs suppresses the expression of Fmr1 protein (FMRP)[24]. Mutations in *FMR1* cause chromosomal fragility, and loss of FMRP causes fragile X syndrome (mental retardation, giant testis, fragile X chromosome findings). FMRP is expressed in the central nervous system and testis (Sertoli cells, spermatogonia) and is considered involved in translational regulation as an RNA-binding protein[23,91]. The *FMR1* orthologs are widely conserved in animals (ORTHOSCOPE). As mentioned above, Fx-mir does not have paralogous clusters[22]. The reason why Fx-mir cannot be compensated may be that the benefit (speciation) for selfish genes[92] is greater than the loss for the species. However, male sterility due to malfunction of the MMI system is indeed a loss for species preservation, and it seems that some defense system coexists. Fmr1 is involved in spermatogenesis, axoneme synthesis, and the Warburg effect[93,94], and its function is very similar to that of SPAG1, so it may control SPAG1-2 expression as an RNA-binding translational regulatory protein. If the SPAG1-2/Eri15 axis runs out of control due to widespread loss of Fx-mir, it may be that the misexpressed FMRP suppresses the translation of SPAG1-2 mRNA. The two events in the testis by SPAG1/Eri15/Fx-mir axis and FMR1/Fx-mir axis may be the scene of the antinomy conflict of species evolution and conservation.

The relationship between miRNA and mRNA is many-to-many[27]. If the proteome is formed around miRNA cluster, attention should be paid to selecting miRNA to knockout and interpret the results. DMI shows quantitative traits that exhibit phenotypes at various stages of sperm and individual (absent~rare) count. Quantitative traits are usually the result of the combined action of many genes. The fuzzy relationship between mRNA and miRNA cluster will likely to be a candidate for the minimal unit of DMI model genes that allows for quantitative traits.

Most Fx-mir is downregulated in cancer cells except for some and is considered inhibitory for cancer growth and malignant transformation[95]. Furthermore, Fx-mir is not expressed in spermatogonia, which shows the Warburg effect as well as cancer cells[33]. It can be said that the SPAG1/Eri15/Fx-mir axis suppresses apoptosis of stem cells, including cancer stem cells, by programmed mitophagy. This axis can be called an anti-apoptosis system.

Infertility and SPAG1/Eri15/Xt-mir axis

Examination of X-chromosome SNPs in non-obstructive azoospermia (NOA) males has detected SNPs associated with the onset of NOA near hsa-miR506/507 and hsa-miR510. SNPs near miR506/507 increased the risk of NOA, and SNPs near miR510 decreased[96]. miR508 adjacent to miR506/507 and miR506 targets SPAG1-2 with 7nt and 6nt matching, respectively (Table S1). Many of the mechanisms of miRNA expression are unknown, but clusters are said to be collectively regulated[95]. Therefore, miR506 and miR508 may be involved in developing NOA via spag1-2. miR510 targets not only SPAG1-2 but also SPAG1-1 with 7nt and 8nt matching, respectively (Table S1). The Fx-mir targeting SPAG1-1 is only miR510, and it seems that Fx-mir does not normally control SPAG1-1, but it cannot be ruled out that the misexpression of miR510 may have an inhibitory effect on the onset of NOA through the suppression of SPAG1-1. Therefore, these SNPs may be associated with the development of NOA by the action of SPAG1 via Fx-mir.

miR888 cluster is released into the peri-sperm fluid in epididymis via exosome, suggesting communication with mature sperm and downstream epithelial cells[97]. An exosome is a small membrane vesicle surrounded by a lipid bilayer, which contains proteins, lipids, mRNAs, microRNAs, etc., and is released extracellularly and has been attracting attention as an intercellular communication medium in recent years[98]. Exosomes are released not only in blood but also in most body fluids[97,99,100]. It has been confirmed that the protein in the exosome in the female reproductive fluid of mice is taken up by sperm[100]. Of the 13 epididymis-derived miRNAs in the semen of patients with asthenozoospermia (AZS), only the miR888 cluster was downregulated, showing a positive correlation between the expression level of the miR888 cluster and sperm motility[101].

