られる消化器症状は，消化管自体の問題以外にも様々な原因で症状の出現を認める。また，MRCDに特徴的な乳酸アシドーシスは，それ自体がgastroparesisや胃腸管のdysmotilityを誘発し，嘔吐や腹部膨満を来すとされる[2]。Gibson[11]らは，新生児ミトコンドリア病の臨床病型として，脳筋型，肝腸管型，心臓型の3病型を提唱しているが，これらにおいても哺乳不良，繰り返す嘔吐，成長障害は共通に認める三大症状であるとしている[11]。MRCDにおいて消化器症状は，多くが1歳以内に出現しており，初発症状として現れるものが多いと考えられ，その原因に関わらず注意すべき症状であるといえる。生後早期の哺乳不良や消化器症状を呈する症例では，それ以外の症状に乏しい場合でも経時的な他臓器の評価や高乳酸血症の評価など，MRCDを念頭に置いた精査が重要である。

また，消化器病変による症状が単独で存在する場合に，MRCDと診断することは非常に困難であると思われる。消化管粘膜の生検における病理像は，ミトコンドリアの浸潤や巨大ミトコンドリアなどが認められる場合には有用[7]であり，免疫染色も重要な参考所見である。MRCDは組織特異性があることが知られており[12]，診断においては罹患臓器の呼吸鎖酵素解析が重要であるが，消化管での呼吸鎖の異常の診断は極めて難しい。我々が報告した，生後より経腸栄養が不可能であった難治性下痢症例で，肝の呼吸鎖Complex Iの著しい低下によりMRCDと診断された症例における，腸管での呼吸鎖酵素活性は正常であった[9]。これは，腸管が粘膜層，筋層といった複数の組織で構成されていることや，障害部位の広範な脱落壊死などにより正確な評価が困難であるなどの理由が考えられる。図7に消化器病変を示す症例が，どのような臓器によって診断されたかをまとめた。特に肝臓において特異性が高く，次いで筋肉が高い。逆に皮膚線維芽細胞での活性は正常となることが多かった。特に肝臓，筋肉においての酵素活性の測定が重要であることが示唆された。

結　論

ミトコンドリア呼吸鎖複合体異常症における消化器症状について検討した。難治性下痢症，胆汁性嘔吐，胃食道逆流はMRCDにおいて頻度の高い消化器症状である。生後早期の原因不明の哺乳不良や消化器症状が持続する場合，他臓器病変とその後の臨床経過を注意深くみていくなど，MRCDを念頭に置いた精査が重要である。

謝　辞

本研究を行うにあたり，日頃大変御尽力いただいている埼玉医科大学小児科技師　原嶋宏子さん，および千葉県こども病院検査科，安嶋まさみさんに深謝いたします。

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Gastroenterological symptoms of mitochondrial respiratory chain complex deficiency

*1Chiba Children's Hospital, Dep of Metabolism
*2Chiba Children's Hospital, Dep of Neonatology
*3Saitama medical University, Dep of Pediatrics
*4Faculty of Medicine, Saga University, Dep of Pediatrics

Emi Kawachi*1*4, Kei Murayama*1, Takuya Fushimi*1, Keiko Ichimoto*2,
Tomoko Tsuruoka*2, Masaki Takayanagi*1, Akira Ohtake*3

Mitochondrial respiratory chain complex deficiency (MRCD) can affect any, in fact all, bodily systems including the gastrointestinal tract. Of the 189 patients diagnosed as having MRCD in our hospital, 56 had gastroenterological symptoms. Of these 56 patients, 25 presented with a main problem of gastroenterological symptoms such as intractable diarrhea, bilious vomiting, gastroesophageal reflux and necrotizing enterocolitis. Mitochondrial gastrointestinal dysfunction occurred within the first year of life and more than half of the patients had a neonatal onset. Diagnosis was based mainly on enzyme assays performed using hepatic and muscle tissues. The other 31 patients had gastroenterological symptoms associated with severe respiratory distress, heart failure, seizures and lactic acidosis. In summary, based on these results, continuous poor sucking and gastroenterological symptoms in infancy are key features suggesting MRCD, necessitating evaluation of damage to other organs and the existence of lactic acidosis.

日本未熟児新生児学会雑誌　第26巻　第1号　131～137頁（2014年）

●原　　著●

受付日：平成25. 06. 13
受理日：平成25. 10. 01

出生直後から代謝性アシドーシス，肝機能障害をきたした新生児期発症のミトコンドリア呼吸鎖複合体異常症の1例

*1神奈川県立こども医療センター　新生児科，*2新潟大学医歯学総合病院　小児科，*3千葉県こども病院　小児科

斎藤朋子*1*2・大山牧子*1・川滝元良*1・猪谷泰史*1・村山　圭*3

Key Words：mitochondrial respiratory chain, mitochondrial disease, newborn, lactic acidosis

要　旨

出生直後から急激に呼吸障害をきたし，高乳酸血症，肝機能障害，播種性血液内凝固，貧血を認めたミトコンドリア呼吸鎖複合体異常症の1症例を経験した。児は透析により一旦軽快したが，再度同様のエピソードを呈し日齢20に死亡した。病理解剖を行い，筋肉と肝臓のミトコンドリア酵素活性を測定し，ミトコンドリア呼吸鎖複合体異常症と診断した。原因不明の多臓器に及ぶ重篤な症状を認めた場合，積極的に本症を疑い，検索する必要がある。新生児期発症のミトコンドリア呼吸鎖異常は核DNA異常であることが多く，遺伝子診断は時に困難であり，本症の診断には，肝生検または剖検で得られた肝臓の組織で，ミトコンドリア呼吸鎖酵素活性などの生化学的検査が有用である。

緒　言

ミトコンドリア呼吸鎖複合体異常症（Mitochondrial respiratory chain disorder：MRCD）は呼吸鎖における電子伝達系の異常により発症する疾患で，その発症率は5,000人に1人といわれている[1]。MRCDはミトコンドリア脳筋症やLeigh脳症など小児期の神経疾患としてよく知られているが，新生児期発症例がMRCD全体の約半数を占め，そのうち6割が死亡しているとの報告があることから[2]，致死率の高い疾患である。本症の症状は非特異的であり，診断がつかないまま亡くなった症例の中にMRCDが含まれている可能性がある。

われわれは，胎児期には異常を指摘されず，出生後急激に致死的な経過をたどり，病理解剖で得られた検体からMRCDと診断した症例を報告する。

別刷請求先：〒951-8510　新潟県新潟市中央区旭町通り1-757
新潟大学医学部小児科
斎藤朋子

症　例

症例：日齢0　女児。

家族歴：母28歳，1妊1産。同胞4歳女児，生来健康。突然死の家族歴なし，近親婚なし。

今回の妊娠経過：近医産院で妊婦健診を受けていたが，経過中異常は指摘されなかった。

出生時所見：児は在胎41週2日，出生体重3,310g，経腟自然分娩で出生した。Apgar score 7/1分，8/5分。出生後啼泣を認めたが，徐々に呼吸状態が悪化し，生後2時間で挿管され，当院へ新生児搬送依頼があった。生後3時間に当院医師到着時，皮膚色蒼白で脈拍は触知されず，心臓マッサージ，adrenaline，sodium bicarbonateを投与され心拍が出現した。肺サーファクタント投与後皮膚色改善し，当院へ搬送された。

入院時現症：SpO_2測定不能，心拍数130/min，血圧30/16mmHg。全身蒼白，筋緊張低下。心雑音なし，両側肺胞呼吸音良好。腹部軟，肝3cm触知。顔貌異常・外表奇形なし。

