

## <Special Article>

# 孤発性パーキンソン病の分子病態機序はどこまで解明されたか

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### 要 目

- 孤発性パーキンソン病の発症機序としては、ミトコンドリア呼吸系酵素の障害、炎症反応、酸化ストレス障害が一因とされてきたが、遺伝性パーキンソン病家系の解析などから6つのメンデル遺伝性原因遺伝子が明らかにされ、蛋白分解異常の重要性が示された。
- ゲノムワイド関連解析による新たな疾患感受性遺伝子の同定や、コーシェ病変異も rare variant として重要である。
- $\alpha$ -シヌクレインのミスフォールド蛋白質が近隣の神経細胞に伝播していくシヌクレインプリオン仮説も提唱されている。
- ミクログリアの活性化を伴う継続的な炎症状態も注目される。

### はじめに

パーキンソン病(PD)は、臨床的には、振戦、筋固縮、寡動、姿勢反射障害を主徴とし、認知症、自律神経障害などのさまざまな随伴症状を呈する神経変性疾患である。わが国には16万人以上の患者が存在するが、今後社会の高齢化に伴いさらなる患者数増加が予想されている。一部は遺伝性であるが、患者の約95%は孤発性であり、本稿では孤発性PDの分子病態機序について、現在主流となっている仮説に加え、最近のトピックスも含めて概観する。

### 孤発性PDは多因子遺伝性疾患である

症例的には大多数(90%以上)の孤発性PDの原因は、現時点では不明であるが、加齢および複

数の影響力の弱い遺伝因子(おそらく数十個からなる)と環境要因の組み合わせにより発症する多因子遺伝性疾患と考えられている。Fig. 1に示すように各遺伝要因と環境要因の総和が閾値を超えたときに発症すると考えられている(Fig. 1)。遺伝要因が関係するということの根拠は、①患者の約10%に家族内発症がみられる、②患者の同胞における有病率の一般集団の有病率に対する比( $\lambda_s$ )は6.7(アイスランドの報告)、③一卵性双生児での疾患一致率(55%)が二卵性双生児での一致率(18%)の約3倍であった、ということから示唆されている。

一方、PD症例の90%以上は孤発性発症であるが、5~10%は家族性(その一部はメンデル遺伝性)に発症する。メンデル遺伝性PD家系の連鎖解析などから、6つのメンデル遺伝性PD原因遺伝子( $\alpha$ -シヌクレイン、パーキン、*LRRK2* 遺伝子

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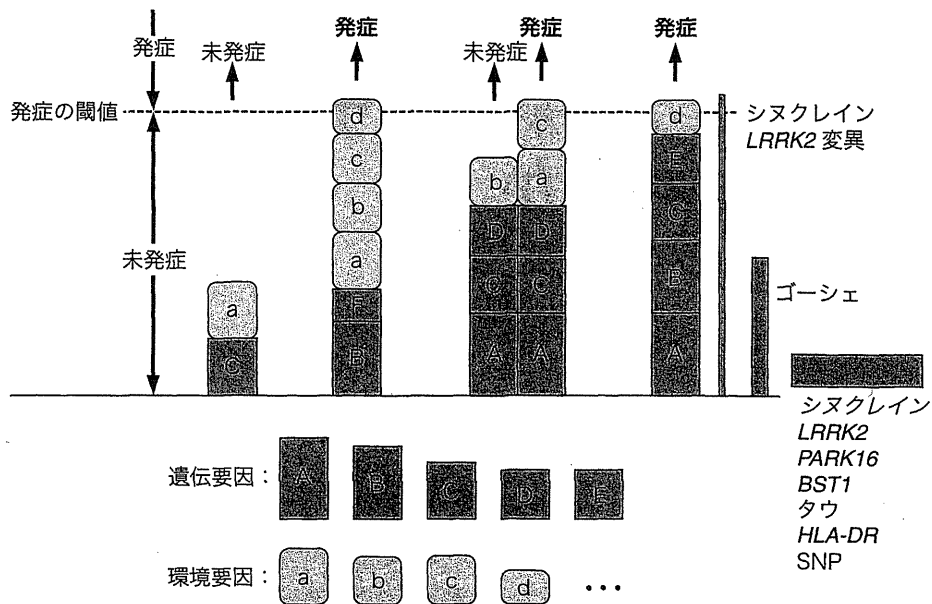


Fig. 1. 孤発性 PD は多因子遺伝性疾患

PD, アルツハイマー病, または生活習慣病を含むほとんどの疾患は, 複数の遺伝要因と複数の環境要因の積み木の総和が, ある閾値を超えたとき発症すると考えられている. メンデル遺伝性変異以外に, common variant として  $\alpha$ -シヌクレイン, PARK16, BST1, LRRK2, タウ, HLA-DR の SNP が, また rare variant としてゴーシェ病遺伝子が重要である.

など)が明らかにされた. これまでにレビー小体の主要構成成分である  $\alpha$ -シヌクレインのほか, プロテアソーム系に関係する遺伝子(パーキン, UCH-L1), ミトコンドリアに関係する遺伝子(PINK1, DJ-1), 酸化ストレスに関係する遺伝子(DJ-1)などが同定されている. 孤発性 PD, メンデル遺伝性 PD とも, 一部共通の発症メカニズムが存在していると考えられ, それらを切り口にして孤発性 PD の病態解明が進んでいる<sup>1)</sup>.

### メンデル遺伝性 PARK 遺伝子の孤発性 PD 病態における立ち位置

1) PARK1, PARK4( $\alpha$ -シヌクレイン)について→1997年に PARK1( $\alpha$ -シヌクレイン)遺伝子が同定されたのを皮切りに, 次々に PARK 遺伝子の同定が進むと, そのほとんどが孤発性 PD の病態メカニズムにも関係する蛋白であることが判明した(Table 1, Fig. 2)<sup>1)</sup>. もっともインパクトが強かったのは, PARK1 遺伝子産物  $\alpha$ -シヌクレイン

蛋白が, メンデル遺伝性 PD, 孤発性 PD 両方のレビー小体の主要成分であったという報告である.  $\alpha$ -シヌクレインはメンデル遺伝性 PD, 孤発性 PD の病態に共通した分子であることにより,  $\alpha$ -シヌクレイン変異の解析は, 孤発性 PD のモデルにもなると考えられる. アミノ酸変異をもつ  $\alpha$ -シヌクレインは *in vitro* で重合化が促進することが知られており, 野生型  $\alpha$ -シヌクレイン蛋白もリン酸化やニトロ化を受けて患者脳で蓄積していることが報告されている<sup>2)</sup>. また, PARK4 が, 野生型  $\alpha$ -シヌクレインを含む 1.6~2.0 Mb の三重重複であったことが報告され, 正常  $\alpha$ -シヌクレインの mRNA, 蛋白発現レベルが増加すれば発症にいたることが示された.

現在,  $\alpha$ -シヌクレイン蛋白は生理的には可溶性単量体(monomer)として存在し, 特定の構造をとらないが, 種々の要因で重合化, 不溶化, 凝集する過程で何らかの神経毒性をもたらすと考えられている. そして, 孤発例では,  $\alpha$ -シヌクレインの

Table 1. 孤発性 PD の機序に関する主な仮説とエビデンス

機序	エビデンス
ミトコンドリア機能障害	患者にミトコンドリア機能低下 パーキンソン様症状を起こす神経毒(MPTP, rotenone など)はミトコンドリア機能を障害 ミトコンドリア機能に関係する遺伝子がメンデル遺伝型家系で同定(PINK1, DJ-1 など)
プロテアソーム機能低下	プロテアソーム機能に関係する遺伝子がメンデル遺伝型家系で同定(パーキン, UCH-L1 など) PD 動物モデルでプロテアソーム活性低下
酸化ストレス	患者で酸化ストレスマーカーの増加 パーキンソン様症状を起こす神経毒(MPTP, rotenone, 6-OHDA など)の作用過程でフリーラジカル産生 メンデル遺伝型家系遺伝子の1つ DJ-1 は酸化ストレス防御作用
$\alpha$ -シヌクレイン蓄積	レビー小体の主成分は $\alpha$ -シヌクレイン メンデル遺伝型家系でみられるアミノ酸変異をもつ合成蛋白の凝集性促進 メンデル遺伝型の正常 $\alpha$ -シヌクレイン遺伝子重複家系で、蛋白・遺伝子発現レベルが上昇 培養細胞、動物モデルで、封入体を伴った神経細胞死