Furthermore, there was no decrease in the amount of mitochondria in AZS patients, but the number of mtDNA was reduced to 9.7% of normal individuals[102]. Usually, mitochondria depleted of mtDNA should lose their action potential and be eliminated during spermatogenesis. However, since they are incorporated into sperm, mtDNA will likely be eliminated during maturation in the epididymis. The male reproductive tract has a defense system using the miR888 cluster against sperm mtDNA depletion, and its target may be SPAG1-2. It is suggested that the failure of the defense system destroys the sperm mtDNA, and the migration ability is reduced due to the depletion of ATP, resulting in AZS.

In an experiment in which the follicular fluid and sperm of a couple undergoing treatment of infertility with intracytoplasmic sperm injection (ICSI) etc. were used to observe sperm migration, a phenomenon that follicular fluid attracted sperm from a specific male was observed. The two are compatible, suggesting that the sperm may react to the chemical signal from the egg, and the egg may have selected sperm[103]. Since the reaction was different depending on the combination of follicular fluid and sperm, it cannot be denied that SPAG1-2 mRNA, which has an incompatibility relationship with Fx-miR in sperm, may be released to suppress sperm migration. Based on the above, some unexplained infertility may be caused by dysregulation or gene mutation of the SPAG1/Eri15/miRNA axis, and infertility patients may stand by the gateway to speciation. The encounter of compatible DMI model genes mutant, which was avoided by infertility of the degree of oligospermia and AZS, may be accelerated by treating infertility with ICSI, etc. It is possible that cryptic species already exist in humankind. From the above, it is suggested that Fx-mir may control the expression of SPAG1-2.

Speciation system

Hybrid sterility There are many fragile sites on the chromosome[91], including the Xq27.3 region in which Fx-mir is located. In the process of evolution, if one of the chromosome pairs breaks at the fragile sites and the Fx-mir is deleted during gametogenesis, the Fx-mir without a paralogous cluster[22] becomes a single copy. In fact, there is no miRNA on the Y chromosome[95]. In this individual, if a mutation that cannot suppress SPAG1-2 expression occurs in Fx-mir, it cannot be compensated, so mtDNA is eliminated, the energy supply is cut off, and gametes cease to mature. This mutation has no effect since SPAG1-1 is not expressed in somatic cells. Mutations in Fx-mir or SPAG 1-2 in male germ cells result in loss of the mutated gene by meiotic arrest, whereas female germ cells that do not express Fx-mir mature without problems and can be carriers for the mutated genes. Gamete maturation is restored if a mutation matching the mutation occurs in the corresponding gene in the male primordial germ cell that inherited the mutated gene from the mother. Even if there is a mutation in the SPAG1-2 coding sequence derived from the mother that cannot bind to SPAG1-1, the MMI system will work, and mtDNA is excluded if wild and mutant type SPAG1-1 is expressed in the mitochondria of F1 male spermatocyte. For this, SPAG1 needs to be co-dominant. In this way, post-reproductive isolation, called hybrid sterility, becomes possible, and new cryptic species covered with an invisible bubble that blocks gene flow is established.

Hybrid inviability As mentioned above, it was speculated that the SPAG1/Eri15 axis is also involved in programmed mitophagy in stem cell mitosis, including spermatogonia. Two (mmu-miR105, 542) of the 11 Xt-mir that are strongly expressed in spermatogonia[33] targeted Spag1-2 (Table S2, Fig.1). Since Xt-mir expressed in spermatogonia is often expressed in testis immediately after birth and in organs other than testis[33], it is highly possible that it is also expressed in stem cells other than spermatogonia. If incompatibility occurs between the Xt-mir and Spag1-2 mRNA, mitophagy will run away in the mitosis stage in stem cells. It seems that homogametic sex also uses the SPAG1/Eri15 axis for programmed mitophagy in stem cells, including oocytes, but the control may be done by putting in and out of SPAG1-1. As mentioned above, according to Orr's observation that hybrid XXY females showed inviability upon introduction of the Y chromosome in attached X fruit flies[12], the genes on the Y chromosome seem to control the testis-specific expression of Xt-mir and SPAG1-1 in heterogametic sex (If DMI can be explained by gonad-specific expression, the dominant theory would not be necessary). However, hybrid XXY females showed inviability rather than sterility, suggesting that the genes on the Y chromosome could not be expressed in the meiosis of spermatocytes due to MSCI and were involved in expressing SPAG1-1 in stem cells.

