Title: Coxfa4l3, a novel mitochondrial electron transport chain Complex 4 subunit protein, switches from Coxfa4 during spermatogenesis

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Abbreviations: AA, Amino acid; BN-PAGE, blue native polyacrylamide gel electrophoresis; CI-CIV, Complex I to IV ;CBB, Coomassie Brilliant Blue; ETC, electron transport chain; IMM, inner mitochondrial membrane; LPS, lipopolysaccharide; Mab, Monoclonal Antibody; OxPhos, oxidative phosphorylation; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, Sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

ABSTRACT

We identified Coxfa4l3, previously called C15orf48 or Nmes1, as a novel accessory protein of Complex IV of the mitochondrial electron transport chain (ETC). Amino acid sequence comparison, the intracellular localization and the protein expression data showed that the protein is the third isoform of Coxfa4 and the expression of Coxfa4 and Coxfa4l3 proteins during spermatogenesis showed a mutually exclusive pattern, implying that Coxfa4 replaces Coxfa4l3 in Complex IV after meiosis. These results may provide some insight into the unique mechanism of ATP production in late spermatogenesis.

1. INTRODUCTION

Spermatogenesis is a process of active cell proliferation and differentiation occurring in the seminiferous tubules. Spermatogonial stem cells divide in a self-renewing state, and after differentiation begins, they progress to spermatocytes, spermatids, and spermatozoa. Spermatogenesis occurs throughout life, producing approximately 4×10^6 sperm cells per day in the male mouse [Thayer et al., 2001]. During a male's reproductive life, a huge amount of energy is continuously consumed to support these extremely active processes.

Spermatogenesis progresses from the basal region of the seminiferous tubules toward the lumen. The blood vessels cover the surface of the seminiferous tubules in a mesh shape but do not penetrate it. Since the glucose and oxygen necessary for ATP production are transported by the blood and spread by diffusion to the lumen, the spermatogenic cells at the late stages of spermatogenesis must produce ATP under low glucose and oxygen conditions. Instead of glucose, the main energy source of the haploid germ cells is lactate, which is supplied by Sertoli cells [Boussouar and Benahmed, 2004]. Lactate is transported to postmeiotic germ cells, converted to pyruvate by lactose dehydrogenase, and used for ATP production in mitochondria. This indicates that the spermatids that are in lumen of the seminiferous tubules mainly produce ATP through mitochondrial oxidative phosphorylation (OxPhos) [Ramalho-Santos and Amaral, 2013]. OxPhos requires oxygen as the final electron acceptor in the electron transport chain (ETC). However, the oxygen partial pressure in the testis lumen is extremely low [Wenger and Katschinski, 2005], suggesting that OxPhos in spermatids has an unusual ATP-producing mechanism adapted to extremely low oxygen pressure.

Mitochondrial morphology changes drastically during spermatogenesis.

Spermatogonia and primary spermatocytes have a normal mitochondrial shape, but the secondary spermatocytes and early spermatids show condensed forms, and the morphology changes to an intermediate form in late spermatids. Moreover, in sperm, mitochondria arrange into a spiral form around the sperm midpiece. The morphological diversity of mitochondria should correlate with function [McCarron et al., 2013], suggesting the morphological changes of mitochondria during spermatogenesis should affect ATP production. Therefore, ATP synthesis during spermatogenesis is regulated dynamically, and it supports the active processes of spermatogenesis.

ATP is produced in three steps involving the cytoplasmic glycolysis system, TCA circuit, and OxPhos coupled with ETC in mitochondria. The ETC consists of four complexes, complexes I (CI), II (CII), III (CIII), and IV (CIV), and CI, CIII, and CIV act as proton pumps into the inner mitochondrial membrane (IMM). This produces a proton concentration gradient potential across the IMM, and mitochondrial ATP synthase produces ATP by using the potential energy. Each complex of ETC is composed of many subunit proteins. ETC CIV contains three core subunits and 11 accessory proteins. It is believed that the Mt-Co1 and Mt-Co2 among mitochondria-coded three subunits proteins perform catalytic activities, and all nuclear-coded accessory proteins are assumed to have regulatory functions [Sinkler et al., 2017].