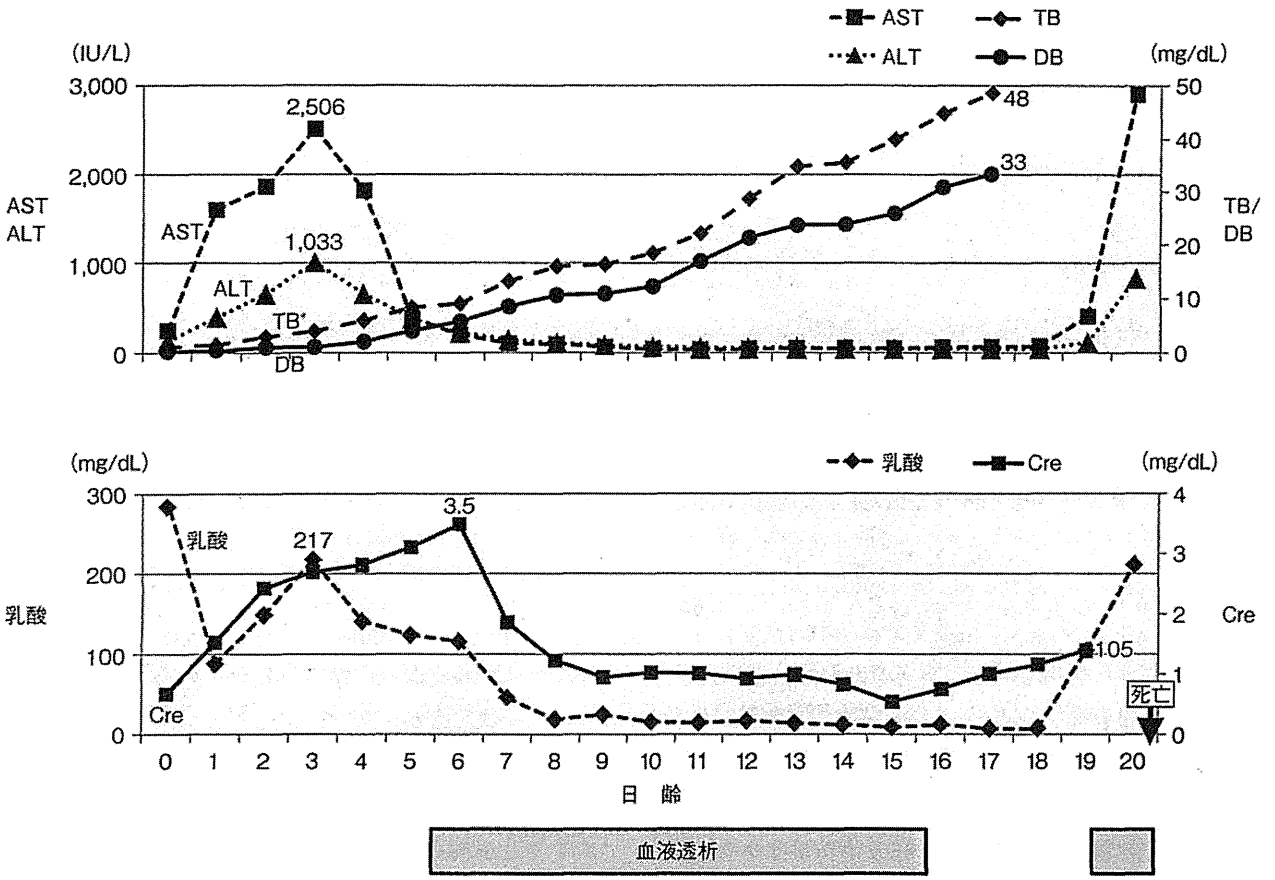

図1 入院後経過

入院時検査所見

〈血液ガス分析〉

pH 6.66, $PaCO_2$ 50mmHg, PaO_2 29mmHg, HCO_3^- 5.3mmol/L, BE −28mmol/L

〈血液学的検査〉

RBC 234万/μL, Hb 7.6g/dL, WBC 17,200/μL, Plt 14.7万/μL, APTT > 200sec, PT 64.2sec, PTINR 8.12, Fg<50mg/dL, D-dimer>250μg/mL, AT Ⅲ 23%

〈生化学的検査〉

AST 243 IU/L, ALT 127 IU/L, LDH 4,281 IU/L, CK 2,825 IU/L, ALP 546 IU/L, γGTP 145 IU/L, TB 1.1mg/dL, BUN 7.7mg/dL, Cre 0.56mg/dL, Na 143mEq/L, K 5.3mEq/L, Cl 103mEq/L
血糖 128mg/dL, ピルビン酸 3.37mg/dL, 乳酸 282mg/dL (L/P比 83), NH_3 66μmol/L

〈血清学的検査〉

CRP <0.10mg/dL, フェリチン 14,865ng/mL
Cytomegalovirus：IgM (EIA)(−), PCR (−),
Herpes Simplex Virus：IgM (EIA)(−), PCR (−),
Epstein − Barr Virus：IgM (ELISA)(−)
ParvovirusB19：IgM (EIA)(−), PCR (−),
Measles Virus：IgM (EIA)(−)
新生児マス・スクリーニング検査（タンデム・マス含む）：異常なし。
アミノ酸分析・尿中有機酸分析：特異的な異常所見なし。
骨髄検査：血球貪食像なし。
母体血 Hb F：0.0%

入院後経過：(図1)

入院時検査で，低酸素血症，貧血，凝固異常，肝機能障害，代謝性アシドーシス，高乳酸血症，高フェリチン血症を認めた。100%酸素で呼吸管理を開始し，大量輸血，sodium bicarbonate 補正を行った。乳酸値は一旦低下したが日齢2に再上昇した。日齢1，貧血，凝固異常に加え，血小板減少も出現したため，播種性血管内凝固（DIC）と判断し新鮮凍結血漿（FFP）に加

表1　ミトコンドリア呼吸鎖複合体（Ⅰ～Ⅳ，Ⅱ＋Ⅲ）の酵素活性

Liver

	Co Ⅰ	Co Ⅱ	Co Ⅲ	Co Ⅳ	CS
% of normal	1	36	8	45	146
CS ratio（%）	0	25	6	30	
Co Ⅱ ratio（%）	1		23	121	

Muscle

	Co Ⅰ	Co Ⅱ	Co Ⅱ＋Ⅲ	Co Ⅲ	Co Ⅳ	CS
% of normal	43	81	41	17	22	68
CS ratio（%）	62	117	59	26	32	
Co Ⅱ ratio（%）	52		50	22	27	

Co Ⅰ：complex Ⅰ，Co Ⅱ：complex Ⅱ，Co Ⅲ：complex Ⅲ，Co Ⅳ：complex Ⅳ，
CS：citrate synthase

えてnafamostat mesilate，danaparoid sodium，human antithrombin Ⅲ concentratedを併用した。日齢1の頭部CTで硬膜下出血を認め，日齢2に痙攣，肺出血を発症し，頭部MRIで硬膜下出血の拡大，および正中偏位を認めた。その後，DICの改善傾向を認めた。日齢6に腎機能障害による溢水に対し血液透析を導入した。透析導入後，血清Cre値，血清乳酸値は速やかに低下した。全身浮腫が軽減し呼吸障害は軽快し，日齢16に血液透析から離脱した。一方，徐々に直接ビリルビン優位の高ビリルビン血症が進行し，日齢17にTB 48mg/dL，DB 33mg/dLまで上昇した。透析中止後48時間に急激に呼吸障害が進行し，代謝性アシドーシス，高乳酸血症を認めた。ただちに血液透析を再開しsodium bicarbonateの大量投与を行ったが，日齢20に児は永眠した。家族の希望があり，病理解剖を施行し，原因検索のため腸腰筋・肝を凍結保存した。

剖検所見

肝臓：肉眼的には腫大し，表面は不整で結節状を呈した。組織学的には線維化が著しく，肝小葉構造は多くの部分で保たれておらず，広範囲な肝細胞障害を示唆した。胆汁うっ滞，炎症細胞浸潤，ヘモジデリン沈着あり，偽胆管の増生を認めた。

筋肉：明らかな筋線維の変性なし。Ragged red fiberは明らかでなかった。

他臓器：両側の腎臓は腫大し（平均重量の＋3.3SD），組織学的には腎乳頭壊死を示した。心臓は肥大し（＋3.5SD），心筋線維の走行の乱れや変性は認めず，骨髄の造血細胞密度，分布に著しい異常は認めなかった。

ミトコンドリア呼吸鎖複合体の酵素活性および比較定量

－80℃で凍結保存した肝・筋を用いて，ミトコンドリア呼吸鎖複合体（以下complex）の酵素活性およびミトコンドリアと核のDNA比較定量を行った。酵素活性は吸光度計を用い生化学的に呼吸鎖のcomplex Ⅰ～Ⅳを測定した[3]。ミトコンドリアマーカーとしてコハク酸合成酵素（CS）を測定し，CSおよびcomplex Ⅱとの比を示した。

肝ではcomplex Ⅰ，Ⅲ，Ⅳの有意な低下を認め，complex ⅡもCS比で低下していた。筋ではcomplex ⅢおよびⅣの有意な低下を認めた。（表1）

ミトコンドリアDNA比較定量および変異検索

呼吸鎖酵素活性の結果，複数の呼吸鎖複合体の活性の低下を認めたため，ミトコンドリアDNA枯渇症候群を疑い，本検査を施行した。ミトコンドリア（complex Ⅰ ND4サブユニット）と核（膵腺胞性線維症遺伝子エクソン）のDNAをreal time PCRにて増幅し，比較定量した[4]。ミトコンドリアDNA（mtDNA）/核DNAのPCR定量は肝で正常コントロール群の78.5%，筋で101.6%と，mtDNA量は正常であり，ミトコンドリアDNA枯渇症候群は否定的であった。また，mtDNA全周性シーケンスを施行したが，病因となる変異や欠失は認めなかった。核DNAについては現在検索中である。

考　察

ミトコンドリアは糖や蛋白，脂質を燃料としてadenosine triphosphateを産生する役割があり，その中心的存在がミトコンドリア呼吸鎖である。呼吸鎖をつかさどる

蛋白はmtDNAと核DNAの両方にコードされているが，多くは核DNAに依存している。MRCDはこの呼吸鎖の酵素活性が低下し，エネルギーの産生が障害されるため発症する疾患である。様々な症状を呈し，いかなる臓器・組織，いかなる年齢にでも発症する[5]。ミトコンドリア呼吸鎖はcomplex ⅠからⅤまであり，新生児期発症のMRCDはcomplex Ⅰ欠損と複数の複合体欠損がそれぞれ4割ずつ占めている[6]。

