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", but did not show the mechanism by which 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 to be the most reliable as 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 these observation and prediction are considered speciation genes. Here, I 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.

Keywords

speciation, postmating reproductive isolation, Haldane's rule, hybrid sterility, hybrid inviability, Dobzhansky-Muller incompatibilities model, maternal mitochondrial DNA inheritance, programmed mitophagy, meiotic arrest, Warburg effect

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" (Darwin, 1859), which dramatically and revolutionarily advances the understanding of life. However, due to the limitations of genetics at the time, it was not possible to explain the 'origin of speciation', how evolved populations are fixed and maintained as new species. There are many definitions of species, but it would be simplest and clearest to make it a population in which genes are not mixed by mating with other populations. Reproductive isolation, especially post-mating reproductive isolation, is considered to be the most reliable guarantee of gene flow blockage (Mallet, 1995).

It is not uncommon to form hybrids by mating between species that were considered morphologically heterogeneous and to maintain their hybrids (i.e., syngameon) (Seehausen, 2004). Furthermore, there are species that 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 (Trontelj, 2009). Morphological differences seem to have nothing to do with speciation. Haldane, 1922 proposed that heterogametic sex (XY male or ZW female, etc.) was absent, rare, or sterile in interspecific hybrid F1. (i.e., Haldane's rule) . In mammals, hybrid males develop spermatogenic deficiency (i.e., hybrid male sterility, HMS) and inviability (absent or rare). As a result, hybrid F2 does not occur and the species is conserved. Dobzhansky, 1934 and Muller et al., 1942 predicted that reproductive isolation was caused by mutations occurring at two or more interacting loci, and the gene functionally diverged in each individual and show incompatibility only in hybrid (i.e., Dobzhansky-Muller incompatibilities model, DMI model). The observations and predictions about post-mating reproductive isolation presented by Haldane, Dobzhansky, and Muller seem to be 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 type male and ZW type female), 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 (Kimura, 1968) 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 exactly 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) (Safran, 2013). 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 considered carefully whether they are speciation genes or mutations that occur after speciation. It is difficult to imagine that the system involved in the extremely essential event of biological evolution is different 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 taxon of the species, indicating incompatibility. Here, I considered the conditions of the speciation genes by reviewing subsequent findings based on the DMI model. Many speciation genes have been reported so far, but none of them satisfy these conditions, so I would like to present my hypothesis.

Two karyotypes

Haldane, 1922 showed that the phenomenon that causes reproductive isolation in hybrid is observed in heterogametic sex (two karyotypes, XY, XO male and ZW, ZO female). 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, 1922 showed that there are two phenotypes of sterility or inviability (absent or rare) in the hybrid heterogametic sex. Hybrid sterility is observed in mice with a meiotic arrest in primary spermatocytes rather than mitotic arrest in spermatogonia (Imai, 1981). Hybrid inviability is due to impaired embryogenesis, and Orr et al., 1997/1 have shown that it is caused by mitotic arrest in Drosophila. The two phenotypes are meiotic arrest or mitotic arrest, suggesting that it is caused by cell division disorders.

Orr, 1993 recognizes inviability in hybrid XXY females by crossing females with attached two X chromosomes and heterogeneous males in two species of fruit flies that normally exhibit hybrid male sterility. For this reason, Orr argues that the genetic causes of Haldane's rule differ between sterility and inviability. It shows that there are 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, and they show 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 (loci)

In the DMI model, at least two genes (loci) can be functionally diverged by mutations that do not cause incompatibility, and when two mutated genes meet in a hybrid, it is said that incompatibility occurs in heterogametic sex, causing sterility or inviability. This 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 gene and effector gene. 2) Disorders by misexpression (or overexpression) of harmful functions due to incompatibility of suppressive epistatic gene and effector gene. 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 the males.

Large-X effect and large-Z effect

X chromosome replacement by backcrossing between heterogeneous has a greater disproportionate effect on hybrid fitness than autosomal chromosomes (i.e., large-X effect) (Bhattacharyya, 2014). Haldane’s rule predicts that even if a harmful mutation occurs in recessive X-linked allele, it is injurious in heterogametic hybrids, but in homogametic hybrids, it is masked by harmless dominant allele and no disorder appears (i.e., dominance theory) (Turelli et al., 1995). 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 (Dobzhansky, 1937). Both have shown the importance of the X chromosome in hybrid sterility. Furthermore, a large Z effect (Ellegren, 2009) 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) (Forejt et al., 1974) on chromosome 17 and hybrid sterility X2 (Hstx2) (Heiden et al., 2009; Bhattacharyya et al., 2014) on chromosome X are mapped as hybrid sterility loci by

quantitative trait locus analysis using consomic strain using mouse sperm count and testis weight as indicators. There are 6 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 to be the causative gene of hybrid sterility (Mihola et al., 2009). However, the Prdm9 knockout mouse shows infertility not only in males but also in females (Hayashi et al., 2005), furthermore, it is shown that it is not essential for meiosis (Mihola et al., 2019), so it is hard to believe that Prdm9 is the effector gene of the DMI model.