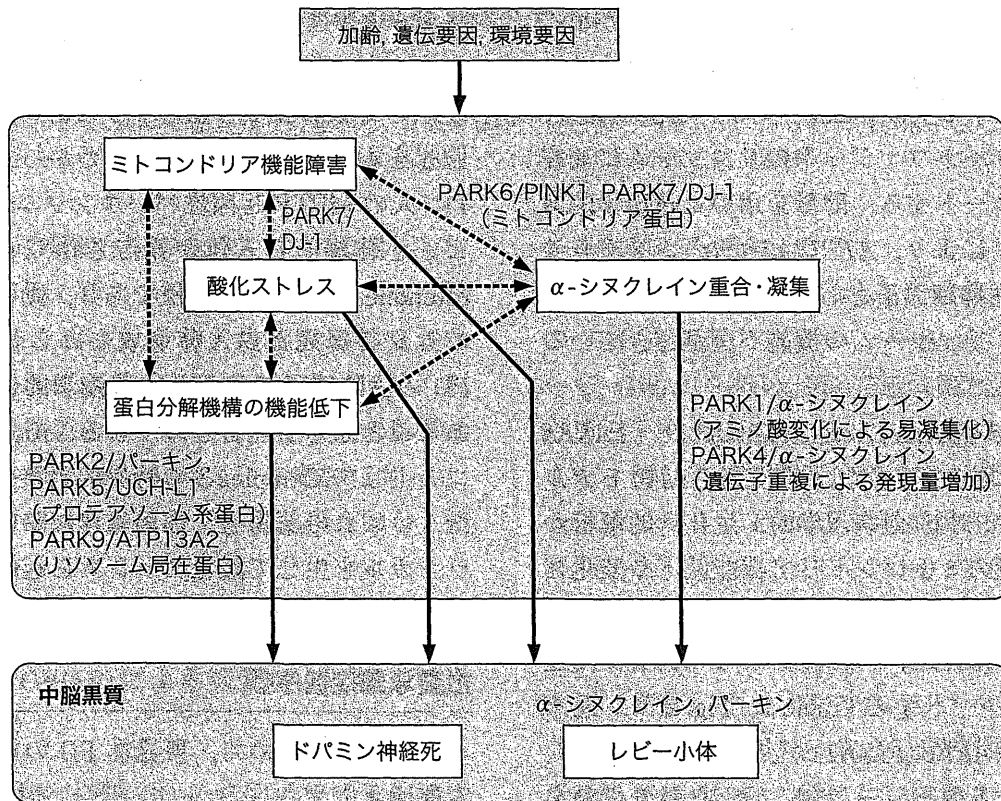


Fig. 2. 孤発性 PD 発症機序と PARK 遺伝子

重合が促進されるような、変異以外の要因が働いて、発症にいたると考えられている<sup>3)</sup>。その際には $\alpha$ -シヌクレインが小胞体-ゴルジ体の小胞輸送を障害し、神経変性を起こすことが報告されている<sup>4)</sup>。

2) PARK2 (パーキン), PARK9 (ATP13A2) について→孤発性 PD の病理では、レビー小体がユビキチン抗体で染まる、脳でオートファジーの亢進がみられる、という報告がある。このことと、蛋白分解機構に関する PARK 遺伝子の存在によ

り、プロテアソーム系、オートファジー系の蛋白分解機構の破綻がPDの病態に関係するという仮説がたてられた(Table 1, Fig. 2)<sup>5,6)</sup>。PARK2(パーキン)蛋白の機能については、ユビキチン・プロテアソーム系蛋白分解において、基質にユビキチンを結合させるE3ユビキチンリガーゼであることが明らかにされた。ユビキチンリガーゼはプロテアソームにより分解を受けるべき蛋白質にユビキチンという目印をつける酵素であり、ユビキチン・プロテアソーム系の破綻によって本来分解される蛋白質(すなわちパーキンの基質)が蓄積することで神経変性を引き起こしていると考えられている。このパーキンの基質はさまざまな報告があるが、真の基質はどれであるか結論が出ていない。

PARK9(ATP13A2)は近年発見され、進行が早く核上性上方注視麻痺を呈するなど非典型的である。原因遺伝子はlysosomal type 5 P-type ATPaseと呼ばれ、オートファジー系のリソソームに局在する蛋白をコードする。酵母を用いた遺伝学的スクリーニングにより、ATP13A2が $\alpha$ -シヌクレインの細胞毒性抑制因子として同定された<sup>7)</sup>。劣性遺伝であり、リソソームの機能不全が起きていると考えられる。

3) PARK6(PINK1), PARK7(DJ-1), PARK8(LRRK2)について→また、剖検脳や動物モデル(薬物性PDモデル)の病理学的・生化学的解析から、孤発例でのミトコンドリア機能低下や酸化ストレスマーカーの増加が報告されていた。PARK6(PINK1), PARK7(DJ-1)は、これらの機構に関連する蛋白をコードする(Table 1, Fig. 2)<sup>8)</sup>。近年パーキンとPARK6(PINK1)が共同してミトコンドリアのオートファジー(ミトファジー)の役割を担うことが明らかにされ、変異があると機能低下した異常ミトコンドリアの除去ができなくなると考えられる<sup>9)</sup>。PARK7の原因はDJ-1蛋白であり、ミトコンドリアに移行し抗酸化作用をもちドパミン細胞死との関係が注目される。またPARK8は常染色体優性遺伝形式を呈するものの中で最多であり、原因蛋白はLRRK2というキナーゼであり、

疾患型変異はキナーゼ活性の亢進をもたらすことから、そのリン酸化基質がPDにおいて重要な役割を担うと考えられる。シヌクレインに対するキナーゼ活性が注目されているが定かではない。

### 神経毒の解析●

PDの症状を引き起こす薬物として、MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), ロテノン(rotenone), 6-OHDAなどが知られており、これらの作用機序からも、病態を考察することができる。これらの神経毒のうち、病理学的にも孤発性PDと類似する所見を示すのが、MPTPとrotenoneである。MPTPは麻薬の合成過程の副産物であり、脳内でMPP<sup>+</sup>となりドパミントランスポーターを介してドパミン神経に取り込まれ、エネルギー産生場であるミトコンドリア電子伝達系のComplex Iを阻害することにより、神経細胞死をもたらすということがわかっている(Table 1)。人のMPTP起因性パーキンソンニズムでは、レビー小体様の封入体が報告されている。また、rotenoneは、農薬の一種であり、動物モデルでドパミン神経死と封入体形成がみられる。農薬がPDの危険因子であるという疫学的研究の報告があることから注目されている。rotenoneもミトコンドリアComplex Iの特異的阻害薬である。また、いずれの薬物も、作用過程において、フリーラジカルを生じて酸化ストレスを起こすことも知られている(Table 1)。

### 主要な発症機序●

これらをまとめて、孤発性PDの神経死にいたる機序として、①ミトコンドリア機能障害により、細胞内エネルギー産生が低下する、②蛋白分解機構(プロテアソーム系、オートファジー系など)機能低下により、神経細胞内に構造異常をきたした蛋白が貯まって、細胞毒性をもつ、③酸化ストレス(活性酸素物質: reactive oxygen species (ROS))が細胞毒性をもつ、などがさまざまな割合で関与していること、そして④ $\alpha$ -シヌクレイン