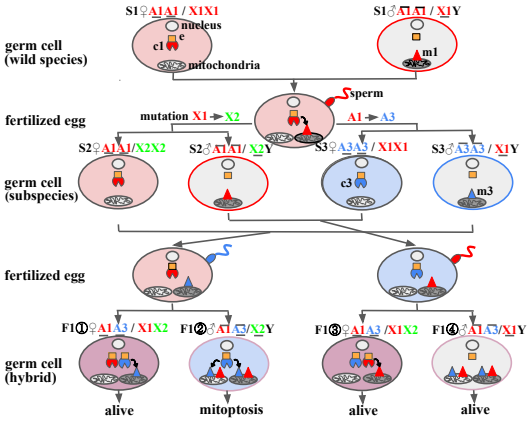
Schema of MMI system and hybrid sterility system

XY-type organisms (Fig. 2) It is considered that SPAG1-2 and SPAG1-1 interact with the TPR domain, and some of the multiple domains are the same due to the splicing variant[52,61]. Therefore, it is predicted that it will not become unresponsive within the same species even if the domain is mutated. However, as mentioned above, SPAG1 is a protein with rapid molecular evolution[28], and it is considered that the TPR domain mutation is progressing among the subspecies. So, it is assumed that the protein-protein interactions of cytoplasm type SPAG1(SPAG1-2) protein (c) and mitochondria type SPAG1(SPAG1-1) protein (m) react only between the same species and not between subspecies. In the fertilized egg of S2♀xS3♂ mating, the relationship is c1 and m3, and in S3♀xS2♂, the relationship is c3 and m1, and mitophagy does not occur, so mtDNA cannot be

eliminated and leaked to F1. S2♀xS3♂ produces F1 ①A1, A3/X1, X2, ②A1, A3/X2, Y. S3♀xS2♂ produces F1③A1, A3/X1, X2, ④A1, A3/X1, Y. Since Xt-mir genes targeting SPAG1-2 are not expressed in the eggs of F1①③♀, both c1 and c3 are expressed, but mitophagy does not occur because m1, m3 is not expressed (leaked mtDNA is eliminated, but only a few do not affect cell function). In F1④♂, both c1 and c3 are suppressed, so mitophagy does not occur. In the spermatocytes of F1②♂, c3 is expressed due to the relationship of A3/X2, so all mitochondria having m3 are excluded. Therefore, only the spermatogenesis of F1②♂ is impaired, and S2 and S3 have an incomplete reproductive isolation relationship. Furthermore, S2 and S3 give rise to subspecies S4 and S5 for each by the same mechanism, and S1, S4, and S5 become a heterogeneous relationship that is entirely hybrid male sterility. Eggs without organ-specific expression of Xt-mir and SPAG1-1 can coexist with A3/X2 in F1♀ and will be a source of incompatibility genes.

Then, is it possible that different species suddenly occur without passing the subspecies? Can heterogeneous A4, A4/X4, X4(Y) evolve from wild species A1, A1/X1, X1(Y), resulting in a reciprocal hybrid in which all males become sterile? For this purpose, A4 must be incompatible with X1, X4 must be incompatible with A1, and A4 and X4 must be compatible with each other simultaneously, so the probability is extremely low. Therefore, subspecies seems unavoidable as a step leading to heterologous. Even if the wild species become rare, the gene is maintained by crossing with subspecies, leaving room to respond to circulating environmental changes. It can be said that the existence of subspecies is valuable because the diversity of species can be obtained via the subspecies. The reason why most species choose hybrid sterility, which produces subspecies after mating, rather than the incompatibility between gametes as a method to prevent gene flow is probably because it is advantageous for species conservation to leave room to maintain species diversity through subspecies.