There are several isoforms in the ATP production pathway during spermatogenesis. In OxPhos, testis-specific cytochrome c, Cox6b2 [Hüttemann et al., 2003], and Cox7b2 [Yue et al., 2014] are expressed specifically in male germ cells. These testis-specific isoforms are substituted by ubiquitous isoforms, and the substitutions might be involved

in the testis-specific regulation of ATP production. However, how they control ATP production during spermatogenesis is largely unknown.

Previously, we identified *nmes1* mRNA as a candidate mRNA regulated by the Dazap1 protein, an RNA-binding protein abundantly expressed in the testis [Sasaki et al., 2018]. Nmes1, previously called C15orf48, was first identified as a downregulated transcript in human esophageal squamous cell carcinoma [Zhou et al., 2002]. The function of the protein is not known, but the AA sequence of the mouse protein was highly homologous to Coxfa4 (previously called Ndufa4), which was recently reported as one of the accessory proteins of ETC CIV [Balsa et al., 2012, Zong et al., 2018]. Coxfa4 has a hypoxia-induced isoform, Ndufa4l2 [Tello et al., 2011]. This suggests that Nmes1 might be a novel and third isoform of Coxfa4.

In this paper, we confirmed that Nmes1 is a novel nuclear-coded accessory protein of ETC CIV and the third isoform of Coxfa4 and Ndufa4l2 and that Coxfa4 and Nmes1 showed mutually exclusive expression during spermatogenesis. These findings suggest the replacement of Coxfa4 with Nmes1 during spermatogenesis is involved in late spermatogenesis-specific ATP production. Based on the localization, expression pattern, and nomenclature [Pitceathly and Taanman, 2018], we propose renaming Ndufa4l2 and Nmes1 as Coxfa4l2 and Coxfa4l3, respectively. In this paper, we use these new protein names.

2. MATERIALS AND METHODS

2.1 Primers and antibodies

All primers used in this study are listed in Table S1. The anti-PSP1 and anti- α Tubulin monoclonal antibodies (Mabs) were described previously [Kuwahara et al., 2006, Akagi

et al., 2018], and anti-Myc (9E10) was purchased from Sigma-Aldrich (MO, USA). All other Mabs used in this study were established in our laboratory using a conventional method and the immunogens, and they are listed in Table S2.

2.2 Construction of prokaryotic and eukaryotic expression vectors

The full-length cDNAs encoding Coxfa4, Coxfa4l2, and Coxfa4l3 expression plasmids were polymerase chain reaction (PCR) amplified using mouse testis cDNA and cloned into NheI-KpnI sites (Coxfa4 and Coxfa4l2) and NheI-EcoRI sites (Coxfa4l3) of the pCAGGS-GST-cMyc-tag vector. These plasmids were used for the overexpression in eukaryotic cells. Coxfa4, Coxfa4l2, and Coxfa4l3 prokaryotic expression vectors were used to produce recombinant proteins to immunize mice for the Mab production. All coding regions were reamplified from the eukaryotic expression vectors by PCR, and the amplified products were cloned into Kpn I-Nhe I sites (Coxfa4 and Oxfa4l3) and Bam HI-Hind III sites (Coxfa4l2) of pCold III-GST or pCold III-MBP prokaryotic expression vectors. The nucleotide sequences of the inserted fragments of all expression vectors were confirmed by DNA sequencing.

2.3 Cell culture

The mouse myeloma cell line SP2/0-Ag14 (RCB0209) was provided by the Riken Bioresources Center (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT Japan. The cells were cultured in RPMI 1640 (Sigma-Aldrich) with 10% heat-inactivated newborn calf serum (Biowest, Nuaille, France) and recombinant mouse interleukin 6 (IL6: 1 ng/mL) purified from IL6-overexpressed *Escherichia coli* [Rees et al., 1999]. HeLa cells were maintained in DMEM (Sigma-Aldrich) supplemented with

10% newborn calf serum.

2.4 Transfection of eukaryotic expression vectors into HeLa cells

Highly purified plasmids for cell transfection were obtained by applying a silica purification protocol [Li et al., 2010], and the contaminated lipopolysaccharide (LPS) were removed by Triton X-114 [Cotton et al., 1994]. Exponentially growing HeLa cells were transfected by the plasmids using the linear polyethylenimine method [Reed et al., 2006] or electroporation (NEPA21, NepaGene Co. Chiba, Japan).