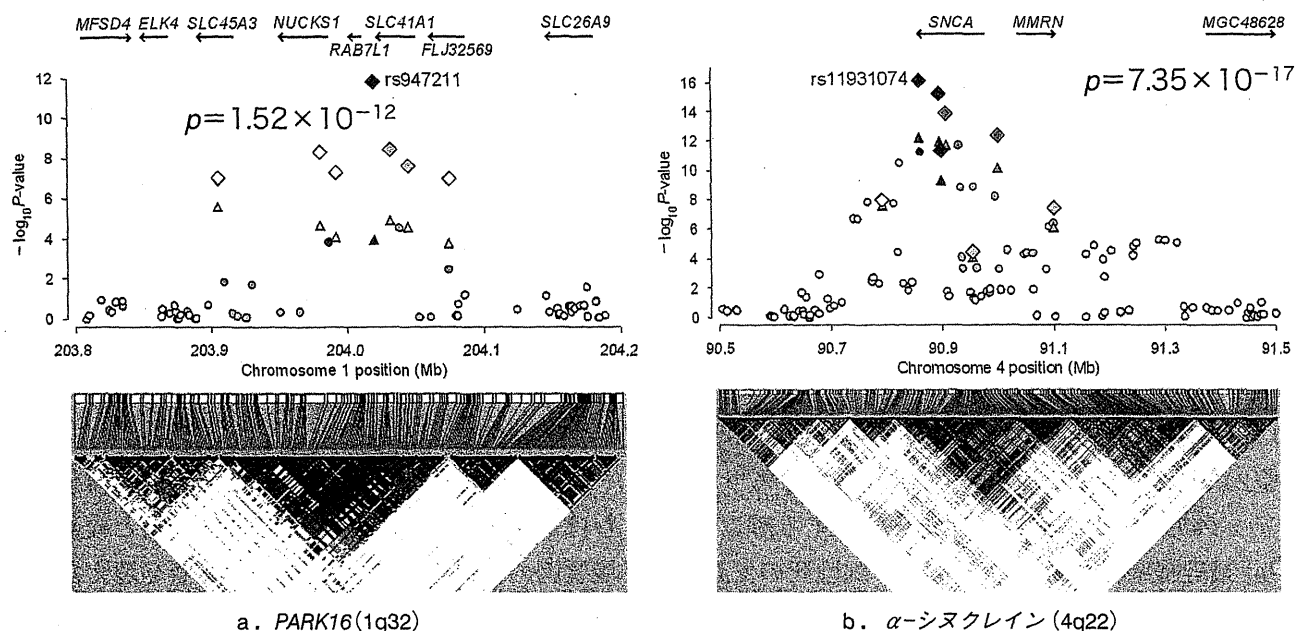


Fig. 3. 日本人 PD 患者 2011 検体, 対照 18381 検体のゲノムワイド関連解析より同定された 4 つの PD 遺伝子座の抜粋

このうち 2 つはまったく新規の領域であった。とくに (a) は非常に強い関連を示し、白人集団でも関連が再現されたことから *PARK16* と名づけた。また残りの 2 つは、常染色体優性遺伝性パーキンソン症の原因遺伝子  $\alpha$ -シヌクレイン (b) と *LRRK2* を含む領域が同定された。縦軸は  $-\log P$ , すなわち上に行くほど関連が強い。 [文献 10) より引用]

蛋白がこれらの経路あるいは別の経路に関与していることについては、あまり異論がない。さらに各機序の関与を裏づける報告も剖検脳の病理学的・生化学的解析から得られている (Table 1)。これらの機序は複数の遺伝要因や環境要因の影響のもとで、それぞれ、あるいは互いに影響しあって、孤発性 PD の病態を形成していくと現在のところ考えられている (Fig. 2)。

### ゲノムワイド関連解析 (Genomewide Association Study : GWAS) による孤発性 PD 感受性遺伝子の同定 ●

ここ 10 年間孤発性 PD の疾患感受性遺伝子の発見を目指した多くの研究がなされてきたが、アルツハイマー病における ApoE4 多型のような確実に発症リスクを高める遺伝因子はなかなか確認されていなかった。ゲノムワイド有意水準 ( $p < 5 \times 10^{-8}$ ) を満たす確実なものは、 $\alpha$ -シヌクレインの 3' 非翻訳領域 SNP (Gasser ら, 筆者らが同定) とゴーシェ病遺伝子 *GBA* の rare variant の 2 つ

の遺伝子のみであった。

われわれは、大規模の患者対照集団と、56 万個の SNP を搭載したイルミナ Hap550 アレイを用いて、GWAS を行い、PD 発症に関わる 2 つの新しい遺伝子座 *PARK16*; *BST1* を同定した (Fig. 3a)。また、常染色体優性遺伝性 PD の原因遺伝子  $\alpha$ -シヌクレイン、*LRRK2* の孤発性 PD への関与を証明した (Fig. 3b)。白人の PD の GWAS 研究を行っていたグループと共同し、 $\alpha$ -シヌクレイン・*PARK16*・*LRRK2* は 2 人種に共通の PD リスクであり、*BST1* のリスク多型の影響は人種特異的であることを示した<sup>10)</sup>。

また孤発性 PD の発症に、常染色体優性遺伝性 PD の原因遺伝子が密に関係していることを示した。原因変異により常染色体優性遺伝性 PD を引き起こす遺伝子が、その SNP を通して、孤発性 PD の遺伝リスクとなったことは興味深い。すなわち、 $\alpha$ -シヌクレイン、*LRRK2* が関与する病理経路が、孤発性を含めた PD 全体の病因の根幹をなすことを示している。一方、微小管の安定化に

働くタウは、アルツハイマー病、前頭側頭型認知症などの原因産物として知られており、PD と他神経変性疾患との共通の病理経路の存在をも示唆している<sup>10)</sup>。

*PARK16* 領域には、3つの遺伝子が存在するが、発現量的形質座解析から、*NUCKS1* が、もっとも有力な責任遺伝子であると考えた (Fig. 3a)。*NUCKS1* は、リン酸化部位を含む核蛋白であるが、神経系における機能は、未知であり、新たなPD発症病態を開拓する可能性がある。*BST1* は、細胞内  $Ca^{2+}$  貯蔵からの  $Ca^{2+}$  放出を誘発するサイクリック ADP リボースの形成を触媒する酵素であり、最近提唱されているドパミン細胞死の  $Ca^{2+}$  ストレス説を想起させ、興味深い。これらは従来のPD病態説からはまったく新規な遺伝子であり、従来説にとらわれず、新規なものを同定できるところにGWASの強みがある。

さらに最近別グループのGWASから、HLA-DR抗原の領域との関連が報告された。PD脳ではDR抗原陽性のミクログリア(後述)が検出されること、非ステロイド抗炎症薬(NSAIDs)は疫学的にPDのリスクを減少させることなどもあり、PDと炎症の関係を示しており興味深い<sup>11)</sup>。

### PDとゴーシェ病とまれな多型(rare variant)●

リピドーシスの常染色体劣性遺伝のユダヤ人ゴーシェ病家系内にPD患者が多いことから、PDでは*GBA* (glucocerebrosidase, 1q21) 変異のヘテロ保因者が有意に多いことが報告された<sup>12)</sup>。東京大学神経内科とわれわれの共同研究グループは、*GBA* 遺伝子の全11エクソンとその近傍をPD患者534人、対照544人リシーケンスして塩基配列変化の有無を調べ、11種類の疾患原性点変異が同定された。ヘテロでもつ保因者はPD患者534人中50人(9.4%)、対照544人中2人(0.37%)であり、PDと*GBA* 変異は強く関連していた( $p = 6.9 \times 10^{-14}$ , オッズ比28.0)<sup>12)</sup>。

さらに全世界での米国人、フランス人、ポルトガル人、台湾人など計約10,000人の患者対照集団

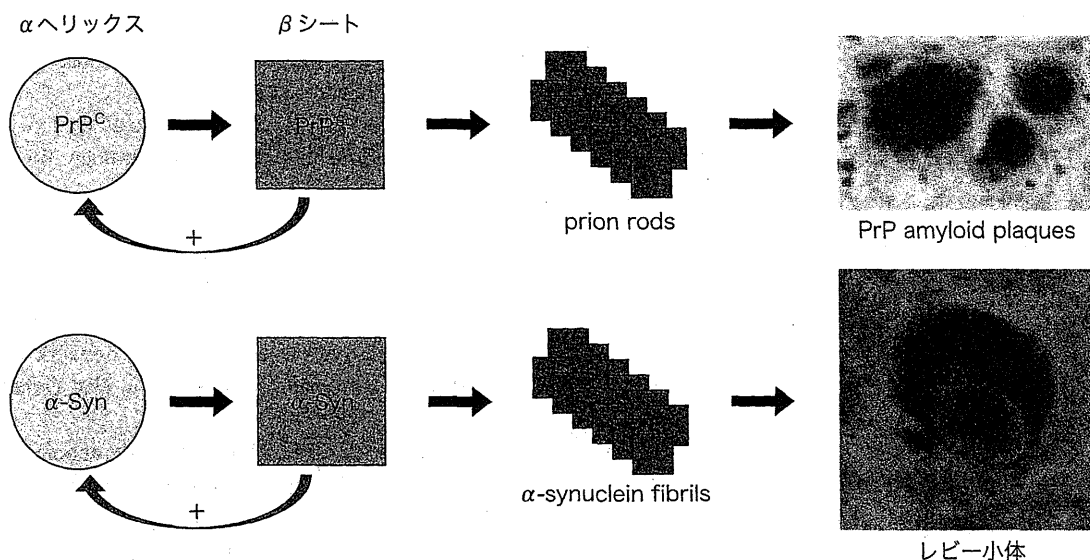
のメタ解析により、どの人種にても*GBA* 遺伝子はリスクとなり平均オッズ比は5である<sup>13)</sup>。*GBA* 変異は確実なPDリスク因子であり、SNPによるCommon Disease-Common Variants 仮説と異なり、Common Disease-Multiple Rare Variant 仮説によるものである。すなわち、頻度は低いが発症へのeffect sizeが大きい。なぜリスクとなるかの原因は不明であるが、単なる酵素活性低下でなく、*GBA* 変異の蛋白分解機構への負担によることも推定されている。