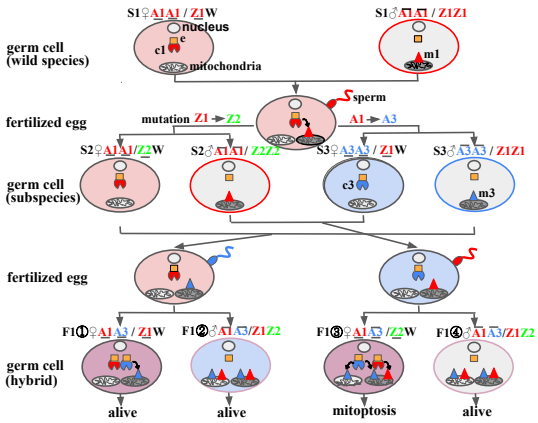
Figure 2. Maternal mtDNA inheritance and hybrid male sterility system in XY-type organisms
S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: SPAG1 gene. X: Xt-mir genes targeting SPAG1-2. c: cytoplasm type SPAG1(SPAG1-2) protein. m: mitochondria type SPAG1(SPAG1-1) protein. e: Eri15 protein. Overlined A: SPAG1-1 (m) expression. Underlined A: SPAG1-2 (c) expression. Underlined X: Xt-mir expression. X and m are expressed only in the testis. X suppresses SPAG1-2. Allele A is co-dominant. X1 mutates to X2, which can suppress A1, and evolves into subspecies S2; A1, A1/X2, X2 (Y). Separately, A1 mutates to A3, which can be suppressed by X1, and evolves into subspecies S3; A3, A3/X1, X1 (Y). However, X2 cannot suppress A3 (SPAG1-2).



ZW-type organisms (Fig. 3) Mitochondria is also maternally inherited in ZW-type birds and butterflies[104]. Considering the same mechanism as the XY type, the DMI system and the MMI system break down. Even in ZW females, if the Z chromosome has Xt-mir (should it be Zo-mir?) (miRNAs also exist on the Z chromosome[32]) and suppresses Spag1-2, male mitochondria cannot be eliminated. TOMM34 also hints at this problem. TOMM34 is the same molecule for both cytoplasm and mitochondria types[63]. The molecular size of SPAG1-1 and SPAG1-2 is reversed in humans and mice. Therefore, the cytoplasm and mitochondria type of SPAG1 are equivalent. Given that Xt-mir on the Z chromosome and the cytoplasm type Spag1-2 has organ (egg)-specific expression, and the mitochondria type Spag1-1 is suppressed by Xt-mir (Spag1-1 is expressed without being suppressed in spermatocytes), this problem will be resolved (genes on W chromosome may be controlling organ-specific expression).

Figure 3. Maternal mtDNA inheritance and hybrid female sterility system in ZW-type organisms
S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: Spag1 gene. Z: Xt-mir genes targeting Spag1-1. c: cytoplasm type Spag1(Spag1-2) protein. m: mitochondria type Spag1(Spag1-1) protein. e: Eri15 protein. Overlined A: Spag1-1 (m) expression. Underlined A: Spag1-2 (c) expression. Underlined Z: Xt-mir expression. Z and c are

expressed only in the eggs. Z suppresses Spag1-1. Allele A is co-dominant. Z1 mutates to Z2, which can suppress A1, and evolves into subspecies S2; A1, A1/Z2, Z2 (Y). Separately, A1 mutates to A3, which can be suppressed by Z1, and evolves into subspecies S3; A3, A3/Z1, Z1 (Y). However, Z2 cannot suppress A3 (Spag1-1).



Sex-determining (SD) system

Since hybrid male sterility is also observed in XO-type organisms[105], the responsible locus for sterility in the heterogametic sex of hybrid F1 lies on the X (or Z) chromosome. A similar composition is found in the sex differentiation system. In the male heterotype, not only the XY type but also the XO type becomes male, and in the female heterotype, not only the ZW type but also the ZO type becomes female[106]. Like the speciation system, the sex differentiation system seems to be caused by the X and Z single copy genes, which are not expressed in diploid but expressed in haploid only in the germline of heterogametic sex. Sex also exists in homozygous gametes of unicellular organisms. It is also conceivable that the primary sexual characteristics seen in multicellular organisms are merely subsequent changes in gametes as appendages. If sex is defined as a system that recognizes whether gametes can fuse, a sex-determining factor may be a protein of the system that gametes recognize and fuse as the opposite sex or a gene that controls it. The gene may also control primary sex characteristics. Since hybrids occur in crosses between closely related species, the sex differentiation system is not a species-specific reaction, and the speciation system may have utilized the existing sex differentiation system. In true slime molds showing uniparental mitochondrial DNA inheritance (UMI), the hierarchy of mitochondrial elimination is determined by the allele of one of the three mating type locus that determines many mating types (sex)[107]. That is, the SD system that determines the gamete's self or others (sex) is also involved in the UMI. The UMI and DMI systems use the SPAG1/Eri15/Xt-mir axis as a common mechanism. Therefore, the SD and DMI systems have a common mechanism, and the epistatic gene in the SD system may also be Xt-mir.