Hstx2 contains 10 protein-coding genes and 22 microRNAs (miRNAs), but the hybrid sterility gene has not yet been identified. Morimoto et al. 2020 confirm that knockout mice of 6 protein-coding genes except for genes, which are known not to be involved in spermatogenesis (there is disagreement about Fmr1 as described later.), do not cause infertility. 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 (Zhang, et al., 2019). This region is easily physically cleaved and was initially noted in studies of fragile sites that are abundant on chromosomes, demonstrating that the fragility lies in the FMR1 mutation (Garber et al., 2008). Recently, X-linked miRNA has been attracting attention from the aspect of 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 (Garber et al., 2008). In mice, 22 genes (44 mature miRNA) are present as Fx-mir (Ramaiah et al., 2019). Some of the mouse Fx-mir are weakly expressed in various organs including the ovary, but most of them are strongly expressed only in the testis (Ramaiah, et al., 2019). 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 (Bartel, 2009). In hybrid male sterility of Nematoda, the interaction between the X chromosome and the autosomal loci was shown to be essential (Bi et al., 2018), 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 (Ramaiah, et al., 2019; Bartel, 2018). 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 (Zhang et al., 2019). 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) (Torgerson et al., 2003; Zhang et al., 2007; Orr, 1997/2). Since the genes on the male X chromosome are single copies, mutations are easily fixed, and strong 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) (Zhang et al., 2007). However, since the faster-Z effect has been confirmed in birds (ZW type) (Mank et al., 2007), this speculation breaks down. 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 next differentiation, so regardless of the neutrality of molecular evolution (Kimura, 1968), 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 said to be an intrinsic, autonomously 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 (Guo et al., 2009). That is, X-linked miR expressed in the testis (X-linked testis miR, 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) (Song et al., 2009). Xt-mir, which is 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 (Ramaiah, et al., 2019), 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 particularly 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 (Berezikov et al., 2005). 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 strongly fixed within the species (Zhang et al., 2007). The evolution of the Fx-mir (or Xt-mir) cluster is very different from other miRNAs and is strongly suspected to be involved in speciation, further strengthening the possibility of DMI model genes.

Infertility

Azoospermia and oligospermia may result if reproductive dysfunction is caused by large mutations or deletions in the DMI model genes that exceed the permissible range in germ cells. Furthermore, as a process of speciation, if both partners are carriers of the incompatible gene, the son will be infertile. Investigating the causes of infertility in humans may provide clues to the speciation genes. 5% of men have infertility, of which 75% are said to have idiopathic sperm dysfunction of unknown cause (Okada et al., 2008). The miR888 cluster was downregulated in the testis of non-obstructive azoospermia (NOA) patients compared to obstructive azoospermia patients (Piryaei et al., 2022). 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 of the reports refer to cell death due to the meiotic arrest of hybrid sterility as apoptosis. However, there are no histological findings characteristic of apoptosis such as nuclear rupture or apoptotic body in hybrid testis (Kaku et al., 1995; Hayashida et al., 2009). 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, it seems that apoptotic cells are 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 very small cytosol/nucleus ratio, so even if cytosol is stained, it is difficult to recognize it overlapping with counterstain in a normal light microscopic image. Hayashida et al. 2009 showed by TUMEL assay using confocal fluorescence microscopic image that spermatocytes of mouse hybrid sterility testis have mtDNA disruption, but not nuclei. Recently, Yu et al., 2022 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 not clear why hybrid gonads cause structural changes (or disruption?) of mtDNA. 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) (Gibeaux et al., 2018). Hybrid sterility and hybrid inviability may be caused by mtDNA depletion rather than apoptosis. The fact that the terminal image of hybrid sterility in very distant taxa, 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.

Candidate conditions for DMI model genes

From the above, DMI model genes candidates can be summarized as follows. 1) DMI model genes may be one set of Fx-mir (or Xt-mir) and effector genes on autosomes controlled by this. 2) Meiotic arrest and mitotic arrest show sterility and inviability, respectively, due to the difference in expression time or cells of two genes incompatibility. 3) DMI model genes may be involved in 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 normally 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 DMI model 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.