PD、アルツハイマー病、または生活習慣病を含むほとんどの疾患は、複数の遺伝要因と複数の環境要因の積み木の総和が、ある閾値を超えたとき発症すると考えられている。これまでみてきたように $\alpha$ -シヌクレインや*LRRK2* の変異はそれ1つだけで閾値に到達し発症するが、対象患者はほとんど存在しないので積み木の幅はとても狭い。ゴーシェ病遺伝子などのrare variantは中等度の高さをもつが10%以下の患者にしか当てはまらないため幅は狭い。一方SNPは、それ自体のオッズは低いが発症にほとんど患者に当てはまるため、積み木の幅は広い。いずれも重要である (Fig. 1)。

### シヌクレインプリオン仮説●

本特集別稿を参照されたいが、Braakらは $\alpha$ -シヌクレインの蓄積部位を多数例のPD脳で詳細に検討し、この蛋白質の異常蓄積はまず嗅球、延髄から始まり、徐々に上行して橋、中脳に及び、最終的に大脳皮質にいたるという仮説を提唱し、Braak仮説として有名である。

一方、PDの治療を目的として胎児の中脳腹側組織を移植し10年以上経過した患者脳を病理学的に検索したところ、驚くべきことに移植された神経細胞にレビー小体が認められた<sup>14)</sup>。また $\alpha$ -シヌクレインを高発現する神経細胞とマウスの幹細胞を共培養する*in vitro* 実験や、 $\alpha$ -シヌクレインを発現するトランスジェニックマウスの海馬に幹細胞を移植する*in vivo* 実験にて、いずれの場合にも幹細胞の細胞質に $\alpha$ -シヌクレインの凝集体が



未知の環境要因→嗅覚器や腸管(外界)→自律神経叢や嗅球で $\alpha$ -シヌクレインのミスフォールディング→放出伝播?

Fig. 4. PDのシヌクレインプリオンモデル

[文献 16)より引用]

形成されること、 $\alpha$ -シヌクレインがエンドサイトーシスによって幹細胞に取り込まれることが示された。これらの知見は、 $\alpha$ -シヌクレインが近接する神経細胞に「伝染」する可能性を示している。またごく最近、細胞内の $\alpha$ -シヌクレインが、他の細胞に移動し凝集し、その凝集体の量が継時的に増加すること、 $\alpha$ -シヌクレイン蛋白の細胞内取り込みに関してエンドサイトーシス阻害薬の同時注入により、取り込みの減少が明らかとなった<sup>15)</sup>。

$\alpha$ -シヌクレインもプリオン蛋白質と同様に、 $\alpha$ ヘリックス構造と $\beta$ シート構造をとりうることから、何らかのきっかけで $\beta$ シート化した $\alpha$ -シヌクレインがシードとなって凝集を開始するとともに、細胞外に放出されて近隣の細胞に取り込まれ、プリオン病と同様の機序で病変が進展するという、PDのプリオンモデルが提唱されている(Fig. 4)。さらにこのモデルと Braak 仮説を合わせてPDの発症機序として、未知の環境要因が嗅覚器や腸管など外界に接した神経組織に作用し、自律神経叢や嗅球において最初に $\alpha$ -シヌクレインのミスフォールド蛋白質が形成され、逆行性に細

胞体まで輸送され、中枢神経内で放出されて近隣の神経細胞に連続的に伝播していく、という大胆な仮説もある(Fig. 4)<sup>16)</sup>。中枢神経内における $\alpha$ -シヌクレインの伝播を防ぐことで(例:エンドサイトーシス阻害薬)PDの進行を抑制するという、新たな治療戦略が考えられる。

#### ミクログリアと炎症反応●

孤発性PD患者剖検脳や家族性PD患者剖検脳、さらにMPTP誘導性PDモデル動物脳の黒質線条体部において共通にみられる病理変化として、ミクログリアの活性化と集積がある。ミクログリアは活性化するとHLA-DRやiNOSやCOX-2を発現し、インターロイキン(IL)-1 $\beta$ やIL-6などの炎症性サイトカインを含む種々のサイトカインを産生し、炎症反応の特徴の多くを示す。また、活性化したミクログリアは炎症性サイトカイン以外に、活性酸素やNO、プロテアーゼ類などの神経傷害性因子も産生する。とくに活性酸素やNOなどのフリーラジカルはPDにおける神経変性の進行機序に関与するため、ミクログリ



アの活性化や脳内炎症反応は PD の発症や進行に密接に関わっている<sup>17)</sup>。MPTP 曝露 16 年後の死後、脳においても活性化ミクログリアが存在し、ミクログリアの活性化を伴う長期間にわたる継続的な炎症状態が PD の病態の進行において重要な意味をもつ可能性を示唆している。GWAS で HLA-DR 抗原の領域が同定されたことも興味深い<sup>11)</sup>。

### おわりに

PD のメカニズムの解明にあたっては、メンデル遺伝型 PD の原因遺伝子の研究成果が大きく貢献してきた。今後は未知のメンデル遺伝型遺伝子の同定のみならず、孤発性 PD において大規模な GWAS, rare variant 解析から、新規の感受性遺伝子が同定され、新しい機序、創薬の手がかりがみつかる可能性がある。次世代シーケンサーによるエクソームリシーケンスも開始され、今後はパーソナルゲノム解析が医学研究に応用されよう。

また、プリオン仮説は異常蛋白凝集を特徴とする神経変性疾患に共通した病態機序である可能性がある。同時に、異常蛋白の細胞間伝播は神経変性疾患に共通の治療ターゲットとなる可能性を秘めており、今後に期待する。

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## Mitochondrial membrane potential decrease caused by loss of PINK1 is not due to proton leak, but to respiratory chain defects

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### ABSTRACT

Mutations in *PTEN-induced putative kinase 1* (*PINK1*) cause a recessive form of Parkinson's disease (PD). *PINK1* is associated with mitochondrial quality control and its partial knock-down induces mitochondrial dysfunction including decreased membrane potential and increased vulnerability against mitochondrial toxins, but the exact function of *PINK1* in mitochondria has not been investigated using cells with null expression of *PINK1*. Here, we show that loss of *PINK1* caused mitochondrial dysfunction. In *PINK1*-deficient (*PINK1*<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs), mitochondrial membrane potential and cellular ATP levels were decreased compared with those in littermate wild-type MEFs. However, mitochondrial proton leak, which reduces membrane potential in the absence of ATP synthesis, was not altered by loss of *PINK1*. Instead, activity of the respiratory chain, which produces the membrane potential by oxidizing substrates using oxygen, declined. H<sub>2</sub>O<sub>2</sub> production rate by *PINK1*<sup>-/-</sup> mitochondria was lower than *PINK1*<sup>+/+</sup> mitochondria as a consequence of decreased oxygen consumption rate, while the proportion (H<sub>2</sub>O<sub>2</sub> production rate per oxygen consumption rate) was higher. These results suggest that mitochondrial dysfunctions in PD pathogenesis are caused not by proton leak, but by respiratory chain defects.