If the SPAG1 molecule is also expressed in the gamete plasma membrane, the relationship between the recognition of mating types in slime mold and the UMI hierarchy can be easily explained. SPAG1 was originally a molecule identified by a blood antibody that reacts with the sperm plasma membrane of infertile men[54]. The recombinant Spag1 antibody that Hayashida et al. used did not respond to the sperm plasma membrane. The Spag1-1 protein undergoes post-translational modification at maturity in the epididymis[51]. There may be differences in post-translational modifications on the outer mitochondrial membranes and plasma membranes. This difference may have changed the antigenicity and acquired a species-specific response to Spag1-2 in MMI. The existence of multiple TPR domains formed by 2~3 TPR motifs in Spag1 protein[51] may ensure recognition among multiple sex gametes of slime mold. It will be necessary to revalidate with an antibody to post-translational modified Spag1 protein instead of recombinant Spag1 protein as an antigen. Xt-mir may be a material proof for gender, a keyword common to MMI, DMI, and SD systems.

Wolbachia and the last boss

The progenitor of mitochondria is said to be rickettsia, which belongs to alphaproteobacteria[108]. Observing the effects of other rickettsia infecting the environment of eukaryotic cells constructed by mitochondria and competing with or interfering with mitochondria on the host may highlight the hidden functions that mitochondria bring to the host. Wolbachia (WO) is a rickettsia that lives symbiotically with arthropods and filarial nematodes and exerts various effects on the host[109]: 1) Maternal inheritance: WO can infect oocytes but is eliminated in sperm during maturation, resulting in maternal inheritance. 2) Male killing: Mating WO-infected females with non-infected males produces only females, not males. 3) Cytoplasmic incompatibility: Oocytes cannot be produced by mating WO-infected males with non-infected females. 4) Feminization: WO-infected genetic males

generated by crossing WO-infected females and non-infected males change into morphological females. 5) Parthenogenesis: WO-infected females can produce offspring without needing males. 6) Females moth (ZW female type) whose WO-infected larvae have been sterilized with antibiotics produce only males, but not females[110]. 1) closely resembles maternal mtDNA inheritance, 2) hybrid male inviability, and 3) DM incompatibility. 4) 5) 6) may also be related to the SD system by the SPAG1/Xt-mir axis mentioned above. Conversely, it is suggested that Xt-mir are involved not only in gamete sex but also in primary sex characteristics. None of the phenotypes 2) 3) 4) 6) are expressed in adult infection and appear during embryonic development after mating infected and uninfected parents, imagining post-mating reproductive isolation and DMI. In other words, the phenotypes of WO-infected hosts suggest that mitochondria are involved not only in the MMI system but also in the DMI and SD systems.