To date, many speciation genes have been identified in yeast, thale cress, fruit fly, mouse, etc., and many are involved in transcriptional or translational regulation (Mack et al., 2018), but the underlying common mechanism remains unclear. Much hybrid sterility or inviability genes have been identified in

the fruit flies. Among them, the lethal hybrid rescue (Lhr) and the hybrid male rescue (Hmr) are reported as two distinct interacting genes (Brideau et al., 2006), but both recognize few orthologs except Brachycera (fly) （ORTHOSCOPE, http://yurai.aori.u-tokyo.ac.jp/orthoscope/Actinopterygii.html）. Below, I would like to present my hypothesis, which almost matches these conditions.

HYPOTHESIS

Maternal mitochondrial DNA inheritance (MMI) and hybrid male sterility(HMS)

MMI system In most sexually reproducing eukaryotes, the mtDNA of one gamete is eliminated after mating between gametes (i.e., uniparental mtDNA inheritance, UMI) (Birky, 1995). 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（maternal mitochondrial DNA inheritance, MMI) (Szollosi, 1965). The MMI system is very strict and is completely eliminated among allogeneic species (Birky, 1995). Based on speculation that this system is for the processing of sperm mtDNA damaged by reactive oxygen species (ROS), there are theories that male sperm mitochondria are selectively processed by the ubiquitin-proteasome system or autophagy of the fertilized egg (Sutovsky et al., 1999; Al Rawi et al., 2011). 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 (Shitara et al., 2000), but sperm mitochondria were eliminated by microinjection into somatic cells (Manfredi et al., 1997). This means 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 in the same way as eggs. In most eukaryotes, most of the mtDNA has been transferred to the nucleus, but only a few genes remain in mitochondria, including genes essential for maintaining the function of mitochondria (ATP synthase, etc.) (Lang et al., 1999). In mice, a phenomenon that sperm mtDNA disappears before the mitochondrial membrane potential is lost was observed in the fertilized egg (Kaneda et al., 1995). Hayashida et al., 2005, 2008 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-isoform 2 (Spag1-2, cytoplasm type) protein transports endogenous retroviral integrase 15kDa (Eri15) (new accession No. LC627956.1), which has endonuclease activity in the egg cytoplasm, and Spag1-isoform 1 (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 (mitophagy) (Hayashida et al., 2005, 2008).

HMS system The MMI system is said to avoid competition with heterosexual mtDNA and parasites brought in by sperm mitochondria (Birky, 1995), but it is easily disrupted by intersubspecific and interspecific hybrid, and paternal mtDNA is detected in the somatic cells of F1 individuals in mice (Kaneda et al., 1995). The intermolecular reactions involved in MMI can be said to be species-specific. For this reason, Hayashida et al., 2009 thought that this system may have evolved rapidly at the forefront of speciation. Spermatocytes of mutant mice lacking part of mtDNA have been shown to cause meiotic arrest (Nakada et al., 2006). Since Eri15 is also expressed in the spermatocytes (Hayashida et al., 2008), 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., 2009 showed that not only Spag1-1 but also Spag1-2 was expressed in the intersubspecific and interspecific hybrid testis in mice, and 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 (Hayashida et al., 2009). 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 system.

SPAG1 SPAG1 was discovered as one of the target proteins of anti-sperm antibodies in unexplained infertile females (Bohring et al., 2001). In mice, Spag1-1 (114 kDa) is expressed only on the outer mitochondrial membrane of the testis, but in epididymal sperm, it is post-translationally modified and detected at 166 kDa (Hayashida et al., 2005), 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 (Torgerson et al., 2003). SPAG1 may be target gene that have a molecular co-evolutionary relationship with Xt-mir (Ramaiah et al., 2019) 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 (Hayashida et al., 2005, 2008). 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 (Neesse et al., 2007; Kanazawa et al., 2003; Smith, et al., 2022). SPAG1 is expressed in both types in cancer cells and undifferentiated respiratory epithelial cells (Neesse et al., 2007; Smith, et al., 2022), and only 50 kDa isoform in sperm (Kanazawa et al., 2003), and is considered to be a cancer-testis antigen (CTA) (Siliņa et al., 2011). SPAG1-2 provides a platform for quaternary protein folding of proteins via the TPR domain (Takaishi et al., 1999; Allan et al., 2011) 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 (Smith, et al., 2022; Maurizy et al., 2018) (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, and its assembly function is strongest at the proper temperature of the testis, 32 °C, and is optimized for the testis environment (Maurizy et al., 2018). This 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 (Wade et al., 1999). The SPAG1/Eri15 axis may also be related to hybrid inviability.