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### Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. Mitochondrial dysfunction has been proposed as a major factor in the pathogenesis of sporadic and familial PD (Abou-Sleiman et al., 2006). In particular, the identification of mutations in *PTEN-induced putative kinase 1* (*PINK1*) has strongly implicated mitochondrial dysfunction owing to its loss of function in the pathogenesis of PD (Valente et al., 2004). *PINK1* contains an N-terminal mitochondrial targeting sequence (MTS) and a serine/threonine kinase domain (Valente et al., 2004). *PINK1* kinase activity is crucial for mitochondrial maintenance via TRAP

phosphorylation (Pridgeon et al., 2007). Loss of *PINK1* function induces increased vulnerability to various stresses (Exner et al., 2007; Haque et al., 2008; Pridgeon et al., 2007; Wood-Kaczmar et al., 2008). However, silencing of *PINK1* has only been partial and only one study has been performed to assess mitochondrial functions in steady and artificial states with complete ablation of *PINK1* expression (Gautier et al., 2008).

Several studies have shown that *PINK1* acts upstream of parkin in the same genetic pathway (Clark et al., 2006; Park et al., 2006) and co-overexpressed *PINK1* and parkin both co-localized to mitochondria (Kim et al., 2008). Overexpression of *PINK1* promotes mitochondrial fission (Yang et al., 2008). Fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy (Twig et al., 2008). *PINK1* loss-of-function decreases mitochondrial membrane potential (Chu, 2010) and the *PINK1*-parkin pathway is associated with mitochondrial elimination in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization (Geisler et al., 2010; Kawajiri et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010; Vives-Bauza et al., 2010). However, the exact mechanism underlying the mitochondrial depolarization induced by *PINK1* defects leading to mitochondrial autophagy has not been examined in detail.

**Abbreviations:**  $\Delta\psi$ , mitochondrial membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MEFs, mouse embryonic fibroblasts; PD, Parkinson's disease; *PINK1*, *PTEN*-induced putative kinase 1; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; TPMP, triphenylmethylphosphonium.

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Here, we describe a detailed characterization of mitochondria in PINK1-deficient cells. We show that PINK1 deficiency causes a decrease in mitochondrial membrane potential, which is not due to proton leak, but to respiratory chain defects.

## Materials and methods

### *PINK1 knock-out mouse embryonic fibroblasts (MEFs)*

PINK1 knock-out MEFs were prepared and cultured as described previously (Matsuda et al., 2010). Mouse embryonic fibroblasts (MEFs) were derived from E12.5 embryos containing littermate 4 mice of each genotype. Embryos were mechanically dispersed by repeated passage through a P1000 pipette tip and plated with MEF media containing DME, 10% FCS, 1× nonessential amino acids, 1 mM L-glutamine, penicillin/streptomycin (invitrogen). The  $\psi$ 2 cell line, an ecotropic retrovirus packaging cell line, was maintained in Dulbecco's modified Eagle medium (DMEM, Sigma) with 5% fetal bovine serum and 50  $\mu$ g/ml kanamycin. Transfection of the  $\psi$ 2 cells with pMESVTS plasmids containing an SV40 large T antigen was performed by lipofection method according to the manual provided by the manufacturer (GIBCO BRL). Five micrograms of the plasmids was used for each transfection. Transfectants were selected by G418 at the concentration of 0.5 mg/ml, and 10 clonal cell lines were established. The highest titer of  $5 \times 10^4$  cfu/ml was obtained for the conditioned medium of a cell line designated  $\psi$ 2SVTS1.  $10^6$  MEFs were plated onto a 10-cm culture dish and kept at 33 °C for 48 hours. Then medium was replaced with 2 ml supplemented with polybrene-supplemented medium conditioned by the  $\psi$ 2SVTS1 cells at confluency for 3 days. Infection was continued for 3 hours, and the medium was replaced with a fresh one. The infected MEFs were cultured at 33 °C until immortalized cells were obtained.

We confirmed that the differences we detected in this study were due to the PINK1 deficiency, not to artificial effects by immortalization, by measuring cellular respiration rates of not immortalized MEFs from other littermates (Supplemental figure). The respiration rates of not immortalized MEFs were slightly slower than those of immortalized MEFs, but the differences between PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs were consistent (Fig. 2A).

### *Cell growth*

Cells were seeded in 12-well plates at density of  $3\text{--}6 \times 10^3$  cells/well and incubated in DMEM high glucose medium (4.5 g/l glucose and 1 mM sodium pyruvate) supplemented with 10% fetal bovine serum. After a day, the medium was replaced with DMEM glucose-free medium supplemented with 1 g/l galactose, 1 mM sodium pyruvate and 10% fetal bovine serum (DMEM galactose medium) at 37 °C in an incubator with a humidified atmosphere of 5% CO<sub>2</sub>. Cells were trypsinized and live cells were assessed by trypan blue dye exclusion.

### *Mitochondrial morphological changes*

Cells were seeded in 6-well plates at  $2.0 \times 10^5$ /well and incubated in DMEM high glucose medium (4.5 g/l glucose and 1 mM sodium pyruvate) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. After a day, the medium was replaced with DMEM glucose-free medium supplemented with 1 g/l galactose, 1 mM sodium pyruvate and 10% fetal bovine serum (DMEM galactose medium) at 37 °C in an incubator with a humidified atmosphere of 5% CO<sub>2</sub>. 24 hours later, cells were fixed and immunostained with anti-Tom20 antibody to visualize mitochondria according to a protocol as previously described (Kawajiri et al., 2010). All images were obtained using an Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany).

### *Cellular ATP levels*

Intracellular ATP levels were determined by a cellular ATP assay kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer's instructions using a Lumat LB9507 luminometer (Berthold Technology, Bad Wildbad, Germany).

### *Membrane potential*

Fluorescence images were recorded using a multi-dimensional imaging workstation (AS MDW, Leica Microsystems, Wetzlar, Germany) with a climate chamber maintained at 37 °C. Fluorescence was quantified with a CCD camera (CoolSnap HQ, Roper Scientific, Princeton, NJ) using a 20× objective. Cells were stained for 1 hour with a non-quenching concentration (20 nM) of tetramethylrhodamine methyl ester (TMRM) in a 96-well plate. The cell-permeable cationic dye TMRM accumulates in mitochondria according to the Nernst equation. Nuclei were stained with 250 nM Hoechst 34580. Mitochondrial TMRM fluorescence was integrated in a 40- $\mu$ m diameter circular area around the nucleus, and the minimum fluorescence in this area was subtracted as background fluorescence.

### *Cell respiration*

Cell respiration was measured at 37 °C using the Oxygen Meter Model 781 and the Mitocell MT200 closed respiratory chamber (Strathkelvin Instruments, North Lanarkshire, United Kingdom). Cells were cultured in DMEM with 4.5 g/l of glucose supplemented with 10% FBS. Cells were then trypsinized and resuspended in Leibovitz's L-15 medium (Invitrogen) at density of  $8.0 \times 10^6$  cells/ml. The oxygen respiration rate was measured under each of the following three conditions: basal rate (no additions); State 4 (no ATP synthesis) [after addition of 1  $\mu$ g/ml oligomycin (Sigma)], uncoupled [after addition of 3  $\mu$ M FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Sigma)] using Strathkelvin 949 Oxygen System. After sequential measurements, the endogenous respiration rate was determined by adding 1  $\mu$ M rotenone + 2  $\mu$ M myxothiazol.

### *Mitochondrial respiration and membrane potential*

Mitochondria were prepared from cultured MEFs as previously described (Amo and Brand, 2007). Mitochondrial oxygen consumption with 5 mM succinate as a respiratory substrate was measured at 37 °C using a Clark electrode (Rank Brothers, Cambridge, United Kingdom) calibrated with air-saturated respiration buffer comprising 0.115 M KCl, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM HEPES (pH 7.2), 2 mM MgCl<sub>2</sub>, 1 mM EGTA and 0.3% (w/v) defatted BSA, assumed to contain 406 nmol atomic oxygen/ml (Reynafarje et al., 1985). Mitochondrial membrane potential ( $\Delta\psi$ ) was measured simultaneously with respiratory activity using an electrode sensitive to the lipophilic cation TPMP<sup>+</sup> (triphenylmethylphosphonium) (Brand, 1995). Mitochondria were incubated at 0.5 mg/ml in the presence of 80 ng/ml nigericin (to collapse the pH gradient so that the proton motive force was expressed exclusively as  $\Delta\psi$ ) and 2  $\mu$ M rotenone (to inhibit complex I). The TPMP<sup>+</sup>-sensitive electrode was calibrated with sequential additions of TPMP<sup>+</sup> up to 2  $\mu$ M, then 5 mM succinate was added to initiate respiration. Experiments were terminated with 2  $\mu$ M FCCP, allowing correction for any small baseline drift.  $\Delta\psi$  was calculated from the distribution of TPMP<sup>+</sup> across the mitochondrial inner membrane using a binding correction factor of 0.35 mg protein/ $\mu$ l. Respiratory rates with 4 mM pyruvate + 1 mM malate as a substrate in State 3 (with 0.25 mM ADP) and State 4 (with 1  $\mu$ g/ml oligomycin) were determined using the Oxygen Meter Model 781 and the Mitocell MT200 closed respiratory chamber (Strathkelvin Instruments).