For the examination of subcellular localization of the Coxfa413 protein, the cells were seeded onto slide glass with flexiPERM (Sarstedt, Nümbrecht, Germany) and cultured for 50–60 hrs. The cells were fixed with 4% paraformaldehyde, permeabilized by methanol, and immunostained by anti-Myc Mab and Alexa Fluor 546 goat anti-mouse IgG H+L (Life Technologies), and the nuclei and mitochondria were stained by DAPI and MitoTracker Green FM (Invitrogen), respectively. The cells were observed and photographed under an Axiophot 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

2.5 Tissue and cell sample preparation for SDS-PAGE and Western blot

Mouse tissues from adult BALB/cAJCl male and female mice and cells (HEK293T and HeLa) collected from culture dishes were placed in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 2 mM NaH₂PO₄, pH 7.4). The cells were homogenized and sonicated and then centrifuged at 12,000 rpm for 3 mins at 4°C. The supernatant was used for SDS-PAGE. Western blot was performed by conventional protocols, as described previously [Kuwahara et al., 2006].

2.6 Subcellular fractionation

The protocol was a modified version of the original procedures described [Candas et al., 2016]. Briefly, HeLa cells harvested from a culture dish were suspended in IBc buffer (10 mM Tris-MOPS, 20 mM Tris-EGTA, and 200 mM sucrose, pH 7.4). The cell suspension was homogenized in Teflon Dounce homogenizer and centrifuged at 100 g for 10 mins at 4°C. The precipitate was used as a nuclear fraction, and the supernatant was recentrifuged at 7,000 g for 10 mins at 4°C. The supernatant was used as a cytoplasmic fraction, and the pellet was used as a mitochondrial fraction.

Adult testes were excised from BALB/cAJCl mice and homogenized in IBc buffer.

After centrifugation at 1,000 g for 10 mins at 4°C, the supernatant was recentrifuged at 10,000 g for 15 mins at 4°C. The supernatant and the pellet were used as a cytoplasmic fraction and a mitochondrial fraction, respectively.

2.7 Sample preparation and blue native polyacrylamide gel electrophoresis (BN-PAGE)

The protocol was essentially the same as described previously [Wittig et al., 2006] with minor modifications. In brief, freshly prepared mitochondrial pellets from mouse (BALB/cAJCl) testes and HeLa cells were suspended and mildly mixed in solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA pH 7.0), and appropriate quantities of digitonin (digitonin : protein ratios were 6:1 for HeLa cells and 12:1 for testis samples) were added. After centrifugation at 15,000 rpm for 30 min at 4°C, the supernatants were mixed with 0.15 volume of 5% (w/v) CBB (Fujifilm Wako Pure Chemical Co. Japan) and separated by BN-PAGE (3.5–16% gel). After the detections of proteins, the filters were washed by

2% SDS, 100 mM 2-mercaptoethanol and 62.5 mM Tris-HCl pH6.7 and probed with anti-mt-Co1 Mab for the evaluation the relative amounts of the loading samples.

3. RESULTS

3.1 Amino acid sequences of Coxfa4l3 are similar to those of Coxfa4 and Coxfa4l2

We noticed that the AA sequences of Coxfa4l3 showed significant homologies to those of Coxfa4 and Coxfa4l2 (Fig. 1A, B). Coxfa4 has been known as an accessory protein of ETC CIV, and Coxfa4l2 is the hypoxia-induced isoform of Coxfa4 [Tello et al., 2011]. Recently, the protein structure of ETC CIV has been solved [Zong et al., 2018]. According to the structure, 15 N-terminal AA sequences are exposed to the matrix region, the next 21 AA sequences are the transmembrane domain, and the following region faces the intermembrane space. Assuming that the overall structure is similar to that of Coxfa4l3, each region was estimated to those shown in Fig.1C. Interestingly, N-terminal half of the intermembrane space region(Coxfa4: AA 42–54) showed significantly high homologies between three proteins. In addition, Coxfa4 and Coxfa4l2 showed high homology in the proximal C-terminal region, but had low homology with those of Coxfa4l3, indicating that unique functions of Coxfa4l3 should reside in these regions.