はじめに，本児の診断について述べる。本児は胎児期には異常は指摘されておらず，出生直後から著明な代謝性アシドーシス，高乳酸血症，肝機能障害，凝固異常，貧血をきたした。透析を行い一度回復したものの，透析中止後2日で再び同様のエピソードを呈し死亡した。本児の多様な臨床像の鑑別診断として，1番目に大量失血による多臓器不全が挙げられる。しかし，分娩前後で大量出血のエピソードはなく，また胎児母体間輸血症候群については，HbFが0%であることと，透析中止後に再度代謝性アシドーシス，高乳酸血症のエピソードが起こったことから，否定した。2番目に，入院時から認めた高フェリチン血症・貧血・肝機能異常などから，先天感染およびそれに引き続く血球貪食症候群を疑ったが，各種ウイルス検査，細菌培養は陰性であり，骨髄検査でも血球貪食像を認めなかった。3番目に，尿素サイクル異常症や有機酸代謝異常症などの先天代謝異常症も鑑別に挙げられたが，血中アミノ酸分析，尿中有機酸分析，アシルカルニチン分析（タンデムマス）等の結果から否定的であった。

本児において，多臓器にわたる致死的な障害を認め，出血，感染，代謝異常が否定的であったことから，MRCDを疑い，病理解剖検査で得られた肝臓および筋肉のミトコンドリア呼吸鎖酵素活性測定を行った。その結果，肝ではcomplex Ⅲの著明な低下を認めた。また，complex Ⅱも軽度低下していた。筋でも呼吸鎖Ⅲ，Ⅳの有意な低下を認めたため，Bernierらの診断基準に基づき，MRCD確定例と診断した。

さらに，ミトコンドリア病の中でもいくつかの鑑別診断が考えられた。本症例は著明な貧血，血小板減少を認めたことから，ミトコンドリア病のうち汎血球減少を主症状とし母系遺伝形式をとるPearson病も疑った[7]。Pearson病はmtDNA遺伝子の異常であることが知られているが，本症例はmtDNAの変異や欠失はなく，Pearson病は否定的であった。また，複数の呼吸鎖複合体欠損，特に核にコードされているcomplex Ⅱ以外のcomplex Ⅰ，Ⅲ，Ⅳの活性低下を認める場合，mtDNA枯渇症候群が鑑別に挙がる。本症例では，mtDNA自体の減少を認めなかったので，mtDNA枯渇症候群は否定した。先に述べたように核DNA異常が示唆されるため，現在エクソーム解析を含む系統的な遺伝子解析を施行中である。

一方，本症例では出生時フェリチン14,865ng/mLと高フェリチン血症を認め，大量輸血後ではあるが，日齢3には130,000ng/mLまで上昇した。その後低下したものの1,500～2,000ng/mL程度で推移していた。病理所見で，肝臓にヘモジデリン沈着を認めているが大量輸血後であり有意な所見とはいえず，さらに膵での鉄沈着は目立たなかった。近年，ミトコンドリア呼吸鎖異常で鉄代謝異常をきたすとの報告があり[8]，本児でも鉄代謝異常の結果，高フェリチン血症をきたした可能性が考えられる。もしそうであれば，出生時からフェリチンが上昇していたことから胎内ですでに発症していたことが示唆される。

次に胎児期発症のMRCDについて述べる。MRCDと診断された児の一部は，胎児期からなんらかの症状を発現する。Bernierらの診断基準には，胎動が乏しく流産に至った既往の有無についても参考所見として記載されている。胎児期の所見としては，子宮内発育遅延を約3割で認め，他に羊水過多／過少，心筋肥厚，不整脈，胎児水腫，脳室拡大などが挙げられる[9][10]。胎児期に症状が出現するか否かは，胎芽胎児期の呼吸鎖酵素遺伝子発現時期や組織特異性と関連していると考えられているが，解明されていない[10]。

本児は胎児期に異常は指摘されていなかったが出生直後に急激に症状が出現しているため，おそらく胎児期から酵素活性の低下は起こっていたと考えられ，分娩・出生による急激なエネルギー消費の増加により症状が顕著になったと推測される。

最後に，新生児期発症のMRCDの診断の重要性および診断基準について述べる。MRCDは新生児期に発症する頻度が高く，しかも新生児期発症例は致死率が高い。Gibsonらは，1975年から2006年に診断されたミトコンドリア病107例のうち新生児期発症は27%で，そのうち87%が死亡したと報告している[9]。本邦では，伊藤らによると，2010年までにMRCDと診断された132例のうち45%の59例が新生児期に発症で，うち日齢1までの発症は78%であった[2]。初発症状として新

表2　MRCDの診断基準

〈大基準〉

Ⅰ．臨床症状

以下の3つをすべて満たすミトコンドリア脳筋症もしくはサイトパチー

①他の原因では説明できない多臓器にまたがる症状が出現

神経・筋・心臓・腎臓・消化器・肝・内分泌・造血器・耳・眼・皮膚・奇形症候群

②エピソードが再燃し進行する臨床経過，もしくは母系遺伝を疑わせる家族歴

③他に考えられる代謝性もしくは非代謝性異常の除外を確実に行う

Ⅱ．病理組織所見

骨格筋のragged red fiber（赤色ぼろ線維）が2%以上

Ⅲ．酵素活性

①抗体染色：COX陰性のfiberが2%以上（50歳以下）

②酵素活性：1つの臓器で20%以下，もしくは2つ以上の臓器で30%以下

1つの培養細胞で30%以下

Ⅳ．機能解析

線維芽細胞のATP合成能＞平均－3SD

Ⅴ．DNA解析

核またはミトコンドリアの明らかな病原遺伝子を同定

〈小基準〉

Ⅰ．臨床症状

ミトコンドリア呼吸鎖異常を示唆する症状※が1つ以上あること

Ⅱ．病理組織所見

- ragged red fiber：1～2%（30～50歳），少しでもあればよい（＜30歳）
- 筋線維膜下のミトコンドリアの異常蓄積：＞2%（＜16歳）
- 臓器を問わないミトコンドリアの電顕異常

Ⅲ．酵素活性

①抗体染色による呼吸鎖複合体欠損の証明

②酵素活性：1つの臓器で20～30%，2つ以上の臓器で30～40%

1つの培養細胞で30～40%

Ⅳ．機能解析

線維芽細胞のATP合成能：平均2～3SD

線維芽細胞がガラクトース培地の上で成育できない

Ⅴ．DNA解析

核またはミトコンドリアの可能性のある遺伝子異常の同定

Ⅵ．生化学検査

呼吸鎖異常を示唆する検査所見が1つ以上ある

※小児では特に，胎動減少，新生児死亡，動きが乏しい，重度の体重増加不良，新生児筋緊張低下・亢進を加えて考慮する。成人では筋・神経症状が必須であるが，小児では必ずしも当てはまらない。

「Definite」：大基準2つ，もしくは大基準1つ＋小基準2つ
「Probable」：大基準1つ＋小基準1つ，もしくは小基準3つ
「Possible」：大基準1つ，または小基準のⅠ＋他の小基準1つ

生児仮死，呼吸障害，代謝性アシドーシス，心筋肥厚，腹部膨満・嘔吐，低血糖などが挙げられ，新生児期発症例の死亡率は63%と高率であった[2]。59例中5例は高乳酸血症を認めておらず[2]，新生児期発症のMRCDが必ずしも高乳酸血症を呈するとはいえない。

一方，堂本らは，超低出生体重児でMRCD（complex Ⅰ deficiency）の2症例を報告した[11]。2症例とも，壊死性腸炎や腸穿孔など消化器症状で発症し，その後の胆汁うっ滞，肝障害に対し肝生検を行い，本症と診断された[11]。これらの症状は，超低出生体重児としては決して稀ではなく，早産児においては診断に苦慮する可能性がある。

MRCDの診断基準には，Bernierらの診断基準が有用とされる（表2）[12]。そこには他の代謝疾患を除外す

ることの重要性が記載されている。尿素サイクル異常症，有機酸代謝異常症，脂肪酸代謝異常症はミトコンドリアの二次的障害を伴うことが非常に多く[13]，アミノ酸分析，尿中有機酸分析，アシルカルニチン分析は必ず行う必要がある。病理診断基準として，大基準では，2%以上のragged red fiberが唯一挙げられているが，小児のミトコンドリア病ではあまり一般的ではない。本症例でもミトコンドリア病に特徴的なragged red fiberなどの病理学的な変化は認めなかった。

また，遺伝子異常を同定することも，診断の大基準の1つである。新生児期発症のMRCDは，核DNA異常によるものが多い[9]。しかし，既知の病因遺伝子だけでも現時点で110種類を超えている。最近Calvoらは，MRCD症例の40例についてのエクソーム解析を報告している[14]。この中で，24%が既知の病因遺伝子として同定され，29%は新規候補遺伝子として推定されている。一方でエクソーム解析でも同定されないものが45%も占めており，核の病因遺伝子同定の難しさがうかがえる。

一方で，呼吸鎖酵素活性は検体を適切に採取・保存することにより，正確に測定・評価が可能で，呼吸鎖機能の生化学的評価として非常に有用である。本症例も臨床所見および酵素活性により大基準2つを満たし，診断に至ることができた。

新生児期に多臓器不全や重篤な経過で死亡し診断がついていない症例の中にも本疾患が含まれている可能性がある。原因不明の新生児仮死や代謝性アシドーシス，肝機能障害などを呈した症例に対し，MRCDを積極的に疑い，病理解剖および肝臓・心臓をはじめとした該当臓器の凍結材料を用いた呼吸鎖酵素活性および遺伝子解析を行うことで診断や病態の解明が可能となり，治療法の開発に結びついてくるものと考える。