TOMM34 (translocase of outer mitochondrial membrane 34) is a protein with the highest homology to SPAG1 (Hayashida et al., 2005). TOMM34 also has 2 TPR domains, is localized in the cytoplasm and outer mitochondrial membrane, and is mainly expressed in the testis (Faou et al., 2012). 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 (Trcka et al., 2014). However, Tomm34 knockout mouse does not show any obstacles (Terada et al., 2003). Some compensation function 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 (Hayashida et al., 2008). This can explain the above-mentioned observation that sperm mitochondria were eliminated by microinjection into somatic cells (Manfredi et al., 1997). In the mitochondria matrix, the pH is alkaline, the Mn^{2+} concentration is high (Hayashida et al., 2008), and the Zn^{2+} concentration is three orders of magnitude lower than that of the cytoplasm (Park et al., 2012). The activity of recombinant Eri15 is strongest 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 (Zheng et al., 1996), whereas Eri15 is truncated in this portion and endonuclease activity is preserved, but on the contrary, the activity is suppressed by Zn^{2+} (Hayashida et al., 2008). 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 (Vo et al., 2021). 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 (Safran et al., 2007). Xia et al., 2014 has been 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. This 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 (Nakada et al., 2006). 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 (Yu et al. 2022). Dmc1 that causes meiotic arrest due to synaptic failure by the defect is ATP-dependent (Pittman et al., 1998). 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 mitophagy for passive quality control.

It is said that 8-10 % of mammalian genomes are occupied by the endogenous retrovirus genes (Crowell et al., 2007), and some are used by the host, such as syncytin, which is involved in human placental formation (Mi et al., 2000). It is quite 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 mitochondrial autophagy (i.e., mitophagy) has been pointed out (Pickles et al., 2018), but most of 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 for the homogenization of mtDNA (Jansen et al., 1998). It was suggested that Spag1 is involved in mitochondria synthesis and dynamic regulation during meiosis in mouse oocytes (Huang et al. 2016). The copy number of mtDNA is actively reduced during spermatogenesis (Kasashima et al., 2014). In erythroblasts differentiated from hematopoietic stem cells, mtDNA degradation by ANKLE1 protein with endonuclease activity causes mitophagy to become mature erythrocytes. ANKLE1 is normally expressed only in erythroblasts, but is also expressed 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) (Warburg, 1956) and avoiding apoptosis (Przanowski et al., 2021).

Most stem cells, including cancer stem cells, have few mitochondria and exhibit the Warburg effect (Zhang et al., 2018). Aurrière et al., 2021 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. 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 important role in mitosis, are ATP-dependent (Vignali et al., 2000). The aforementioned observation by Xia et al. 2014 that ATP depletion by the measles virus in lung cancer cells led to necrosis 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 (Kaku et al., 1995; Li et al., 2009) is that when the mitotic arrest is caused in spermatogonia, it becomes mitotic arrest in the whole stem cells at the same time, and 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, both SPAG1-1 and SPAG1-2 are expressed in cancer cells (Neesse et al. 2007). In lung adenocarcinoma cells, the overexpression of SPAG1-2 increased autophagy and decreased cell proliferation and colony formation (Li et al., 2021). 94, 106 kDa SPAG1 isoform expression increases with the differentiation of human respiratory epithelial cells, whereas only 60 kDa isoform shows a constitutive expression (Smith et al., 2022). This may indicate expression in stem cells, which exist in a certain number of cultured cells. Expression of multiple SPAG1 proteins of ~ 50 and ~ 100 kDa is observed in iPS cells during differentiation induction (Horani et al. 2018). 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 (Heiden et al., 2009). From the above, it is possible to explain that mitotic arrest or meiotic arrest is caused by cells expressing one set of DMI model genes, and show two phenotypes, inviability and sterility, respectively.

Eukaryotic cells are thought to have developed in the symbiosis of heterotrophic eubacteria in autotrophic archaea (Lang et al., 1999). Before the symbiosis, it seems that there was 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.

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) (Bartel, 2018). The relationship between miRNA and mRNA is complex and uncertain, and it seems difficult to discuss the suppressive effect with only the match of the seed sequence, so experimental verification by gene knockout, etc. is required (Bartel, 2018). Since miRNA and miRNA cluster have evolved through genomic duplication events (Sun et al., 2013), and miRNA and mRNA are in a co-evolutionary relationship (Ramaiah et al., 2019), 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) (Bartel, 2018) 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 the formation of the epididymis and sperm maturation (Li et al., 2010). According to

TargetScan online software (https://www.targetscan.org/), 5 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 of the 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., 2014, including parts of other than the Hstx2 (Fx-mir) locus.