3.2 Overexpressed Coxfa4l3 is localized to mitochondria

Originally, Coxfa4l3 was identified as a nuclear protein [Zhou et al., 2002]. However, based on the significant sequence homologies to those of Coxfa4 and Coxfa4l2, we reexamined the subcellular localization of Coxfa4l3.

Immunocytochemistry using anti-Coxfa4l3-specific Mab showed that overexpressed

Coxfa4l3 in HeLa cells was colocalized with mitochondria stained by MitoTracker dye (Fig. 2A). Next, in order to show the intracellular localization of the Coxfa4l3 protein biochemically, mitochondrial fractions were extracted from HeLa cells that were transfected by the Coxfa4l3 expression vector, and the subcellular localization of the Coxfa4l3 protein was examined by Western blot (Fig. 2B). First, the validities of the subcellular fractions were confirmed by a cytoplasmic marker, α Tubulin, and a nuclear marker, PSP1 [Kuwahara et al., 2006]. In addition, the mitochondrial marker Cox6c was mainly present in the mitochondrial fractions, indicating that the protocol used in this study gave satisfactory subcellular fractionation. By using these fractions, we showed that Coxfa4 was localized in the mitochondria, as reported. The exogenous Coxfa4l3 protein derived from the Myc-tagged Coxfa4l3 expression vector was detected strongly in mitochondria fractions by anti-Myc and anti-Coxfa4l3 antibodies, indicating that the Coxfa4l3 protein was a mitochondrial protein.

Next, the subcellular localization of endogenous Coxfa4l3 was examined by mouse testis extracts (Fig. 2C). The validity of the fractionation of testis extracts was confirmed by the same method described for the fractions of HeLa cells. Western blot analysis using these fractionated samples from mouse testis revealed that endogenous Coxfa4 was observed in mitochondrial fractions as expected, and Coxfa4l2 and Coxfa4l3 were observed in the mitochondrial fractions of HeLa cell (Fig.2S) and mouse testis. These results demonstrated that all three proteins were mitochondrial proteins.

3.3 Tissue expression of Coxfa4l3, Coxfa4, and Coxfa4l2

Because the anti-Coxfa4, anti-Coxfa4l2 and anti-Coxfa4l3 antibodies established in this study could distinguish each isoform, as shown in Fig. 1S, the tissue expression of these

three proteins in mice was examined using these antibodies.

Western blot using the anti-Coxfa4 antibody demonstrated that there were large differences in the expression levels between tissues but that the antibody was reacted in all tissues examined (Fig. 3). Coxfa4l2, a hypoxia-induced isoform, was not detected in any of the mouse tissues examined but was expressed in HeLa cells (Fig. 2S). Previously, it was reported that Coxfa4l2 is not expressed in HeLa cells under normoxia, and induced upon hypoxia [Tello et al., 2011]. Our data demonstrated the protein and its mRNA is expressed in the cells in normoxic and chemically induced hypoxic condition. We do not have any reasonable explanation on the discrepancy, but one possibility may be that the cells maintaining by two groups had small difference during long cell cultures. Coxfa4l3 was strongly expressed in testis and epididymis extracts but could not be detected in other tissues. To distinguish the cell types of the epididymis that expressed Coxfa4l3, sperm cells from the epididymis were subjected to Western blot to examine the expression (Fig. 3S) and were confirmed to express Coxfa4l3.