WO could easily adapt to the system created by mitochondria derived from rickettsia, thus making symbiosis possible. However, the changes in the host caused by WO seem to control the physiological phenomena of the host beyond the symbiosis. The 6) phenotype indicates that the effects of WO remain even after sterilization, suggesting that some genes brought in by WO remain. Epistatic genes in the MMI and DMI system are thought to be miRNA (Xt-mir). It is thought that competing miRNAs or higher epistatic genes can control this system. As mentioned above, according to Orr's observation[12], the testis-specific expression of Xt-mir seems to be controlled by the gene on the Y chromosome. Although there is no miRNA on the Y chromosome[46], Long non-coding RNA (lncRNA) was present, and it was shown that KO of Y-linked lncRNA upregulated miRNA and suppressed apoptosis[111]. LncRNA, which is non-coding RNA with a length of 200 bases or more, exists widely in diverse species, including viruses, prokaryotes, and eukaryotes, and is often expressed organ-specifically in animals, especially in the testis[112]. LncRNA is considered a regulatory molecule involved in genetic regulatory processes[113]. In a competitive endogenous RNA (ceRNA) network consisting of lncRNA, miRNA, and mRNA, lncRNA interacts with miRNA to reduce the action of miRNA through its decoy effect and interact with transcription factors to be involved in the activation and repression of transcription[113]. Xt-mir is expressed in meiosis to escape MSCI[33]. Some lncRNAs are involved in gene imprinting and X-chromosome inactivation[114], and its KO mice show reduced sperm counts and decreased males in offspring[115]. Recently, it was shown that among lncRNAs differentially expressed in WO-infected mosquitoes, upregulated aae-lnc-7598 induces an antioxidant gene, and downregulated aae-lnc-0165 upregulates miRNA via the ceRNA network to reduce intracellular mitochondrial ROS (mtROS) and ensure endosymbiosis in the host [116]. Damaged mitochondria, which release cytochrome c, mtROS, and mtDNA that are detrimental to cellular homeostasis, are processed by mitophagy[117]. The previously mentioned herpes simplex virus used its endonuclease to induce mitophagy and prevent host cell apoptosis for infection[69], whereas WO appears to prevent ROS release due to mitochondrial damage for symbiosis. Both suggest that host cells use mitochondria to block invaders. Alternatively, mitochondria may guard their niche in the cell. The findings of aae-lnc-0165 and the aforementioned Y-linked lncRNA suggest the existence of miRNAs that suppress the disruption of mitochondria and the existence of Y-linked lncRNAs that suppress these miRNAs. The factors by which WO controls the host's ceRNA network are unknown. However, by manipulating the host's lncRNA to control the Xt-mir, WO may enable symbiosis and exert sexually biased influences on the host. LncRNAs on heterologous chromosomes may be the last boss of the MMI, DMI, and SD systems.

Conclusions and future perspectives

Genes that match the Haldane/Dobzhansky-Muller model should be one set of an epistatic gene on the X (Z) chromosome and an effector gene on the autosome. The two genes cause incompatibility in gonads or stem cells in hybrid heterogametic sex and exhibit sterility and inviability due to ATP depletion by mitoptosis, respectively. The author proposed SPAG1 and Xt-mir as a candidate for the two genes. SPAG1/Xt-mir axis is not only involved in the maternal mitochondrial DNA inheritance system, infertility, sex determining system, and carcinogenesis, but may also be involved in the cause of unexplained diseases involving mitophagy, so further consideration will be needed.

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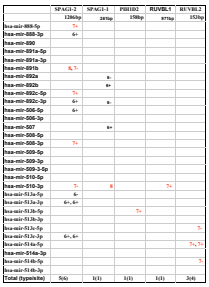
Supplementary informations

Table S1. Human Fx-mir and target genes (*SPAG1-1* and R2SP complex member)
8: 8mer matching. 7+: 7mer-m8. 7-: 7mer-A1. 6+: 6mer. 6-: offset 6mer[27]. 7 and 8mer searched by TargetScan. 6mer searched only *SPAG1* by Multiple sequence alignment (https://www.genome.jp/tools-bin/mafft). The total number indicates the miRNA type (mRNA 3' UTR target site) of 7 or 8mer matching (red numbers).



	SPAG1-2 1206bp	SPAG1-1 2070bp	PHF10D2 1508bp	RUVBL1 977bp	RUVBL2 1528bp
hsa-miR-888-5p	7+				
hsa-miR-888-3p	6+				
hsa-miR-890					
hsa-miR-891a-5p					
hsa-miR-891a-3p					
hsa-miR-891b	8, 7-				
hsa-miR-892a		8-			
hsa-miR-892b		8+			
hsa-miR-892c-5p	7+				
hsa-miR-892c-3p	6+	8-			
hsa-miR-506-5p	6+				
hsa-miR-506-3p					
hsa-miR-507		8+			
hsa-miR-508-5p					
hsa-miR-508-3p	7+				
hsa-miR-509-5p					
hsa-miR-509-3p					
hsa-miR-509-3-5p					
hsa-miR-510-5p					
hsa-miR-510-3p	7-	8		7+	
hsa-miR-513a-5p	6-				
hsa-miR-513a-3p	6+, 6+				
hsa-miR-513b-5p			7+		
hsa-miR-513b-3p					
hsa-miR-513c-5p					7-
hsa-miR-513c-3p	6+, 6+				
hsa-miR-514a-5p					7+, 7+
hsa-miR-514a-3p					
hsa-miR-514b-5p					7-
hsa-miR-514b-3p					
Total (typesites)	5(6)	1(3)	1(1)	1(1)	2(4)