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 (Pittman et al., 1998). Of these two miRNAs, miR743 has a 6nt seed match for SPAG1-2 3'UTR (Table S2). Ota et al. 2019 showed no histological abnormalities in the testes with individual KO mice of mmu-miR741, mmu-miR871, and mmu-miR880, but with miR871 + miR880 or all three KO mice, spermatogenesis is stopped in a part of the seminiferous tubule. Of these three miRNAs, only miR880 has a 6nt seed match to SPAG1-2 3'UTR (Table S2). However, Wang et al., 2020 reported that when 18 of the 21 Fx-mirs of the mice were knocked out at the same time, the mice developed normally and the testes were not histologically affected. If the results of Ota et al., 2019 and Wang et al., 2020 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. 2019 identified 11 Fx-mirs in mice (6 in humans) targeting Fmr1, which are always present downstream of Fx-mir in the fragile X region in mammals, and confirmed that the induction of each of the 4 miRNAs suppresses the expression of Fmr1 protein (FMRP) (Ramaiah et al., 2019). 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 to be involved in translational regulation as an RNA-binding protein (Garber et al., 2008; Feng et al., 2017). The FMR1 orthologs are widely conserved in animals (ORTHOSCOPE). As mentioned above, Fx-mir does not have paralogous clusters (Zhang et al., 2019). The reason why Fx-mir cannot be compensated may be that the benefit (speciation) for selfish genes (Dawkins et al., 2017) 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 kind of defense system coexists. Fmr1 is involved in spermatogenesis, axoneme synthesis, and the Warburg effect (Zhang et al., 2004; Maddalena et al., 2020), 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 (Bartel, 2018), and if proteome is formed around miRNA cluster, it is considered that attention should be paid to the selection of 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 is 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 to be inhibitory for cancer growth and malignant transformation (Yoshida et al., 2021). Furthermore, Fx-mir is not expressed in spermatogonia, which shows the Warburg effect as well as cancer cells (Song et al., 2009). It can be said that 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 NOA men 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 (Ji et al., 2016). 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 (Yoshida et al., 2021). Therefore, miR506 and miR508 may be involved in the development of 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 SPAG1-1 is not normally controlled by Fx-mir, 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 (Belleannée, 2015). An Exosome is a small membrane vesicle surrounded by a lipid bilayer, which contains proteins, lipids, mRNAs and microRNAs, etc., and is released extracellularly, and has been attracting attention as an intercellular

communication medium in recent years (Raposo et al., 2013). Exosomes are released not only in blood but also in most body fluids (Belleannée, 2015; da Silveira et al., 2012; Griffiths et al., 2008). It has been confirmed that the protein in the exosome in the female reproductive fluid of mice is taken up by sperm (Griffiths et al., 2008). 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 (Qing, et al., 2017). Furthermore, there was no decrease in the amount of mitochondria in AZS patients, but the amount of mtDNA was reduced to 9.7% of normal individuals (Kao et al., 2004). Normally, mitochondria depleted of mtDNA should lose their action potential and be eliminated during spermatogenesis, but since they are incorporated into sperm, mtDNA is likely to 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 sperm mtDNA is destroyed by the failure of the defense system 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 (Fitzpatrick et al., 2020). 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 the patients of infertility 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 the treatment of infertility with ICSI, etc. It is quite possible that cryptic species already exist in humankind.

From the above, it is suggested that the expression of SPAG1-2 may be controlled by Fx-mir.

Speciation system

Hybrid sterility There are many fragile sites on the chromosome (Feng et al., 2017), 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 (Zhang et al., 2019) becomes a single copy. In fact, there is no miRNA on the Y chromosome (Yoshida et al., 2021). 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. Since SPAG1-1 is not expressed in somatic cells, this mutation has no effect. 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 block 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 (Song et al., 2009) 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 (Song et al., 2009), 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, a runaway of mitophagy will occur 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 (Orr, 1993), 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 the expression of SPAG1-1 in stem cells.

Simulation 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 (Hayashida et al., 2008; Maurizy et al., 2018). Therefore, it is predicted that within the same species, it will not become unresponsive even if the domain is mutated. However, as mentioned above, SPAG1 is a protein with rapid molecular evolution (Torgerson et al., 2003), and it is considered that the TPR domain mutation is progressing among the subspecies. So, it is assumed that the protein-protein interactions of c and 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, mtDNA cannot be eliminated and leaked to somatic cells. Mating S2♀xS3♂ produces F1 ①A1, A3 / X1, X2, ②A1, A3 / X2, Y, S3♀xS2♂ to F1③A1, A3 / X1, X2, ④A1, A3 / X1, Y. Since X is not expressed in the eggs of ①③♀, 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 F1 ④♂, both c1 and c3 are suppressed and mitophagy does not occur. In the spermatocytes of F1②♂, c3 is expressed due to the relationship of A3 / X2, and all mitochondria having m3 are excluded. Therefore, only the spermatogenesis of ②♂ 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 completely 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.