3.4 Coxfa4l3 is a novel accessory protein of ETC CIV

Since Coxfa4l3 was found to be highly expressed in the testis and localized in the mitochondria *in vivo*, mitochondrial fractions from the testis were examined for complex analysis by BN-PAGE. In addition, BN-PAGE was performed by using mitochondrial fractions from HeLa cells that express Coxfa4l2 but not Coxfa4l3 (Fig. 2SC). Anti-Mt-Co1 (CIV marker), anti-Cox6c (CIV marker), anti-Uqcrc2 (CIII marker), anti-Ndufa9 (CI marker), anti-Coxfa4, and anti-Coxfa4l3 antibodies were used for Western blot (Fig. 4). In the mitochondrial fractions of the testis, Coxfa4l3 was found to be in the ETC CIV, because it showed a similar pattern to those of Mt-Co1 and

Cox6c, unlike Ndufa9 (CI) and Uqcrc2 (CIII). On the other hand, Coxfa4 was weakly expressed in the testis, and in the case of short time exposure, only a thin band was detected in the ETC CIV monomer. Coxfa4l2 was also found to be in the ETC CIV in HeLa cells (Fig. 2S). These data indicate that all three proteins be accessory proteins of ETC CIV.

3.5 Expression of Coxfa4 and Coxfa4l3 during testicular differentiation

Coxfa4l2 is a hypoxia-induced isoform of Coxfa4. The sequence similarity between the two known isoforms and Coxfa4l3, the subcellular localization, and the complex analysis suggest that Coxfa4l3 might be a new isoform of Coxfa4. We examined the possibility by comparing expression patterns in the mouse testis where both proteins were expressed.

The anti-Coxfa4 and anti-Coxfa4l3 Mabs can be applied to Western blot but not to immunohistochemistry (unpublished data). Therefore, we decided to analyze the first waves of spermatogenesis after birth to identify the expressing cells of each protein at different spermatogenic stages (Fig. 5A). The SDS-PAGE samples of the mitochondrial fractions were normalized by the expression level of Mt-Co1. Coxfa4 was observed in the testis immediately after birth and was highly expressed until the 18th day after birth. Male germ cells begin meiosis 20–25 days after birth. The expression of Coxfa4 gradually decreased after meiosis. The expression of Coxfa4l3 began at 25 days after birth, and it was maintained until the end of the first wave. These data indicate that Coxfa4 is expressed in spermatogenic cells, from spermatogonia to spermatocytes, and that Coxfa4l3 starts to be expressed in haploid spermatogenic cells (Fig. 5B). To examine whether the replaced Coxfa4l3 protein might be incorporated into ETC CIV,

we performed BN-PAGE by using mitochondrial fractions from testis samples of mice as indicated (Fig. 5C). As expected, Coxfa4l3 was replaced from Coxfa4 in ETC CIV. We concluded that Coxfa4 and Coxfa4l3 show mutually exclusive expression, that the replacement of Coxfa4 with Coxfa4l3 occurs in ETC CIV of spermatogenic cells after meiosis, and that Coxfa4l3 is a haploid male germ cell-specific isoform of Coxfa4.

4. DISCUSSION

4.1 Coxfa4l3 is a novel isoform of Coxfa4 in ETC CIV complex

Coxfa4l3, previously called C15orf48 or Nmes1, was first identified in a study of human esophageal squamous cells, and the expression was reduced in the carcinoma samples [Zhou et al., 2002]. Many mRNA expression studies have shown that the Coxfa4l3 protein could be a tumor suppressor [Riggs et al., 2005, Sova et al., 2006, Arai et al., 2008, Zimmer et al., 2012, Su et al., 2013, Kim et al., 2018]. However, it was reported that the Coxfa4l3 transcript was a functional primary miR-147 [Zimmer et al., 2012]. In addition, miR-147 was induced in LPS-stimulated mouse macrophages and under *in vivo* conditions in the lungs of LPS-treated mice, suggesting that miR-147 regulates the inflammatory response in macrophages. The previously reported tumor suppressor function of Coxfa4l3 protein may be mistaken for the function of miR-147 from *coxfa4l3* mRNA. This paper cast doubt on the tumor suppressor functions of the Coxfa4l3 protein, and the function of the Coxfa4l3 protein needs to be readdressed.

The Coxfa4l3 antibody used in the previous study [Zhou et al., 2002] detected signals in the nuclei of alimentary tract cells. Since then, there has been no report confirming the nuclear localization. By using newly established Mabs specific for Coxfa4, Coxfa4l2, and Coxfa4l3 that distinguished three isoforms (Fig. 1S), we showed that

Coxfa4l3 was an accessory protein of mitochondrial ETC CIV. Although we do not exclude the possibility of nuclear localization, the protein is likely located and functioning mainly in the mitochondria. Our data are also supported by the finding that the exogenously expressed Coxfa4l3 protein interacts with ETC CI and CIV accessory proteins in HEK293 cells and HepG2 cells [Floyd et al., 2016].