結　　語

出生後急激に呼吸不全，貧血，DIC，代謝性アシドーシスをきたし致死的な経過をたどった症例を経験した。病理解剖を行い，肝臓，筋肉のミトコンドリア呼吸鎖複合体酵素活性を測定し，MRCDと診断した。原因不明の新生児仮死や代謝性アシドーシス，肝機能障害などを呈した症例では，MRCDを鑑別診断として念頭におく必要がある。

本症の診断にあたり，病理解剖を担当した当院病理科　田中祐吉先生，田中水緒先生，凍結材料のmtDNA解析を行っていただいた埼玉医科大学小児科　大竹　明先生，自治医科大学小児科　森　雅人先生に深謝いたします。

すべての著者に日本未熟児新生児学会の定める利益相反に関する開示事項はありません。

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A Case of Mitochondrial Respiratory Chain Complex Disease with Metabolic Acidosis and Liver Dysfunction Just After Birth

*1 Department of Neonatology, Kanagawa Children's Medical Center
*2 Department of Pediatrics, Niigata University Medical & Dental Hospital
*3 Department of Pediatrics, Chiba Children's Hospital

Tomoko SAITO *1*2, Makiko OYAMA *1, Motoyoshi KAWATAKI *1
Yasufumi ITANI *1, Kei MURAYAMA *3

We report an infant with biochemically confirmed mitochondrial respiratory chain complex disease (MRCD) who developed severe respiratory difficulty, with profound lactic acidemia, liver dysfunction, disseminated intravascular coagulation, and severe anemia just after birth. The symptoms subsided after dialysis was introduced. However, she died of a second attack 2 days after weaning from dialysis. Multiple mitochondrial respiratory chain complex activities were decreased in the liver and muscle specimens taken at autopsy. Molecular analysis was undertaken.

Symptoms suggesting multiple organ failures may suggest a mitochondrial disorder. Enzyme assay of the mitochondrial respiratory chain using affected tissue is important for diagnosis of MRCD in the neonatal period, since the major etiology of neonatal onset MRCD is reported to be mutation of nuclear DNA, which can be difficult to analyze.

Exp. Anim. 63(1), 21–30, 2014

—Original—

Selection of Rodent Species Appropriate for mtDNA Transfer to Generate Transmitochondrial Mito-Mice Expressing Mitochondrial Respiration Defects

Shunkei ENOKI[1)]*, Akinori SHIMIZU[1)]*, Chisato HAYASHI[1)], Hirotake IMANISHI[1,2)], Osamu HASHIZUME[1)], Kazuyuki MEKADA[3)], Hitoshi SUZUKI[4)], Tetsuo HASHIMOTO[1)], Kazuto NAKADA[1)], and Jun-Ichi HAYASHI[1)]

[1)]*Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1–1–1, Tsukuba, Ibaraki 305-8572, Japan*
[2)]*Japan Society for the Promotion of Science (JSPS), 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan*
[3)]*RIKEN BioResource Center, Koyadai 3–1–1, Tsukuba-shi, Ibaraki 305-0074, Japan*
[4)]*Laboratory of Ecology and Genetics, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-0810, Japan*

Abstract: Previous reports have shown that transmitochondrial mito-mice with nuclear DNA from *Mus musculus* and mtDNA from *M. spretus* do not express respiration defects, whereas those with mtDNA from *Rattus norvegicus* cannot be generated from ES cybrids with mtDNA from *R. norvegicus* due to inducing significant respiration defects and resultant losing multipotency. Here, we isolated transmitochondrial cybrids with mtDNA from various rodent species classified between *M. spretus* and *R. norvegicus*, and compared the O_2 consumption rates. The results showed a strong negative correlation between phylogenetic distance and reduction of O_2 consumption rates, which would be due to the coevolution of nuclear and mitochondrial genomes and the resultant incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genome from the other rodent species. These observations suggested that *M. caroli* was an appropriate mtDNA donor to generate transmitochondrial mito-mice with nuclear DNA from *M. musculus*. Then, we generated ES cybrids with *M. caroli* mtDNA, and found that these ES cybrids expressed respiration defects without losing multipotency and can be used to generate transmitochondrial mito-mice expressing mitochondrial disorders.

Keywords: interspecies mtDNA transfer, multipotency, *Mus caroli* mtDNA, respiration defects, transmitochondrial ES cybrids

Introduction

Accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations that induce respiration defects has been proposed to be responsible for mitochondrial diseases, aging, and age-related disorders [20, 21]. Generation of transmitochondrial mito-mice expressing respiration defects by the introduction of exogenous mtDNA with pathogenic mutations would provide an ideal system for precise investigation of the pathogenesis of these disorders. However, it is impossible to generate transmitochondrial mito-mice carrying artifi-

(Received 20 May 2013 / Accepted 1 July 2013)
Address corresponding: J.-I. Hayashi, Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1–1–1, Tsukuba, Ibaraki 305-8572, Japan
**Both authors contributed equally to this study.*

cially mutagenized mtDNA, because no procedures have yet been established for introducing exogenous mtDNA into mitochondria.

One way of generating transmitochondrial mito-mice expressing respiration defects is to detect mtDNA with a somatic mutation that induces respiration defects in cultivated mouse cell lines. Our previous studies generated transmitochondrial mito-mice carrying mtDNA with pathogenic mutations and expressing various disorders by introduction of mitochondria carrying mtDNA with somatic mutations accumulated in mouse tumor cell lines into fertilized mouse eggs [6] or into mouse ES cells [4, 7, 11, 23].

Another procedure used to generate transmitochondrial mito-mice expressing respiration defects is to introduce mtDNA from different rodent species. Because most mitochondrial respiratory complexes consist of subunits encoded by both nuclear DNA and mtDNA [21], transmitochondrial cybrids with nuclear DNA from mice (*M. musculus*) but with mtDNA from a different rodent species express respiration defects owing to incompatibility between the nuclear and mitochondrial genomes from different rodent species [2, 13, 14, 22]. Similar incompatibility has been reported in transmitochondrial cybrids with human nuclear DNA but with mtDNA from different primate species [1].

However, no reports have succeeded in obtaining transmitochondrial mito-mice expressing respiration defects by introducing mtDNA from different rodent species. For example, transmitochondrial mito-mice with nuclear DNA from mice (*M. musculus*) but with mtDNA from a different mouse species (*M. spretus*) do not express respiration defects and disease phenotypes [11, 15], whereas transmitochondrial mito-mice with mtDNA from rats (*R. norvegicus*) cannot be generated [11]. The latter failure is due to the induction of significant respiration defects and the resultant loss of multipotency in mouse ES cybrids with rat mtDNA [11]. Therefore, we need to find a rodent species of which we can use its mtDNA to induce respiration defects but not induce loss of multipotency in mouse ES cells.

Here, we addressed the issue by isolating transmitochondrial cybrids with nuclear DNA from *M. musculus* and mtDNA from rodent species that are phylogenetically classified between *M. spretus* and *R. norvegicus*. We found that one of these rodent species was an appropriate mtDNA donor for generating mito-mice expressing respiration defects and mitochondrial disorders.

Materials and Methods

Cells and cell culture

Mouse mtDNA-less (ρ^0) B82 cells derived from fibroblasts of *M. musculus* [5], transmitochondrial cybrids B82mtB6, B82mtSpr, B82mtRat [22], B82mtCOI^M [11], B82mtCar, and B82mtAsp isolated in this study were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum, 50 ng/ml uridine, and 0.1 mg/ml pyruvate. Mouse ES cells (TT2-F, an XO subline established from XY TT2 cells) [11] and mtDNA-repopulated ES cybrids were cultivated on mitomycin C-inactivated feeder cells derived from mouse embryonic fibroblasts, in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% KNOCKOUT™ Serum Replacement (Invitrogen), 1× non-essential amino acids (MP Biomedicals LLC, OH, USA), leukemia inhibitory factor (10^5 units/ml, Invitrogen), and 100 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA).

Isolation of transmitochondrial cybrids

Platelets of *M. caroli*(RBRC00123) were provided from RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Platelets of *Apodemus speciosus* were provided from Dr. Hitoshi Suzuki (Hokkaido University, Japan). Platelet mtDNA was introduced into ρ^0 B82 cells by the fusion of the platelets and ρ^0 B82 cells in the presence of 50% (w/v) polyethylene glycol (PEG) as described previously [9]. The fusion mixture was cultivated in selection medium RPMI1640 without pyruvate and uridine, in which unfused ρ^0 B82 cells without mitochondrial respiratory function were unable to grow [8].

Isolation of transmitochondrial ES cybrids

Mouse ES cybrids with mtDNA from *M. caroli* were isolated based on the procedure as reported previously [11]. Briefly, the host ES cells were pretreated with rhodamine 6G (R6G; 0.38–1.5 μg/ml in 3% ethanol) for 48 h in medium supplemented with uridine (50 ng/ml) and pyruvate (0.1 μg/ml) to eliminate endogenous mitochondria and mtDNA [17]. Then, they were washed with phosphate-buffered saline (PBS) and suspended in R6G-free medium for 2 h to allow recovery. The mtDNA donor B82mtCar cybrids pretreated with cytochalasin B (10 μg/ml) for 10 min were centrifuged at 15,000×*g* for

30 min at 37°C for enucleation. The resultant cytoplasts were fused with R6G-pretreated ES cells using polyethylene glycol, and the fusion mixture was cultivated in selective medium with HAT (hypoxanthine aminopterin thymidine). Due to the absence of thymidine kinase activity of nuclear donor B82 cells, B82mtCar cybrids carrying nuclear genome from B82 cells could not survive in the presence of HAT. Seven days after fusion, growing colonies were picked up for further examination.