Is it possible for all hybrid males to become sterile with reciprocal crossing by evolving from wild species A1, A1 / X1, X1 (Y) to heterogeneous A4, A4 / X4, X4 (Y)? For this purpose, it is necessary for A4 to be incompatible with X1 and X4 to be incompatible with A1 and for A4 and X4 to be compatible with each other at the same time, so the probability is extremely low. Therefore, the existence of subspecies seems to be unavoidable as a step leading to heterologous. Even if the wild species become a rare species, 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.

ZW-type organisms (Fig. S1) Mitochondria is also maternally inherited in ZW-type birds and butterflies (Berlin et al., 2004). 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 (Guo et al. 2009)) and suppresses Spag1-2, male mitochondria cannot be eliminated. This problem is also hinted at by TOMM34. Both cytoplasm and mitochondria types of TOMM34 function with the same molecule (Faou et al., 2012). The molecular size of SPAG1-1 and SPAG1-2 is reversed in humans and mice. Therefore, the cytoplasm and mitochondria type of SPAG1 are considered to be 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).

Sex-determining system

Since hybrid male sterility is also observed in XO-type organisms (Wu et al., 1993), 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 (Ellegren, 2011). 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 or not, 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. Primary sex characteristics may also be controlled by the gene. 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) (Meland et al., 1991). That is, the SD system that determines the gamete's self or others (sex) is also involved in the UMI. The UMI system and DMI system use the SPAG1 / Eri15 / Xt-mir axis as a common mechanism. Therefore, the SD system and the DMI system 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 women (Bohring et al., 2001). The recombinant SPAG1 antibody that Hayashida et al., 2005 used did not respond to the sperm plasma membrane. The SPAG1-1 protein undergoes post-translational modification at maturity in the epididymis (Hayashida et al., 2005). 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 (Hayashida et al., 2005) 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, which is a keyword common to MMI, DMI, and SD systems.

Wolbachia and the last boss

The progenitor of mitochondria is said to be a rickettsia, which belongs to alphaproteobacteria (Roger et al., 2017). Analyzing the effects on the host as a result of competition or interference with mitochondria due to infecting the environment of eukaryotic cells constructed by mitochondria with the same rickettsia is possible to illuminate hidden functions that mitochondria bring to their hosts. Wolbachia (WO) is a rickettsia that lives symbiotically with arthropods and filarial nematodes and exerts various effects on the host (Kaur et al., 2021). 1) WO can infect oocytes but is eliminated in sperm during maturation, resulting in maternal inheritance. 2) Male killing: Mating of WO-infected females with non-infected males produces only females, but 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 type) whose WO-infected larvae have been sterilized with antibiotics produces only males, but not females (Sugimoto et al., 2012). 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 of 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 was able to 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 system 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 (Orr, 1993), 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 (Ji et al., 2016), Long non-coding RNA (lncRNA) was present, and it was shown that KO of Y-linked lncRNA upregulated miRNA and suppressed apoptosis (Hao et al., 2021). 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 (Hong et al., 2018). LncRNA is considered a regulatory molecule involved in genetic regulatory processes (Paraskevopoulou et al., 2016). 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 (Paraskevopoulou et al., 2016). Xt-mir is expressed in meiosis to escape MSCI (Song et al., 2009). Some lncRNAs are involved in gene imprinting and X-chromosome inactivation (Sahlu et al., 2020), and its KO mice show reduced sperm counts and decreased males in offspring (Hong et al., 2021). 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 (Mao et al., 2022). Damaged mitochondria, which release cytochrome c, mtROS, and mtDNA that are detrimental to cellular homeostasis, are processed by mitophagy (Ma et al., 2020). The previously mentioned herpes simplex virus used its endonuclease to induce mitophagy and prevent host cell apoptosis for infection (Saffran et al., 2007), whereas WO appears to prevent releasing ROS 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, but 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 system, DMI system, and SD system.

Conclusion

Genes that match the HDM model should be one set of epistatic genes on the X(Z) chromosome and effector genes on the autosomes. 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 Xt-mir and SPAG1 as candidates for the two genes.

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Figure 1. Xt-mir mapping on the mouse X chromosome
colored number, Xt-mir targeting SPAG1-2 mRNA 3' UTR with 7-8mer matching. red number, Xt-mir expressed mainly in spermatocytes ~ spermatids. orange number, Xt-mir expressed in spermatocytes and organs other than the testis. blue number, Xt-mir expressed in spermatogonia, spermatocytes, postnatal testis, and organs other than testis (Song, 2009).