Coxfa4 (formerly called Ndufa4) was first identified as an accessory protein of ETC CI, but later, it was reported that Coxfa4 was an accessory of ETC CIV [Balsa et al., 2012]. The three-dimensional structure of human ETC CIV was solved, and Coxfa4 was included in this stereostructure, confirming that Coxfa4 is an accessory protein of ETC CIV [Zong et al., 2018]. Coxfa4 has a hypoxia-induced isoform, Coxfa4l2. The sequence similarity between the two known isoforms and Coxfa4l3, the subcellular localization, and the complex analysis indicated that Coxfa4l3 is the third isoform of Coxfa4 and Coxfa4l2.

Based on the present data and the nomenclature by Pitceathly and Taanman, 2018, we propose renaming Ndufa4l2 and Nmes1, as Coxfa4l2 and Coxfa4l3, respectively.

4.2 Coxfa4l3 may be involved in the regulation of ETC activity

Stereostructure of intact and monomer fourteen subunit CIV indicates that cardiolipin is inserts into a pocket formed by MT-CO1, MT-CO3, and the transmembrane domain and the C-terminal region of Coxfa4. And cardiolipin interacting AAs are indicated as blank boxes in Fig. 1A [Zong et al., 2018]. All the corresponding AAs in Coxfa4l2 are identical or similar to those of Coxfa4, but some of the AAs in Coxfa4l3 are not (blue arrows). Cardiolipin is the phospholipid of mitochondria, present in high amounts in the IMM [Mileykovskaya and Dowhan, 2009, 2014, Milenkovic et al., 2017]. and is

considered to stabilize respirasome. Unfavorable substitutions of cardiolipin binding sequence in Coxfa4l3 might influence the respirasome stability.

Because the Mabs used in this study cannot be applied to immunostaining (unpublished data), we could not examine the cell types in the testis that expressed Coxfa4 and Coxfa4l3 immunohistochemically. Therefore, the protein expression was examined by the first-wave analysis of spermatogenesis. The data suggested an inverse correlation with the expression, indicating Coxfa4 is replaced to Coxfa4l3 in CIV after meiosis. Similar replacements for the isoforms can also be seen in other ETC CIV accessory proteins. For example, it has been reported that under hypoxic conditions, Cox4l1 is replaced with Cox4l2 [Pitceathly et al., 2013] and Coxfa4 is replaced with Coxfa4l2 [Tello et al., 2011]. The ATP demand varies depending on changes in the external environment surrounding the cells in the living body, changes in tissues, and cell types. In mitochondrial ETC CIV, subunit replacement influence the stability and respirasome formation in respirasome and controls levels of ATP synthesis.

4.3 Coxfa4l3 is involved in unique ATP metabolism during late spermatogenesis

Our data strongly suggest that Coxfa4 in ETC CIV of testicular cells before meiosis is replaced by Coxfa4l3 in post-meiotic cells, and that the replacement regulates ATP production in testis.

There are several ETC proteins which express specifically during spermatogenesis. Haploid spermatogenic cells express testis-type cytochrome c with high antioxidant activity that degrades ROS to prevent cell damage. Electron transfer CI and CIII are the major ROS sources, and the activity of CIV changes under the expression of testicular cytochrome c, which differs from the activity of the somatic-type. Cox6b2, isoform of

somatic-type Cox6b1, is expressed in germ cells at all stages of spermatogenesis [Esakky et al., 2013], whereas Coxfa4l3 starts to be expressed in haploid germ cells after meiosis. Coxfa4l3 is the first subunit protein of ETC that showed stage specific expression and subunit replacement after meiosis.