## Modular kinetic analysis

To investigate differences in oxidative phosphorylation caused by PINK1 knock-out, we applied a systems approach, namely modular kinetic analysis (Amo and Brand, 2007; Brand, 1990). This analyzes the kinetics of the whole of oxidative phosphorylation divided into three modules connected by their common substrate or product,  $\Delta\psi$ . The modules are (i) the reactions that produce  $\Delta\psi$ , consisting of the substrate translocases, dehydrogenases and other enzymes and the components of the respiratory chain, called 'substrate oxidation'; (ii) the reactions that consume  $\Delta\psi$  and synthesize, export and dephosphorylate ATP, consisting of ATP synthase, the phosphate and adenine nucleotide translocases and any ATPases that may be present, called the 'phosphorylating system'; and (iii) the reactions that consume  $\Delta\psi$  without ATP synthesis, called the 'proton leak' (Brand, 1990). The analysis reports changes anywhere within oxidative phosphorylation that are functionally important but is unresponsive to changes that have no functional consequences. Comparison of the kinetic responses of each of the three modules to  $\Delta\psi$  obtained using mitochondria isolated from PINK1<sup>+/+</sup> and PINK1<sup>-/-</sup> MEFs would reveal any effects of PINK1 on the kinetics of oxidative phosphorylation. Oxygen consumption and  $\Delta\psi$  were measured simultaneously using mitochondria incubated with 80 ng/ml nigericin and 4  $\mu$ M rotenone. Respiration was initiated by 5 mM succinate. The kinetic behavior of a ' $\Delta\psi$ -producer' can be established by specific modulation of a  $\Delta\psi$ -consumer and the kinetics of a consumer can be established by specific modulation of a  $\Delta\psi$ -producer (Brand, 1998). To measure the kinetic response of proton leak to  $\Delta\psi$ , the State 4 (non-phosphorylating) respiration of mitochondria in the presence of oligomycin (0.8  $\mu$ g/ml; to prevent any residual ATP synthesis), which was used solely to drive the proton leak, was titrated with malonate (up to 8 mM). In a similar way, State 4 respiration was titrated by FCCP (up to 1  $\mu$ M) for measurement of the kinetic response of substrate oxidation to  $\Delta\psi$ . State 3 (maximal rate of ATP synthesis) was obtained by addition of excess ADP (1 mM). Titration of State 3 respiration with malonate (up to 1.1 mM) allowed measurement of the kinetics of the  $\Delta\psi$ -consumers (the sum of the phosphorylating system and proton leak). The coupling efficiencies of oxidative phosphorylation were calculated from the kinetic curves as the percentage of mitochondrial respiration rate at a given  $\Delta\psi$  that was used for ATP synthesis and was therefore inhibited by oligomycin. Note that any slip reactions will appear as proton leak in this analysis (Brand et al., 1994).

## Mitochondrial ROS production

Mitochondrial ROS production rate was assessed by measurement of H<sub>2</sub>O<sub>2</sub> generation rate, determined fluorometrically by measurement of oxidation of Amplex Red to fluorescent resorufin coupled to the enzymatic reduction of H<sub>2</sub>O<sub>2</sub> by horseradish peroxidase using a spectrofluorometer RF-5300PC (Shimadzu, Kyoto, Japan). The H<sub>2</sub>O<sub>2</sub> generation rate was measured in non-phosphorylating conditions (= State 4) using either pyruvate/malate or succinate as respiratory substrates. Mitochondria were incubated at 0.1 mg/ml in respiration buffer. All incubations also contained 5  $\mu$ M Amplex Red, 2 U/ml horseradish peroxidase and 8 U/ml superoxide dismutase. The reaction was initiated by addition of 5 mM succinate or 4 mM pyruvate + 1 mM malonate and the increase in fluorescence was followed at excitation and emission wavelengths of 560 and 590 nm, respectively. Appropriate correction for background signals and standard curves generated using known amounts of H<sub>2</sub>O<sub>2</sub> were used to calculate the rate of H<sub>2</sub>O<sub>2</sub> production in nmol/min/mg mitochondrial protein. The percentage free radical leak, which is a measure of the number of electrons that produce superoxide (and subsequently H<sub>2</sub>O<sub>2</sub>) compared with the total number of electrons which pass thorough the respiratory chain, was calculated as the rate of H<sub>2</sub>O<sub>2</sub> production divided by the rate of O<sub>2</sub> consumption (Barja et al., 1994).

## Statistics

Values are presented as means  $\pm$  SEM except Fig. 2D, in which error bars indicate SD. The significance of differences between means was assessed by the unpaired Student's *t*-test using Microsoft Excel; *P* values < 0.05 were taken to be significant.

## Results

### Cell growth and mitochondrial morphology

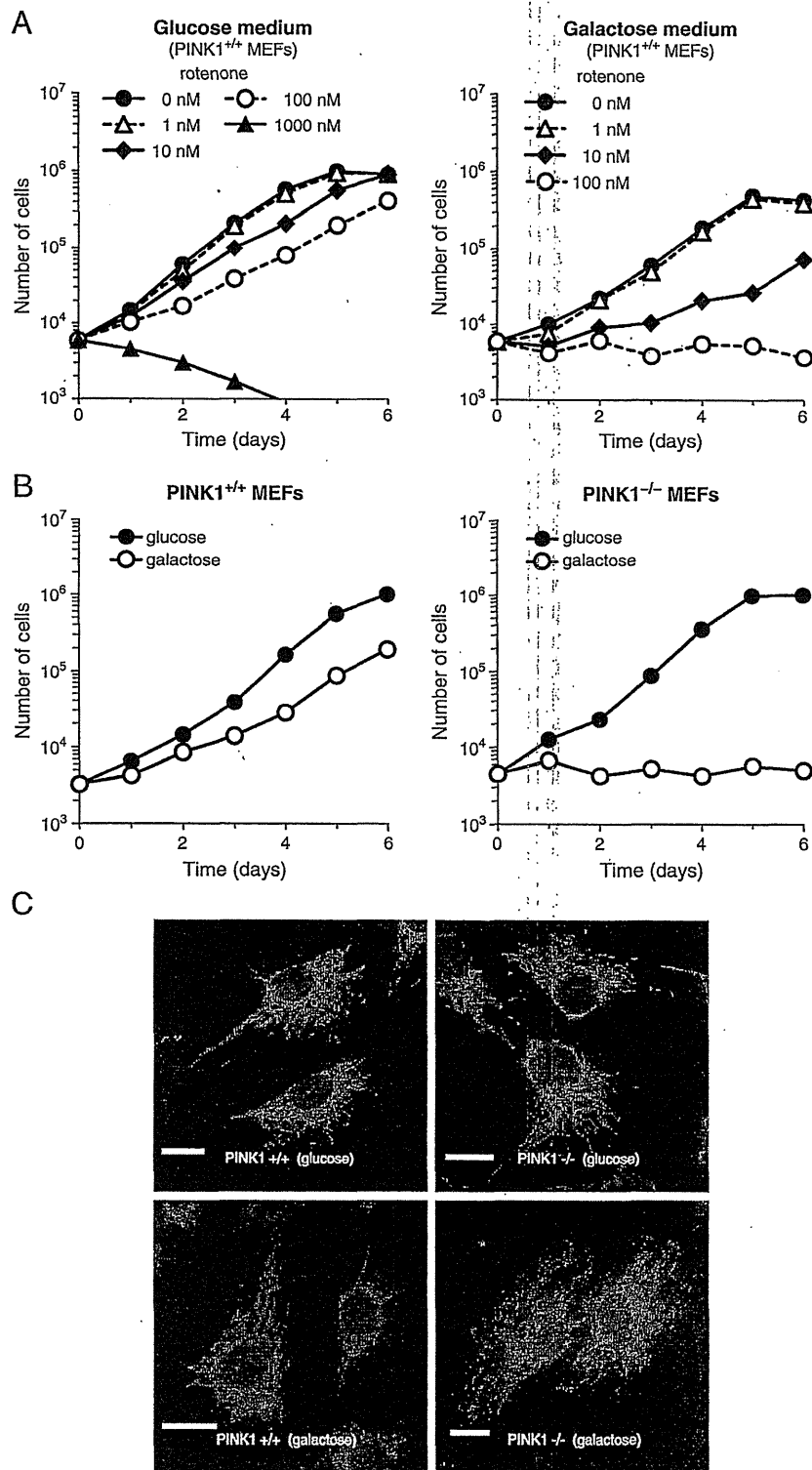
In general, cultured cells gain their energy mostly from glycolysis. Therefore, cells deficient in respiratory function can grow in normal medium, although possibly at a slower rate, relying predominantly on glycolysis (Hofhaus et al., 1996). Actually,  $\rho^0$  cells, which lack mitochondrial DNA completely, can grow producing energy exclusively through glycolysis (King and Attardi, 1989). On the other hand, galactose metabolism via glycolysis is much slower than glucose metabolism (Reitzer et al., 1979). Therefore, cells in galactose medium are forced to oxidize pyruvate through the mitochondrial respiratory chain for energy required for growth. Consequently, cells with defects in their mitochondrial respiratory chains show growth impairments in galactose medium. To evaluate this phenomenon is also observed in our cells, we examined growth retardation by addition of mitochondrial complex I inhibitor, rotenone (Fig. 1A). In glucose medium, 10 nM rotenone had only a slight effect on the growth of PINK1<sup>+/+</sup> MEFs and slower growth was observed even in the presence of 100 nM rotenone. However, in the galactose medium, 10 nM rotenone significantly inhibited the growth of PINK1<sup>+/+</sup> MEFs and 100 nM rotenone completely arrested the growth. Therefore, we could confirm that the growth impairment of our cells in the galactose medium was due to mitochondrial respiratory chain defects.