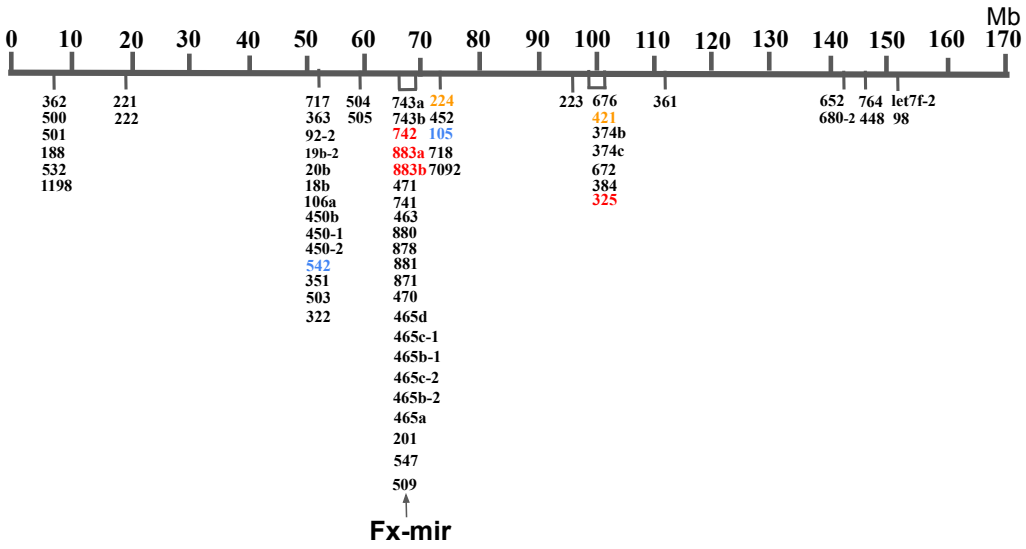

Figure 2. Maternal mtDNA inheritance and hybrid male sterility system in XY-type organisms
S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: SPAG1 gene. X: Xt-mir genes targeting SPAG1-2. c: cytoplasm type SPAG1(SPAG1-2) protein. m: mitochondria type SPAG1(SPAG1-1) protein. e: 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).