Since the blood vessels do not invade the seminiferous tubules of the testis, the central regions of the seminiferous tubules are in the lowest oxygen partial pressure in all tissues [Velickovic and Stefanovic 2014]. After meiosis, lactate is supplied as an energy substrate from Sertoli cells to haploid germ cells, and it is oxidized to pyruvic acid by lactose dehydrogenase [Boussouar and Benahmed, 2004]. Therefore, it is postulated that ATP production of male haploid germ cells is not mediated by the glycolytic pathway and that ATP production in haploid cells depends on the mitochondrial pathways [Santos-Ramos and Amaral, 2013]. The switch of this energy source may be related to the expression of post-meiotic Coxfa4l3. The reduction of oxygen as the final electron acceptor causes electron traffic jam in the respiratory chain and electron leak, inducing oxidative stress. This can be regarded that haploid spermatogenic cells are exposed to oxidative stress. Approximately 25% in semen samples of male infertile patients have high levels of reactive oxygen species (ROS). Therefore, normal spermatogenesis proceeds with a high level of balance between oxidative and antioxidant effects and the unbalance may cause excessive ROS leading to male infertility, sperm dysplasia and sperm health. Dysfunction of Coxfa4 causes Leigh syndrome [Pitceathly et al., 2013], and Coxfa4l2 is related to cancer malignancies [Sinkler et al., 2017, Piltti et al., 2018, Li et al., 2018, Lucarelli et al., 2018, Meng et al., 2019], suggesting that Coxfa4 and its isoforms are important regulators for ATP synthesis. Whether the dysfunction of Coxfa4l3 is involved in disease is currently

unknown, but it should be important to study the relationship between male infertility and Coxfa4l3 expression.

4. CONCLUSION

In conclusion, Coxfa4l3, previously called C15orf48 or Nmes1, is a novel accessory protein of CIV of the ETC. Interestingly, the expression of the Coxfa4 and Coxfa4l3 proteins during spermatogenesis showed a mutually exclusive pattern, implying that Coxfa4 replaces COXfa4l3 in CIV after meiosis. The lumen of the seminiferous tubules where the replacement occurs is under extremely low oxygen partial pressure, suggesting there might be a unique mechanism or regulation of ATP synthesis. The replacement may provide some insight into the unique ATP synthesis in late spermatogenesis.

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FIGURE LEGENDS

Fig. 1. Comparison of amino acid sequences and predicted secondary structure of Coxfa4 isoforms. A, B) AA sequences of mouse Coxfa4 and its isoforms are compared by CLUSTALW program. These secondary structures and homology comparisons are estimated by the latest stereostructure of ETC C IV [Zong et al., 2018] and Jpred 4, respectively. Predicted α -helices and β -sheets are marked as black and gray boxes are, respectively. Blank boxes and blue arrows indicate cardiolipin binding sequence deduced from the stereostructure [Zong et al., 2018] and AAs of Coxfa4l3 which has different chemical properties from the counterparts of Coxfa4, respectively. Red letters indicate common AAs in the three proteins.

Fig. 2. Coxfa4l3 is a mitochondrial protein. A) Mitochondrial localization of overexpressed Coxfa4l3 in HeLa cells. The cells were transfected with the pCAGGS-Coxfa4l3-GST-myc vector. After 60 hrs of transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with cold methanol, and blocked with 5% skim milk for 1 hr at room temperature. The anti-Coxfa4l3 Mab and Alexa Fluor 546 goat anti-mouse IgG (H+L) (Life Technologies) were used for primary and secondary antibodies, respectively. After extensive washing, the cells were stained by MitoTracker Green FM (200 nM) according to the manufacturer's protocol. Then, the cells were observed by fluorescent microscopy. B) Subcellular localization of exogenously expressed Coxfa4l3. HeLa cells were transfected with the pCAGGS-Coxfa4l3-GST-myc vector and cultured for 72 hrs. After subcellular fractionation as described in the Materials and Methods, the fractions were subjected to SDS-PAGE and Western blot by using Mabs for the fraction-specific markers. C) Subcellular localization of endogenously expressed Coxfa4l3. The testis was collected from an adult BALB/cAJCl male mouse, and subcellular fractionation was carried out as described in the Materials and Methods. The protein samples were resolved by SDS-PAGE and subjected to Western blot.

Fig. 3. Coxfa4, Coxfa4l2, and Coxfa4l3 expression in mouse tissue extracts. Tissue preparation from adult BALB/cAJCl mice was carried out as described in the Materials and Methods. The extracts were separated by SDS-PAGE and subjected to Western blot by using anti-Coxfa4, anti-Coxfa4l2, and anti-Coxfa4l3 Mabs.