PINK1 acts upstream of parkin, regulating mitochondrial integrity and function; therefore, loss of PINK1 is considered to affect mitochondrial functions. To assess the mitochondrial functions of PINK1<sup>-/-</sup> MEFs, growth capability in a medium in which galactose replaced glucose was examined. As shown in Fig. 1B, PINK1<sup>-/-</sup> MEFs appeared to show clear growth impairments in the galactose medium, whereas PINK1<sup>+/+</sup> MEFs grew slightly slower than in the glucose medium.

No differences of mitochondrial morphology between PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs in the glucose medium were detected (Fig. 1C), consistent with the previous report (Matsuda et al., 2010). However, in the galactose medium, mitochondria of the PINK1<sup>-/-</sup> MEFs were more fragmented compared to the PINK1<sup>+/+</sup> MEFs (Fig. 1C). This is consistent with previous reports, which found mitochondrial morphological changes were more pronounced when PINK1 knock-down HeLa cells were grown in low-glucose medium (Exner et al., 2007) and human PINK1 homozygous mutant fibroblast in galactose medium (Grünewald et al., 2009). In these cells, mitochondrial morphological changes were associated with the mitochondrial functional impairment.

### Assessments of mitochondrial functions at the cellular level

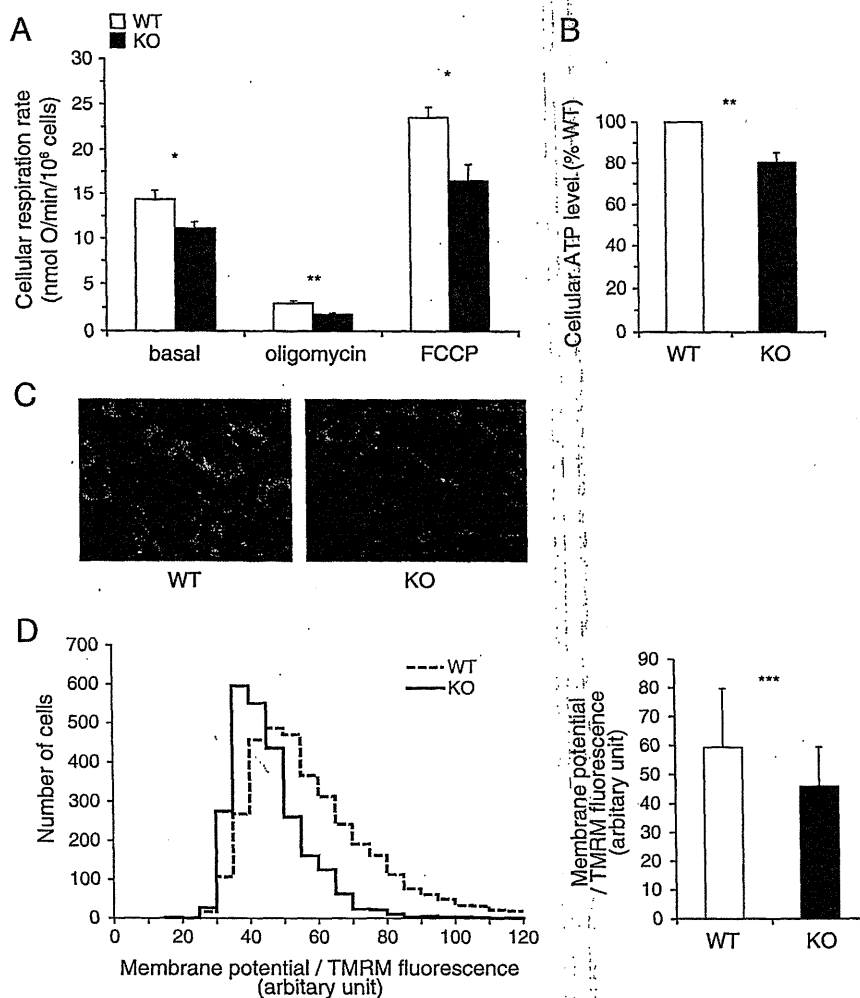
Because PINK1<sup>-/-</sup> MEFs showed severe growth impairments in the galactose medium, the mitochondrial functions of these cells were assessed at the cellular level. First, cellular respiration rates were measured (Fig. 2A). The basal respiration rate was significantly reduced in PINK1<sup>-/-</sup> cells compared with that in PINK1<sup>+/+</sup> cells (11.13  $\pm$  0.71 versus 14.36  $\pm$  1.01 nmol O/min/10<sup>6</sup> cells; *p* < 0.05; *n* = 5 independent experiments), consistent with previous reports using partial knock-down of PINK1 expression (Gandhi et al., 2009; Liu et al., 2009). Oligomycin inhibits ATP synthase, resulting in non-phosphorylating respiration. FCCP uncouples oxidative phosphorylation, leading to maximum respiration rates. In both conditions, the



**Fig. 1.** (A) Growth retardation of PINK1<sup>+/+</sup> MEFs by mitochondrial complex I inhibitor, rotenone in glucose or galactose medium. Closed circles with solid line, 0 nM rotenone; open triangles with dashed line, 1 nM rotenone; closed diamonds with solid line, 10 nM rotenone; open circles with dashed line, 100 nM rotenone; closed triangles with solid line, 1000 nM rotenone. Cells grown in 12-well plates were trypsinized and live cells were assessed by trypan blue dye exclusion. (B) Growth curves of PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs. Closed symbols (*glucose*), growth curve for cells grown in DMEM containing 4.5 g/l glucose and 1 mM sodium pyruvate; open symbols (*galactose*), growth curve for cells grown in DMEM lacking glucose and containing instead 1.0 g/l galactose and 1 mM sodium pyruvate. Cells grown in 12-well plates were trypsinized and live cells were assessed by trypan blue dye exclusion. (C) Mitochondrial morphology of PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs. After incubating cells with the glucose or galactose medium for 24 hours, cells were fixed and immunostained with anti-Tom20 antibody to visualize mitochondria. Scale bar, 20  $\mu$ m.