Construction of phylogenetic trees

Sequences of the cytochrome b (*mt-Cytb*) gene, a *mt-Dcr* region, and the *mt-Rnr1* gene in mtDNA (Supplementary Table 1) were manually aligned using SEAVIEW program (http://pbil.univ-lyon1.fr/software/seaview.html). For each locus pairwise distances were inferred on the basis of Kimura's two-parameter model [12], with among-site rate heterogeneity taken into consideration by assuming discrete distribution with 4 categories. Using the distance matrix obtained phylogenetic tree was constructed by NEIGHBOR program implemented in PHYLIP software (http://www.phylip.com/) under the assumption of evolutionary rate constancy among lineages. On the basis of the phylogenetic tree distances between *M. musculus* and other organisms were calculated.

Genotyping of mtDNA

Total cellular DNA (0.2 μg) extracted from cultivated cells was used as a template. Restriction fragment length polymorphism (RFLP) analysis was carried out for detection of *M. caroli* mtDNA. A 306-bp fragment was amplified by PCR with the following primers 5′-CTCTGGTCTTGTAAACC-3′ and 5′-GACTGTATGGTGTATATCAG-3′, which corresponded to mouse mtDNA sequences (GenBank Accession No. AY172335) from positions 15306 to 15322 and from 15807 to 15787, respectively. The cycle times were 30 s for denaturation at 94°C, 30 s for annealing at 46°C and 30 s for extension at 72°C for 30 cycles. The PCR amplicon contains a region of the mtDNA *mt-Dcr* with a *Dra* I (Takara) restriction site (control mouse mtDNA was not cleaved), and generates 267-bp and 39-bp fragments on *Dra* I digestion. Similarly, detection of *A. speciosus* mtDNA was achieved by RFLP analysis. A 250 bp fragment was amplified by PCR with the following primers 5′-GGTGTCCTAGCCTTAATC-3′ and 5′-CGATAATTCCTGAGAGATTGGT-3′, which corresponded to mouse mtDNA sequences (GenBank Accession No. AY172335) from positions 15012 to 15029 and from 15261 to 15242, respectively. The cycle times were 30 s for denaturation at 94°C, 30 s for annealing at 54°C and 30 s for extension at 72°C for 30 cycles. The PCR amplicon contains a region of the *mt-Cytb* gene with an *Mbo* II (NEB) restriction site (control mouse mtDNA was not cleaved), and generates 219-bp and 31-bp fragments on *Mbo* II digestion. These restriction fragments were separated by electrophoresis in a 3% agarose gel. For quantification of the mtDNA from *M. caroli* and mtDNA from *M. musculus*, we used the NIH IMAGE program.

Analysis of mitochondrial respiratory function

Oxygen consumption rates were measured by trypsinizing cells, incubating the suspension in phosphate-buffered saline, and recording oxygen consumption in a polarographic cell (2.0 ml) at 37°C with a Clark-type oxygen electrode (Yellow Springs Instruments, OH). Cytochemical analysis of cytochrome *c* oxidase (COX) activity was carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome [16].

Analysis of multipotency of ESmtCar cybrids

To test the multipotency of the ESmtCar cybrids, 1×10^6 cells were inoculated subcutaneously into the backs of 6-week-old nude mice (JCL, BALB/c-nu/nu; CLEA Japan). The resulting teratomas (tumors) were fixed in 10% neutral-buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (HE) and examined histologically.

Animal experiments

All animal experiments were performed in accordance with protocols approved by the Experimental Animal Committee of the University of Tsukuba.

Statistical analysis

We analyzed data with the (unpaired or paired) Student's *t*-test. Values with $P<0.05$ were considered significant.

Results

Isolation of mouse transmitochondrial cybrids with mtDNA from different rodent species

On the basis of the phylogenetic trees constructed by

comparing the sequences of the *mt-Cytb* gene in the mtDNA (Fig. 1A), we used *M. caroli* and *A. speciosus*, which are phylogenetically classified between *M. spretus* and *R. norvegicus* [18, 19], as candidate mtDNA donor species. As mtDNA recipients, we used ρ^0 B82 cells without mtDNA and with the nuclear genetic background of *M. musculus* [5].

Cytoplasmic transfer of mtDNA from *M. caroli* and *A. speciosus* into ρ^0 B82 cells was performed by the fusion of ρ^0 B82 cells with platelets from *M. caroli* and *A. speciosus*, respectively. Colonies grown in selective medium to exclude unfused ρ^0 B82 cells were isolated clonally as transmitochondrial cybrids and were named B82mtCar cybrids and B82mtAsp cybrids (Table 1). Genotyping of mtDNA showed that B82mtCar cybrids possessed *M. caroli* mtDNA and B82mtAsp cybrids possessed *A. speciosus* mtDNA (Fig. 1B).

For further examination of mitochondrial respiratory function, we used B82mtB6, B82mtSpr, and B82mtRat possessing mtDNA from *M. musculus*, *M. spretus*, and *R. norvegicus*, respectively [11], as control cybrids (Table 1). We furthermore used B82mtCOI^M cybrids possessing *M. musculus* mtDNA with a pathogenic T6589C mutation in the *mt-Co1* gene as control cybrids (Table 1), because we had already successfully generated transmitochondrial mito-miceCOI^M expressing respiration defects and mitochondrial disease phenotypes by introducing the T6589C mtDNA into a mouse female germ line [11].

Effect of phylogenetic distance on respiratory function in transmitochondrial cybrids

We used transmitochondrial cybrids possessing mtDNA from various rodent species or possessing mouse mtDNA with a pathogenic mutation to compare O_2 consumption rates, which reflected overall mitochondrial respiratory function (Fig. 1C). B82mtCar cybrids with mtDNA from *M. caroli*, which belongs to the same genus *Mus*, had a 35% reduction in O_2 consumption rates, indicating that B82mtCar cybrids expressed relatively mild respiration defects. In contrast, B82mtAsp cybrids with mtDNA from *A. speciosus* belonging to a different genus Apodemus had a 66% reduction in O_2 consumption rates. These results suggest that mitochondrial respiratory function of B82 cybrids with mtDNA of different rodent species is reduced in accordance with the phylogenetic distance from *M. musculus*(Figs. 1A and C).

To explore this idea further, we estimated the phylogenetic distances of the rodent species used here by comparing the sequences of the *mt-Cytb* gene (Fig. 2A), the *mt-Dcr* region (Fig. 2B), and the *mt-Rnr1* gene (Fig. 2C) in the mtDNA. Phylogenetic distance and O_2 consumption rates were well correlated negatively in transmitochondrial cybrids with mtDNA from various rodent species (Fig. 2). Thus, the increase in respiration defects would be due to increased incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genomes from other rodent species.

Selection of rodent species appropriate for generating transmitochondrial mito-mice

Our previous study [11] proposed that failure to obtain transmitochondrial mito-mice with rat mtDNA was due to the induction of significant respiration defects by the introduction of rat mtDNA and the resultant loss of multipotency in mouse ES cybrids with rat mtDNA. Because B82mtAsp cybrids showed a 66% reduction of O_2 consumption rates, which are comparable to the reduction rates observed in B82mtRat cybrids (Figs. 1C and 2), mtDNA from *A. speciosus* would not be appropriate for generating transmitochondrial mito-mice.

In contrast, the 35% reduction in O_2 consumption rates in B82mtCar cybrids appears comparable to the 31% reduction in B82mtCOI^M cybrids with mouse T6589C mtDNA (Figs. 1C and 2). Considering that mouse T6589C mtDNA can be effectively introduced into the female germ line and induces disease phenotypes in transmitochondrial mito-miceCOI^M in the absence of embryonic lethality [11], *M. caroli* is a suitable mtDNA donor species for generating transmitochondrial mito-mice expressing phenotypes related to mitochondrial diseases.

Isolation of transmitochondrial mouse ES cybrids with mtDNA from M. caroli

Next, we isolated transmitochondrial mouse ES cybrids with mtDNA from *M. caroli*. For exclusive isolation of the ES cybrids with mtDNA from *M. caroli*, we used B82mtCar cybrids as mtDNA donors, because unenucleated B82mtCar cybrids can be excluded from the fusion mixture by using HAT selection medium owing to their deficiency in thymidine kinase (Table 1; Materials and Methods). As nuclear DNA donors and mtDNA recipients, we used female-type mouse ES cells, because mtDNA is exclusively inherited via the female germ line [10, 17].