Fig. 4. Coxfa4 and Coxfa4l3 are accessory proteins of ETC CIV. Digitonin-treated

mitochondrial proteins from BALB/cAJCl male mouse testis were used for BN-PAGE. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membranes and analyzed by Western blot. Mt-Co1 and Cox6c are ETC CIV markers, and Uqcrc2 and Ndufa9 are ETC CII and CI markers, respectively.

Fig. 5. Expression of Coxfa4 and Coxfa4l3 during mouse spermatogenesis. A) To estimate the expression pattern of Coxfa4l3 during mouse spermatogenesis, BALB/cAJCl male mouse testis extracts from the first wave of spermatogenesis were analyzed. B) The schematic representation of the expressions of Coxfa4 and Coxfa4l3. Shaded boxes and blank boxes indicate the cell types that expressed Coxfa4l3 and Coxfa4, respectively.

SUPPLEMENTAL DATA LEGENDS

Fig. 1S. Validation of the established Mabs. To generate anti-Coxfa4, anti-Coxfa4l2, and anti-Coxfa4l3 Mabs, the recombinant proteins expressed in *E. coli* listed in Table S2 were used. The proteins were immunized into BALB/cAJCl mice twice. Hybridoma production and screening were essentially as described previously [Kuwahara et al., 2006]. The cell extracts transfected with eukaryotic expression vectors for each protein were analyzed to validate the specificities of established Mabs. Anti- α Tubulin and GST Mabs were used for transfection and loading controls, respectively.

Fig. 2S Expression of Coxfa4l3 in HeLa cells. Tello et al. 2011 reported that Coxfa4l2 was induced only in HeLa cells under hypoxic condition. Our data showed that the protein was expressed in HeLa cells with and without hypoxic induction (Fig. 2SA).

RT-PCR analysis by using cDNA from the uninduced HeLa cells demonstrated that the mRNA was expressed. The primers used were

α Tubulin FW (exon3); ATGCCCCGAGGGCACTACAC

α Tubulin RV (exon 4); AGACGTTCCATGAGCAGCG

COXFA4L3 Fw (exon 2); ATGGCAGGAGCCAGTCTTGG

COXFA4L3 Rv (exon 5); TGGCTTAGAAGTCTGGCCGG.

Fig. 3S. Expression of Coxfa4, Coxfa4l3, and Cox6c in mouse sperm. The epididymis of BALB/cAJCl male mice was minced in PBS buffer and placed for 10 mins at room temperature. The sperm from the epididymis were collected by centrifugation and used for Western blot. The Mabs used for Western blot were anti-Coxfa4, anti-Coxfa4l3, and anti-Cox6c.

Table S1. Primers used for constructing eukaryotic expression vectors in this study

Coxfa4	Fw: ATAGGTACCATGCTCCGCCAGATCC
mouse	Rv: ATAGCTAGCGAAGTCTGGGCCTTC
Coxfa4l2	Fw: ACTGCTAGCATGTCCCCTATAC
mouse	Rv: CGTGAATTCTTAGAAGTCTGGC
Coxfa4l3	Fw: AGTGGTACCATGGGCGTTTTCCAG
mouse	Rv: ATAGCTAGCTCTGGTTGCCCTCCG

Table S3. Monoclonal antibodies used in this study

Antigen	Immunogen amino acid position (species)	Clone name	Donor myeloma
Mt-Co1	recombinant 490-507 (mouse)	H7	P3U1
Cox6c	recombinant 13-88 (mouse)	#12	P3U1
Ndufa9	recombinant 315-377 (mouse)	3F2	SP2
Uqcr2	recombinant 367-454 (mouse)	1D11	SP2
GST	recombinant full-length (<i>Schistosoma japonicum</i>)	1E10	P3U1
Coxfa4	recombinant 55-82 (mouse)	3C3	SP2
Coxfa4l2	recombinant 1-87 (mouse)	#23	SP2
Coxfa4l3	recombinant 52-83 (mouse)	5B11	SP2