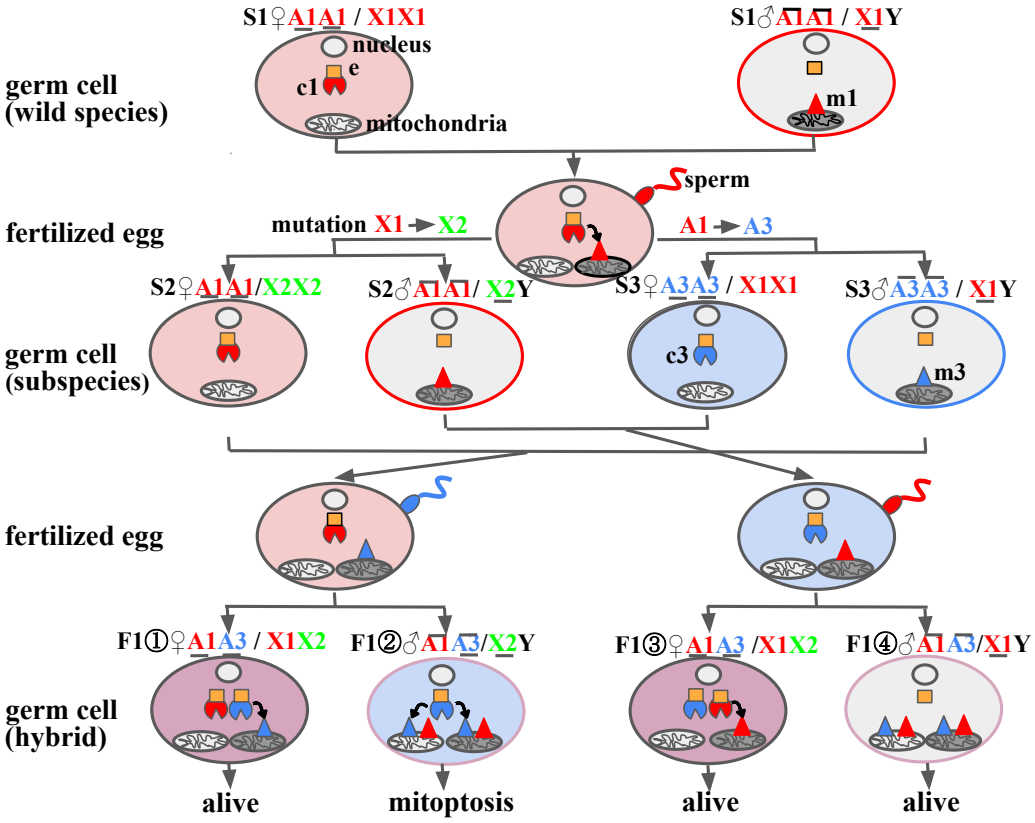

Figure S1. Maternal mtDNA inheritance and hybrid female sterility system in ZW-type organisms
S1: wild-type species. S2, S3: subspecies. F1: first filial generation. A: SPAG1 gene. Z: Xt-mir genes targeting SPAG1-1. c: cytoplasm type SPAG1(SPAG1-2) protein. m: mitochondria type SPAG1(SPAG1-1) protein. 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).

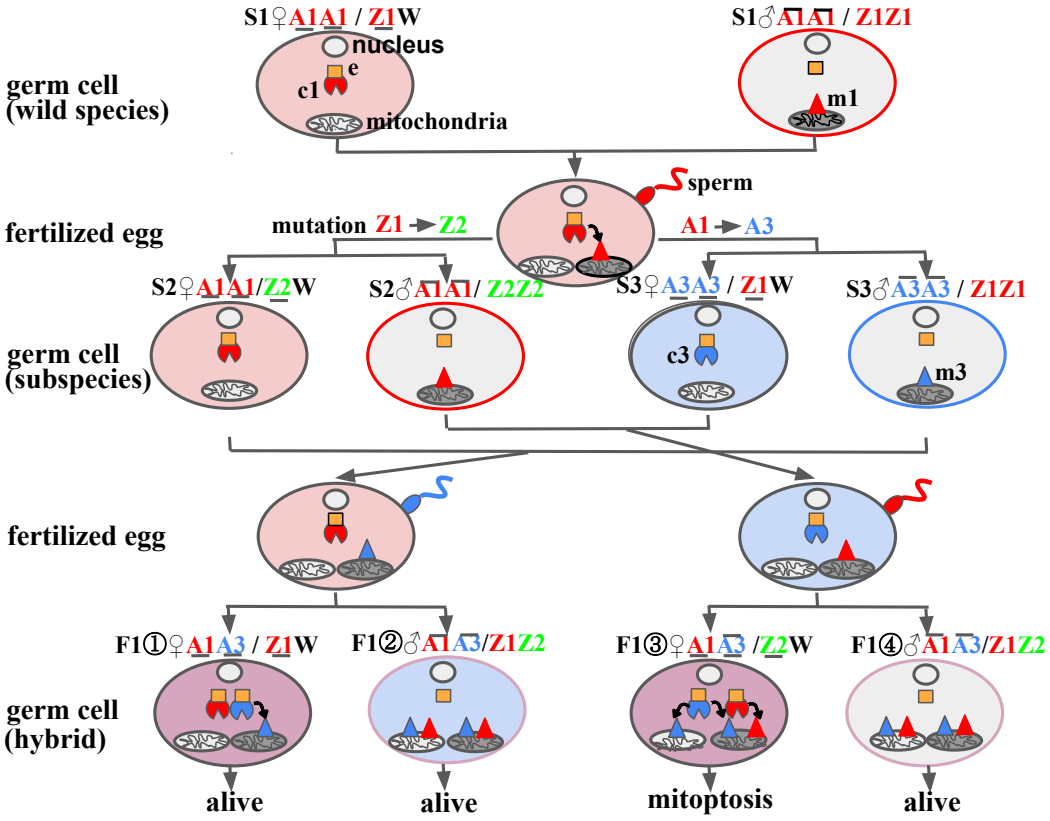

Table S1. human Fx-mir and target genes

8: 8mer matching. 7+: 7mer-m8. 7-: 7mer-A1. 6+: 6mer. 6-: offset 6mer (Bartel, 2018). 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.

	SPAG1-2	SPAG1-1	PIH1D2	RUVBL1	RUVBL2
	1206bp	261bp	158bp	971bp	153bp
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		6-			
hsa-mir-892b		6+			
hsa-mir-892c-5p	7+				
hsa-mir-892c-3p	6+	6-			
hsa-mir-506-5p	6+				
hsa-mir-506-3p					
hsa-mir-507		6+			
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 (type/site)	5(6)	1(1)	1(1)	1(1)	3(3)

Table S2. mouse Fx-mir, Xt-mir, and target genes

8: 8mer matching. 7+: 7mer-m8. 7-: 7mer-A1. 6+: 6mer. 6-: offset 6mer (Bartel, 2018). 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. Xt-mir is quoted from Song, 2009. 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)