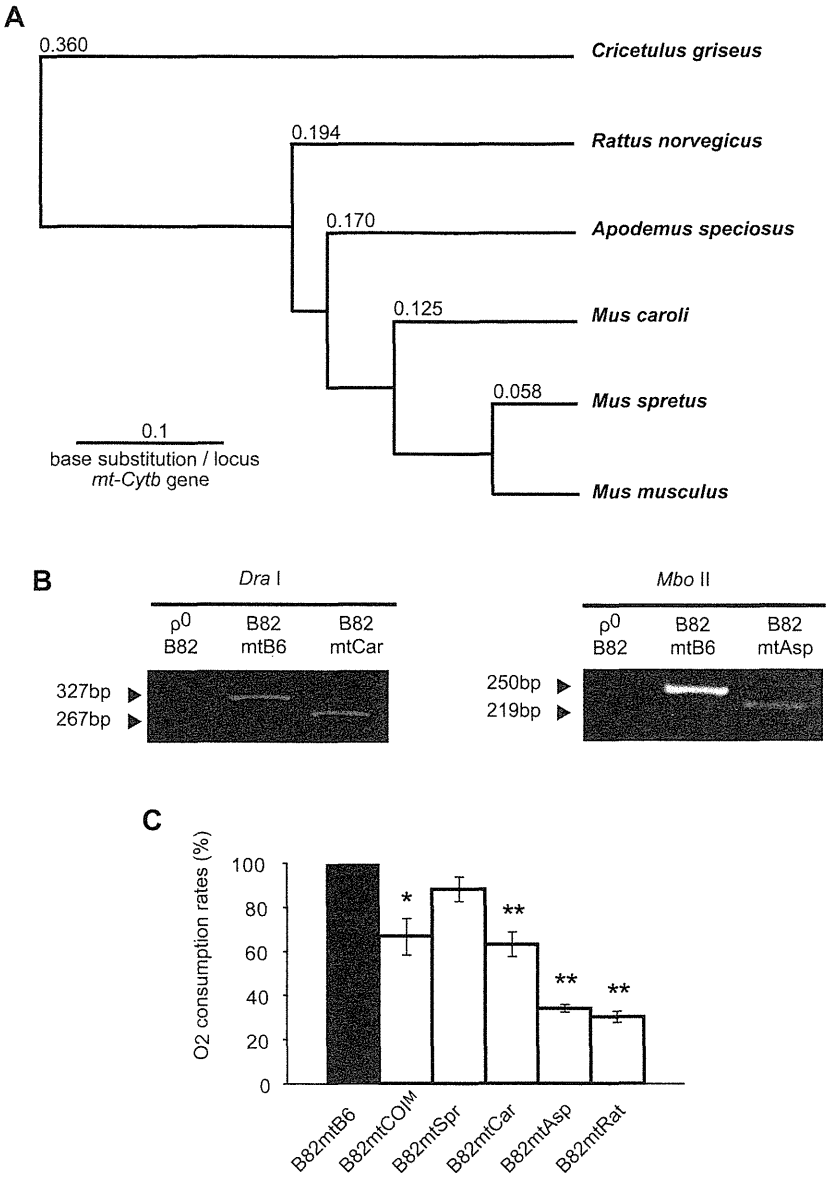

Fig. 1. Characterization of transmitochondrial cybrids with mtDNA from various rodent species. Transmitochondrial cybrids B82mtB6, B82mtSpr, B82mtCar, B82mtAsp, and B82mtRat possessed nuclear DNA from *M. musculus* and mtDNA from *M. musculus*, *M. spretus*, *M. caroli*, *A. speciosus* and *R. norvegicus*, respectively. B82mtCOI^{M} cybrids possessed *M. musculus* mtDNA with a pathogenic T6589C mutation in the *mt-Co1* gene that induces respiration defects [11]. (A) Phylogenetic trees constructed by comparison of the sequence of the *mt-Cytb* gene encoded by mtDNA. On the basis of Kimura's two-parameter model [24], we used *mt-Cytb* gene sequence data (positions 14139 to 15266) to create phylogenetic trees with PHYLIP software (http://www.phylip.com/). Branch lengths show evolutionary distance from *M. musculus*. The tree is rooted using *Cricetulus griseus*(Chinese hamster) sequence data. Values on each branch indicate base substitution in the *mt-Cytb* gene. (B) Genotyping of mtDNA. On *Dra* I digestion of the PCR products, B82mtB6 cells with *M. musculus* mtDNA gave a 327-bp fragment, whereas B82mtCar cells with *M. caroli* mtDNA gave a 267-bp fragment and a 39-bp fragment (not detectable) by a gain of a *Dra* I site and a 21-bp deletion in the *mt-Dcr* region. On *Mbo* II digestion of the PCR products, B82mtB6 cybrids with *M. musculus* mtDNA gave a 250-bp fragment, whereas B82mtAsp cybrids with *A. speciosus* mtDNA gave a 219-bp fragment and a 31-bp fragment (not detectable) by the gain of an *Mbo* II site in the *mt-Cytb* gene. (C) Estimation of O_2 consumption rates. B82mtB6 cells carrying nuclear and mitochondrial genomes from *M. musculus* were used as standards expressing normal respiratory function. Asterisks indicate a *P*-value less than 0.05 and double asterisks indicate a *P*-value less than 0.01.

Table 1. Genome composition of transmitochondrial cybrids with imported mtDNA from various rodent species

Transmitochondrial cybrids	Nuclear genetic marker	Rodent speicies	
		Nuclear genome	Mitochondrial genome
B82mtB6*	HAT sensitive	*M. m. domesticus*	*M. m. domesticus*
B82mtCOI^{M}**	HAT sensitive	*M. m. domesticus*	*M. m. domesticus*
B82mtSpr*	HAT sensitive	*M. m. domesticus*	*M. spretus*
B82mtCar	HAT sensitive	*M. m. domesticus*	*M. caroli*
B82mtAsp	HAT sensitive	*M. m. domesticus*	*A. speciosus*
B82mtRat*	HAT sensitive	*M. m. domesticus*	*R. norvegicus*

*, Established in our previous report study [10]. **, Possessing *M. m. domesticus* mtDNA with a T6589C mutation in the *mt-Co1* gene; established in our previous report study [5].

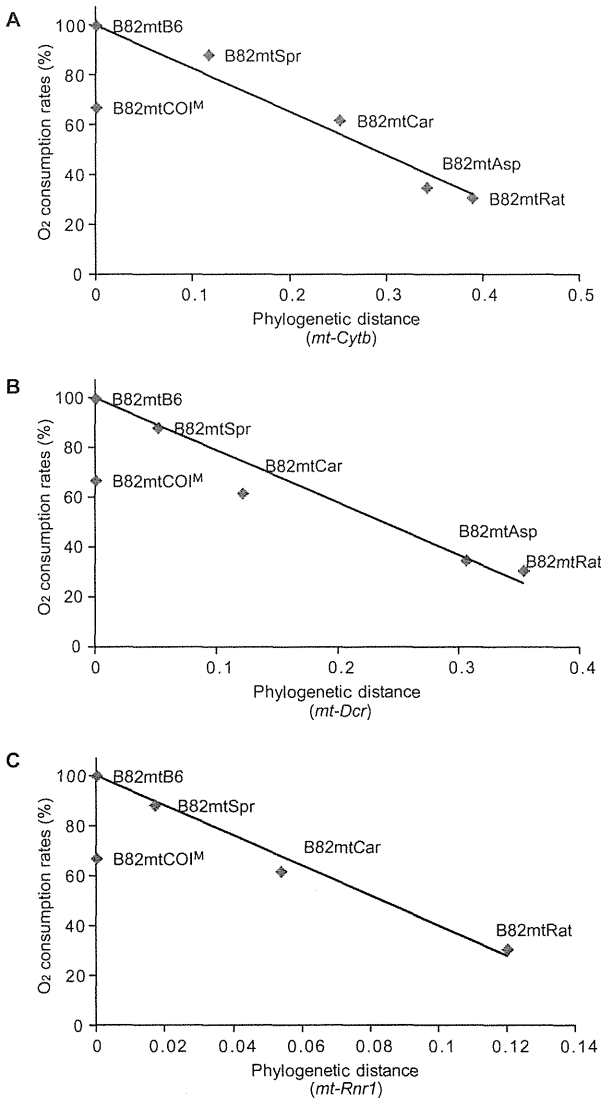

Fig. 2. Correlation between phylogenetic distance and reduction rates of O_2 consumption in transmitochondrial cybrids. Phylogenetic distance was estimated from base substitution in (A) the *mt-Cytb* gene, (B) the *mt-Dcr* region, and (C) the *mt-Rnr1* gene in mtDNA. Correlation coefficients were 0.964, 0.951 and 0.984 in (A), (B) and (C), respectively.

The ES cells were pretreated with rhodamine 6G (R6G) to eliminate endogenous mitochondria and mtDNA, and were then fused with enucleated B82mtCar cybrids. Unfused R6G-pretreated ES cells were unable to grow owing to the absence of mitochondria and mtDNA, and unenucleated B82mtCar cybrids failed to grow in the HAT selection medium. Three colonies growing in the selection medium were isolated clonally. They were named ESmtCar-1, -2, and -3, and mtDNA genotyping showed that they possessed 39%, 42% and 42% mtDNA, respectively, from *M. caroli* (Fig. 3A). The ESmtCar cybrids were therefore transmitochondrial ES cybrids with heteroplasmic mtDNA consisting of *M. caroli* mtDNA from the B82mtCar cybrids and *M. musculus* mtDNA in the host ES cells, probably due to incomplete elimination of their own mtDNA by the R6G pretreatment.

Considering that the ESmtCar cybrids possessed nuclear DNA exclusively from *M. musculus*, it is possible that the *M. caroli* mtDNA would eventually be excluded from the ESmtCar cybrids during cultivation by preferential replication of *M. musculus* mtDNA. We examined this possibility by mtDNA genotyping after prolonged cultivation of the ESmtCar-2 cybrids for 6 weeks after cloning. The results showed that the proportion of *M. caroli* mtDNA did not change substantially (Fig. 3B). Thus, *M. caroli* mtDNA can replicate and propagate stably into subsequent generations, even in the presence of host *M. musculus* mtDNA in ESmtCar cybrids.