Table S2. Mouse Fx-mir, Xt-mir, and target genes (*Spag1* and *Fmr1*)
8: 8mer matching. 7+: 7mer-m8. 7-: 7mer-A1. 6+: 6mer. 6-: offset 6mer[27]. 7 and 8mer searched by TargetScan. 6mer searched only Spag1/Fx-mir by Multiple sequence alignment. The total number indicates the miRNA type (mRNA 3' UTR target site) of 7 or 8mer matching (red numbers). Xt-mir (except Fx-mir) is quoted from Song et al., 2009[33]. The 3'UTRs of mouse Spag1-1 and Spag1-2 are almost the same.

Fx-mir	Spag1	Fmr1	Xt-mir	Spag1	Fmr1
	726bp	2299bp		726bp	2299bp
mmu-mir-743a-5p			mmu-mir-105	7-	
mmu-mir-743a-3p	6-	7+	mmu-mir-106a		
mmu-mir-743b-5p			mmu-mir-1198		
mmu-mir-743b-3p	6-	7+	mmu-mir-18b		7-
mmu-mir-742-5p	8		mmu-mir-188		7+
mmu-mir-742-3p	6-		mmu-mir-19b-2		8, 7-
mmu-mir-883a-5p		7-	mmu-mir-1906-2		
mmu-mir-883a-3p	7+, 6+, 6+		mmu-mir-20b		
mmu-mir-883b-5p			mmu-mir-2137		
mmu-mir-883b-3p	7+, 6+, 6+		mmu-mir-221		8
mmu-mir-471-5p			mmu-mir-222		8
mmu-mir-471-3p	6-		mmu-mir-223		
mmu-mir-741-5p			mmu-mir-224	7+, 7+	
mmu-mir-741-3p			mmu-mir-3112		
mmu-mir-463-5p			mmu-mir-322		7+
mmu-mir-463-3p	6-		mmu-mir-325	7-, 7-, 7-	8, 7+, 7-
mmu-mir-880-5p	6+	8	mmu-mir-3472		
mmu-mir-880-3p		7+	mmu-mir-3473a		
mmu-mir-878-5p	6-	7+	mmu-mir-351		7-
mmu-mir-878-3p			mmu-mir-361		
mmu-mir-881-5p		7+	mmu-mir-362		
mmu-mir-881-3p		7+	mmu-mir-363		8
mmu-mir-871-5p			mmu-mir-374b		7+
mmu-mir-871-3p			mmu-mir-374		
mmu-mir-470-5p			mmu-mir-384		7-
mmu-mir-470-3p			mmu-mir-421	7+	
mmu-mir-465d-5p			mmu-mir-448		7+, 7-, 7-
mmu-mir-465d-3p			mmu-mir-450a		
mmu-mir-465c-1-5p			mmu-mir-450a-2		8
mmu-mir-465c-1-3p			mmu-mir-450b		7+, 7-
mmu-mir-465b-1-5p			mmu-mir-452		8, 7+
mmu-mir-465b-1-3p			mmu-mir-500		
mmu-mir-465c-2-5p			mmu-mir-501		
mmu-mir-465c-2-3p			mmu-mir-503		7-, 7-
mmu-mir-465b-2-5p			mmu-mir-504		
mmu-mir-465b-2-3p			mmu-mir-506		
mmu-mir-465a-5p			mmu-mir-532		7-
mmu-mir-465a-3p			mmu-mir-542	7+	
mmu-mir-201-5p	6+	7-	mmu-mir-652		
mmu-mir-201-3p		8	mmu-mir-672		7-
mmu-mir-547-5p			mmu-mir-676		
mmu-mir-547-3p			mmu-mir-680-2		
mmu-mir-509-5p			mmu-mir-717		7+
mmu-mir-509-3p			mmu-mir-718		
			mmu-mir-764		
			mmu-let-7f-2		7+
			mmu-mir-92a-2		8
			mmu-mir-98		
Total (type/site)	3 (3)	10 (10)		5 (8)	21 (29)