Effects of M. caroli mtDNA on respiratory function and multipotency of ESmtCar cybrids

A question was whether ESmtCar cybrids expressed respiration defects. Because it was difficult to obtain a sufficient number of ESmtCar cybrids (5×10^6 cells) for estimation of O_2 consumption rates without feeder cell

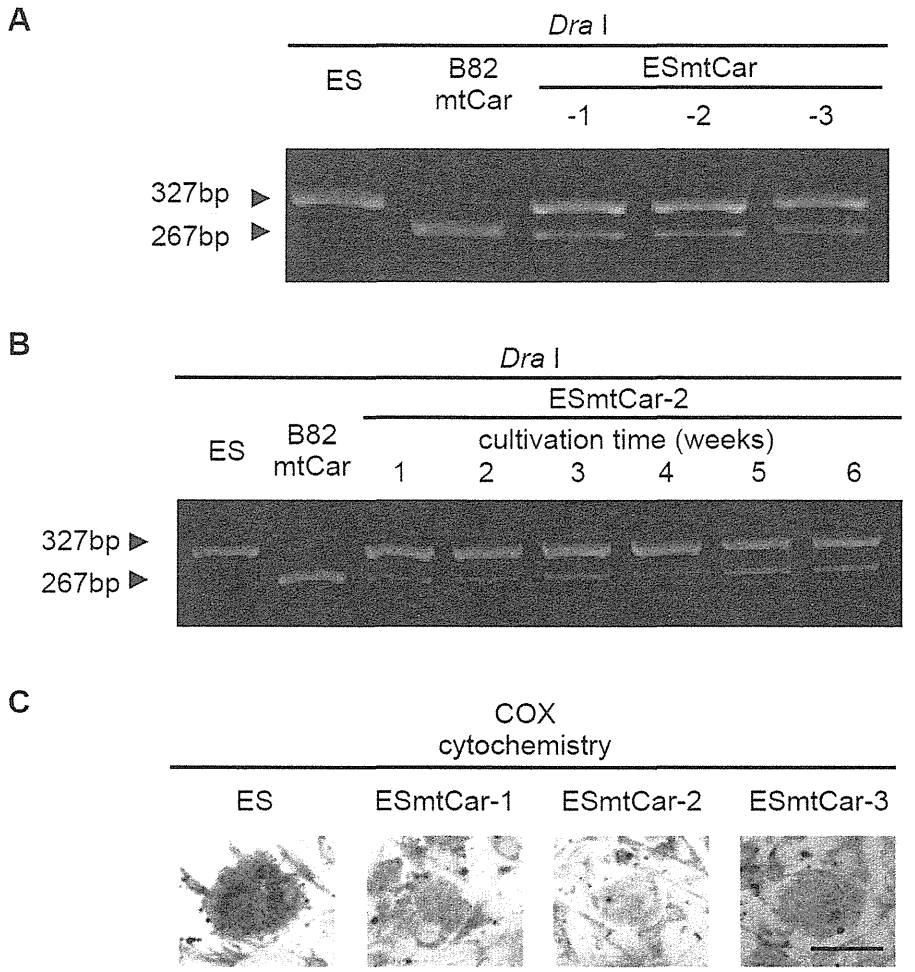

Fig. 3. Characterization of transmitochondrial ESmtCar cybrids. (A) Genotyping of mtDNA from ESmtCar cybrids. On *Dra* I digestion of the PCR products, B82mtB6 cells with *M. musculus* mtDNA gave a 327-bp fragment, whereas B82mtCar cybrids with *M. caroli* mtDNA gave a 267-bp fragment and a 39-bp fragment (not detectable) by the gain of a *Dra* I site and a 21-bp deletion in the *mt-Dcr* region. ES cells with homoplasmic *M. musculus* mtDNA and B82mtCar cybrids with homoplasmic *M. caroli* mtDNA were used as negative and positive controls, respectively. Three ESmtCar cybrid clones showed heteroplasmy of *M. caroli* mtDNA and *M. musculus* mtDNA. ESmtCar-1, -2, and -3 possessed 39%, 42%, and 42% *M. caroli* mtDNA, respectively. (B) Stability of *M. caroli* mtDNA in ESmtCar-2 cybrids cultivated for 1 to 6 weeks after cloning. (C) Analysis of mitochondrial respiratory function of ESmtCar cybrids by COX cytochemistry. Bar, 100 μm.

contamination, we used cytochemical analysis of COX activity. ESmtCar cybrids with *M. caroli* mtDNA showed respiration defects, whereas parental ES cells did not (Fig. 3C). These observations indicated that mtDNA genotypes and respiration phenotypes were transferred simultaneously from mtDNA donor B82mtCar cybrids to ESmtCar cybrids.

The next question was whether ESmtCar cybrids expressing respiration defects retained their multiple differentiation potential. Our previous report [11] showed that mouse ES cybrids with rat mtDNA (ESmtRat cybrids) lost their multipotency phenotypes upon subcutaneous inoculation under the back skin of nude mice, resulting in failure to generate transmitochondrial mice with rat mtDNA. Therefore, we tested whether the ESmtCar cybrids were able to differentiate into various tissues under the back skin of nude mice. Both the ESmtCar cybrids and the parental ES cells formed primary tumor masses within 4 weeks after their inoculation (Fig. 4). Histological analysis of their primary tumors showed

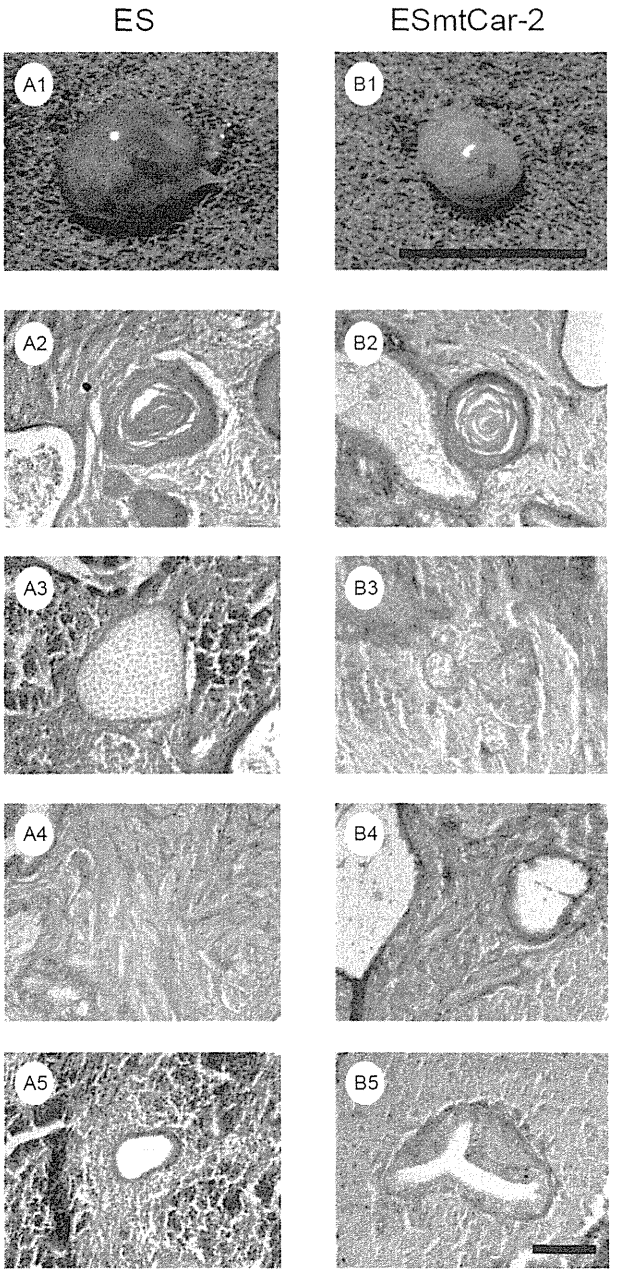

Fig. 4. Effects of respiration defects on multipotency of transmitochondrial ESmtCar cybrids. ES cells and ESmtCar-2 cybrids with 42% *M. caroli* mtDNA were inoculated into nude mice and the resultant primary tumor masses were used for histochemical analysis. (A1–A5), ES cells; (B1–B5), ESmtCar-2 cybrids. A1 and B1 are primary tumor masses formed in nude mice. A2 and B2, A3 and B3, A4 and B4, and A5 and B5 correspond to hair follicles, bone cells, striated muscle and secretory cells, respectively. Bar in B1, 1 cm; bar in B5, 100 μm.

that both ES cells and ESmtCar-2 cybrids were able to differentiate into multiple tissue types, namely secretory cells, bone cells, striated muscle, and hair follicles (Fig. 4). These observations suggest that the ESmtCar cybrids still express multipotency, and could therefore be used in future studies to generate transmitochondrial mito-mice with mtDNA from *M. caroli*.

Discussion

This study determined that *M. caroli* was an appropriate mtDNA donor for generating transmitochondrial mito-mice expressing respiration defects by isolating transmitochondrial cybrids with nuclear DNA from *M. musculus* and mtDNA from *M. caroli* or *A. speciosus*, which are phylogenetically classified between *M. spretus* and *R. norvegicus*.

Comparison of the O_2 consumption rates of transmitochondrial cybrids with mtDNA from various rodent species showed a strong negative correlation between phylogenetic distance and reduction of O_2 consumption rates (Fig. 2). Because most mitochondrial respiratory complexes consist of both nuclear genome-coded and mitochondrial genome-coded subunits [21], mitochondrial respiratory function is controlled by both genomes. Therefore, the respiration defects observed in transmitochondrial cybrids with mtDNA from various rodent species (Fig. 2) would have been due to the coevolution of nuclear and mitochondrial genomes and the resultant incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genome from the other rodent species.

Transmitochondrial cybrids with *A. speciosus* mtDNA (B82mtAsp cybrids) showed a 66% reduction in O_2 consumption rates, which were comparable to the reduction rates observed in B82mtRat cybrids (Fig. 1C). Because transmitochondrial mito-mice with rat mtDNA have not been generated owing to the induction of significant respiration defects by the rat mtDNA and the resultant losing multipotency in mouse ES cybrids with rat mtDNA [11], mtDNA from *A. speciosus* or from *R. norvegicus* would not be appropriate for generating transmitochondrial mito-mice. Similar failures to generate transmitochondrial mito-mice were reported, particularly when mouse embryos possessed mouse mtDNA with pathogenic mutations that induce significant respiration defects [3, 7].

In contrast, B82mtCar cybrids showed a 35% reduction in O_2 consumption rates (Fig. 1C). Although a previous report [13] showed that transmitochondrial cybrids with mtDNA from *M. caroli* did not exhibit reduced

activity of each respiration complex, the O_2 consumption rates we obtained here reflect the overall activity of mitochondrial oxidative phosphorylation. Moreover, a good correlation between reduction rates of the O_2 consumption and phylogenetic distance (Fig. 2) suggests that O_2 consumption rates are reliable for estimating overall activity of mitochondrial respiration. Furthermore, the reduction rates of the O_2 consumption induced by *M. caroli* mtDNA were comparable to those induced by T6589C mtDNA of *M. musculus* (Fig. 1C), which is effectively transferred via the mouse female germ line to the following generations and induces disease phenotypes in transmitochondrial mito-miceCOI^M [11]. Therefore, mtDNA from *M. caroli* is a candidate mtDNA that can carry possible pathogenic mutations in the transmitochondrial mito-mice with nuclear DNA from *M. musculus*.

We also succeeded in isolating transmitochondrial ESmtCar cybrids with *M. caroli* mtDNA expressing respiration defects (Fig. 3C) without loss of multipotency (Fig. 4B). Therefore, we next intend to generate transmitochondrial mito-mice with *M. caroli* mtDNA using the ESmtCar cybrids, and to examine whether they can be used as models of mitochondrial disorders.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research S 19100007 (to J.-I.H.), Scientific Research A 23240058 (to K.N.), and Scientific Research on Innovative Areas 24117503 (to J.-I.H.) from Japan Society for the Promotion of Science.

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Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the *tRNA^{Lys}* gene

Akinori Shimizu[a], Takayuki Mito[a], Chisato Hayashi[a], Emi Ogasawara[a], Ryusuke Koba[a], Issei Negishi[a], Keizo Takenaga[b], Kazuto Nakada[a,c], and Jun-Ichi Hayashi[a,c,1]

[a]Faculty of Life and Environmental Sciences and [c]International Institute for Integrative Sleep Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; and [b]Department of Life Science, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan

Edited by Luca Scorrano, University of Padua, Padua, Italy, and accepted by the Editorial Board January 14, 2014 (received for review September 25, 2013)

We generated transmitochondrial mice (mito-mice) that carry a mutation in the *tRNA^{Lys}* gene encoded by mtDNA for use in studies of its pathogenesis and transmission profiles. Because patients with mitochondrial diseases frequently carry mutations in the mitochondrial *tRNA^{Lys}* and *tRNA$^{Leu(UUR)}$* genes, we focused our efforts on identifying somatic mutations of these genes in mouse lung carcinoma P29 cells. Of the 43 clones of PCR products including the *tRNA^{Lys}* or *tRNA$^{Leu(UUR)}$* genes in mtDNA of P29 cells, one had a potentially pathogenic mutation (G7731A) in the *tRNA^{Lys}* gene. P29 subclones with predominant amounts of G7731A mtDNA expressed respiration defects, thus suggesting the pathogenicity of this mutation. We then transferred G7731A mtDNA into mouse ES cells and obtained F_0 chimeric mice. Mating these F_0 mice with C57BL/6J (B6) male mice resulted in the generation of F_1 mice with G7731A mtDNA, named "mito-mice-tRNA^{Lys7731}." Maternal inheritance and random segregation of G7731A mtDNA occurred in subsequent generations. Mito-mice-tRNA^{Lys7731} with high proportions of G7731A mtDNA exclusively expressed respiration defects and disease-related phenotypes and therefore are potential models for mitochondrial diseases due to mutations in the mitochondrial *tRNA^{Lys}* gene. Moreover, the proportion of mutated mtDNA varied markedly among the pups born to each dam, suggesting that selecting oocytes with high proportions of normal mtDNA from affected mothers with *tRNA^{Lys}*-based mitochondrial diseases may be effective as a primary prevention for obtaining unaffected children.

mutated mtDNA segregation | mtDNA heteroplasmic mutation | preimplantation genetic diagnosis | selection of oocytes

Mitochondrial DNA (mtDNA) carrying a large-scale deletion (ΔmtDNA) and single-point mutations in the *tRNA^{Lys}* gene and in the *tRNA$^{Leu(UUR)}$* gene causes chronic progressive external ophthalmoplegia (CPEO); myoclonic epilepsy with ragged-red fibers (MERRF); and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), respectively—the three most prevalent mitochondrial diseases (1–3). However, there are slight differences among the three disease phenotypes, even though these pathogenic mtDNA mutations all induce mitochondrial respiration defects. Considering that mitochondrial respiratory function is controlled by both mitochondrial and nuclear genomes (1–3), this controversial issue can be clarified by generating transmitochondrial mice (mito-mice) that share the same nuclear genetic background but carry different pathogenic mtDNA mutations corresponding to the mutations found in the three prevalent mitochondrial diseases. However, no well-established, effective protocols are available for introducing mutagenized mtDNA into the mitochondria of mammalian cells.

In our previous studies (4–8) we found mtDNAs carrying pathogenic mutations in mouse cell lines, transferred them into mouse female germ lines, and generated several types of mito-mice, including mito-mice-Δ (4, 5) which harbor ΔmtDNA and therefore are disease models for CPEO. However, mito-mice harboring mtDNA with pathogenic mutations in the *tRNA^{Lys}* and *tRNA$^{Leu(UUR)}$* genes—and therefore prospective disease models of MERRF and MELAS, respectively—have not previously been established owing to the unavailability of mouse cell lines with corresponding *tRNA* mutations in mtDNA.

To complement the paucity of effective technologies required for introducing mutagenized mtDNA into mitochondria of living mouse cells, we developed an alternative strategy involving cloning and sequence analysis to detect small amounts of mtDNA with somatic mutations in the mitochondrial *tRNA* genes. Because pathogenic mutations responsible for mitochondrial diseases occur preferentially in the *tRNA$^{Leu(UUR)}$* and *tRNA^{Lys}* genes of humans (1–3), we sequenced 43 clones generated from PCR products carrying the mitochondrial *tRNA$^{Leu(UUR)}$* and *tRNA^{Lys}* genes of P29 mouse lung carcinoma cells (9). One of the 43 clones had a somatic G7731A mutation in *tRNA^{Lys}*, which enabled the generation of transmitochondrial mito-mice expressing respiration defects for their use as models for diseases caused by mutations in the mitochondrial *tRNA^{Lys}* gene.

Results

Cloning and Sequence Analysis of PCR Products Including *tRNA* Genes. To detect the small proportion of mtDNA with somatic and possibly pathogenic mutations in the *tRNA$^{Leu(UUR)}$* and *tRNA^{Lys}* genes, we used two sets of primers so that the resulting PCR products would include the *tRNA$^{Leu(UUR)}$* or *tRNA^{Lys}* gene of P29

Significance

We generated transmitochondrial mito-mice-tRNA^{Lys7731} as models for precise examination of the pathogenesis and transmission profiles of mtDNA mutations in the *tRNA^{Lys}* genes and have obtained important information regarding primary prevention of the diseases caused by the mtDNA mutations. Although nuclear transplantation from oocytes of affected mothers into enucleated oocytes of unrelated women has been suggested, the methodology carries the technical risk of inducing nuclear abnormalities and prompts ethical concerns regarding the production of three-parent babies with normal mtDNA from unrelated oocyte donors. The current study suggests that the selection of oocytes with high proportions of normal mtDNA from affected mothers can be used to avoid these issues and therefore provides insights into mitochondrial genetics and medicine.

Author contributions: A.S., K.N., and J.-I.H. designed research; A.S., T.M., C.H., E.O., R.K., and I.N. performed research; K.T. contributed new reagents/analytic tools; A.S., T.M., and E.O. analyzed data; and J.-I.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. L.S. is a guest editor invited by the Editorial Board.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AP014540 and AP014541).

[1]To whom correspondence should be addressed. E-mail: jih45@biol.tsukuba.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318109111/-/DCSupplemental.