PINK1<sup>-/-</sup> cells respired significantly slower than the PINK1<sup>+/+</sup> cells ( $1.76 \pm 0.13$  versus  $2.95 \pm 0.27$  ( $p < 0.01$ ;  $n = 5$  independent experiments) and  $16.44 \pm 1.80$  versus  $23.50 \pm 1.18$  nmol O/min/ $10^6$  cells ( $p < 0.05$ ;  $n = 5$  independent experiments), respectively).

The main function of mitochondria is ATP synthesis via oxidative phosphorylation. ATP levels under basal conditions were significantly reduced in PINK1<sup>-/-</sup> MEFs (Fig. 2B), as reported previously for dissociated PINK1<sup>-/-</sup> mouse neurons (Gispert et al., 2009) and PINK1



**Fig. 2.** Mitochondrial functions assessed at the cellular level. Open bars, PINK1<sup>+/+</sup> MEFs; closed bars, PINK1<sup>-/-</sup> MEFs. (A) Cell respiration rate of PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs. The oxygen respiration rate was measured at density of  $8.0 \times 10^6$  cells/ml under each of the following three conditions: basal rate (no additions); State 4 (no ATP synthesis) [after addition of 1  $\mu$ g/ml oligomycin], uncoupled [after addition of 3  $\mu$ M FCCP]. After sequential measurements, the endogenous respiration rate was determined by adding 1  $\mu$ M rotenone + 2  $\mu$ M myxothiazol. Error bars indicate SEM ( $n = 5$  independent experiments). (B) Cellular ATP levels. Data were normalized based on cell numbers and expressed as the percentage of the level in PINK1<sup>+/+</sup> cells. Error bars indicate SEM ( $n = 4$  independent experiments). (C) Live cell images of PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs with TMRM fluorescence. (D) Mitochondrial membrane potential evaluated by live cell imaging of TMRM fluorescence. *Left panel*, the distribution of TMRM fluorescence from 3537 PINK1<sup>+/+</sup> and 2566 PINK1<sup>-/-</sup> cells from 12 wells per cell type; *right panel*, the average value of TMRM fluorescence per cell. Error bars indicate SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

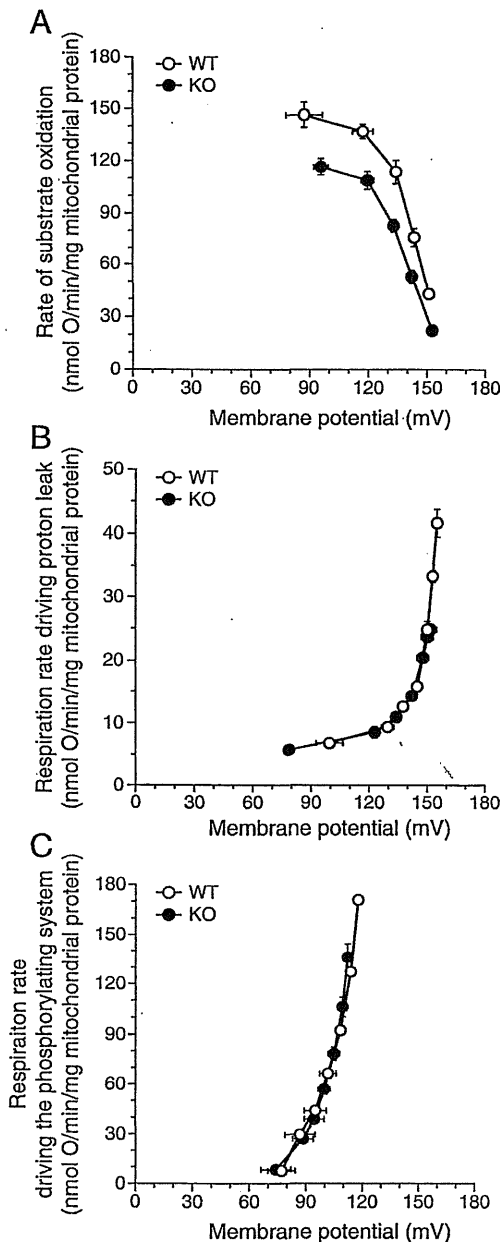
siRNA knock-down PC12 cells (Liu et al., 2009). Mitochondrial membrane potential was also measured by live cell imaging of TMRM fluorescence. Typical images were shown in Fig. 2C. The histogram shows the distribution of TMRM fluorescence from 3537 PINK1<sup>+/+</sup> cells and 2566 PINK1<sup>-/-</sup> cells from 12 wells per cell type and the bar graph indicates the mean  $\pm$  SD of TMRM fluorescence per cell (Fig. 2D). According to the Nernst equation, the ratio of TMRM fluorescence would translate into, on average, 6.88 mV lower mitochondrial membrane potential in the PINK1<sup>-/-</sup> cells if the plasma membrane potentials were not different between PINK1<sup>+/+</sup> and <sup>-/-</sup> cells. Mitochondrial membrane potential decrease was also showed previously in PINK1 knock-down HeLa cells (Exner et al., 2007) and in stable PINK1 knock-down neuroblastoma cell lines (Sandebring et al., 2009).

#### Assessments of mitochondrial functions using isolated mitochondria

To further analyze mitochondrial functions, we measured the kinetics of oxidative phosphorylation using isolated mitochondria from PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs. Fig. 3 shows the kinetics of the three modules of oxidative phosphorylation using succinate as a respiratory substrate (complex II-linked respiration). Fig. 3A shows the kinetic response of substrate oxidation to its product,  $\Delta\psi$ . The

substrate oxidation kinetic curve for PINK1<sup>-/-</sup> cells was clearly shifted lower compared with that for PINK1<sup>+/+</sup> cells, indicating that the loss of PINK1 caused mitochondrial respiratory chain defects. Fig. 3B shows the kinetic response of proton leak to its driving force,  $\Delta\psi$ , and Fig. 3C shows the kinetic response of the ATP phosphorylating pathway to its driving force,  $\Delta\psi$ . Both kinetic curves for PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs (open and closed symbols, respectively) were overlapping, implying that there were no significant differences in those modules.

We also independently measured the mitochondrial oxygen consumption rate using pyruvate/malate as a respiratory substrate instead of succinate to check complex I. Modular kinetic analysis using pyruvate/malate is technically difficult for the following reasons: (1) the oxygen consumption rate with pyruvate/malate is much slower than succinate respiration; and (2) there are no competitive inhibitors of complex I-linked respiration, such as malonate for succinate respiration. As shown in Fig. 4A, the respiration rates in State 3 and 4 with pyruvate/malate of isolated mitochondria from PINK1<sup>-/-</sup> cells (closed symbols) were significantly slower than those of PINK1<sup>+/+</sup> cells (open symbols), as in the case of succinate respiration (Fig. 4B; data derived from the kinetic curves in Fig. 3).



**Fig. 3.** Modular kinetic analysis of oxidative phosphorylation in mitochondria isolated from PINK1<sup>+/+</sup> and <sup>-/-</sup> MEFs. Modular kinetic analysis of the kinetic responses to membrane potential,  $\Delta\psi$ , of respiration driving (A) substrate oxidation ( $\Delta\psi$  titrated with uncoupler, FCCP, starting in State 4), (B) proton leak ( $\Delta\psi$  titrated with malonate, starting in State 4) and (C) the phosphorylating system, calculated by subtracting respiration driving proton leak from respiration driving the  $\Delta\psi$ -consumers ( $\Delta\psi$  titrated with malonate starting in State 3; not shown) at each  $\Delta\psi$ . Open symbols, PINK1<sup>+/+</sup> MEFs; closed symbols, PINK1<sup>-/-</sup> MEFs. Error bars indicate SEM ( $n=4$  independent mitochondrial preparations).

#### Mitochondrial ROS production

Mitochondrial ROS production rate was assessed by measurement of the H<sub>2</sub>O<sub>2</sub> generation rate. Mechanisms of mitochondrial ROS production were well described elsewhere (Fig. 1 of Lambert et al., 2010). Pyruvate and malate generate NADH, which induced forward electron transport and generate ROS mainly from complex I and III. For pyruvate/malate respiration, the basal H<sub>2</sub>O<sub>2</sub> generation rate (measured in the absence of respiratory chain inhibitors) was not different between PINK1<sup>+/+</sup> and <sup>-/-</sup> mitochondria (Fig. 4C). The addition of antimycin A and further addition of rotenone, which inhibited forward electron transport at complex III and I, respectively,

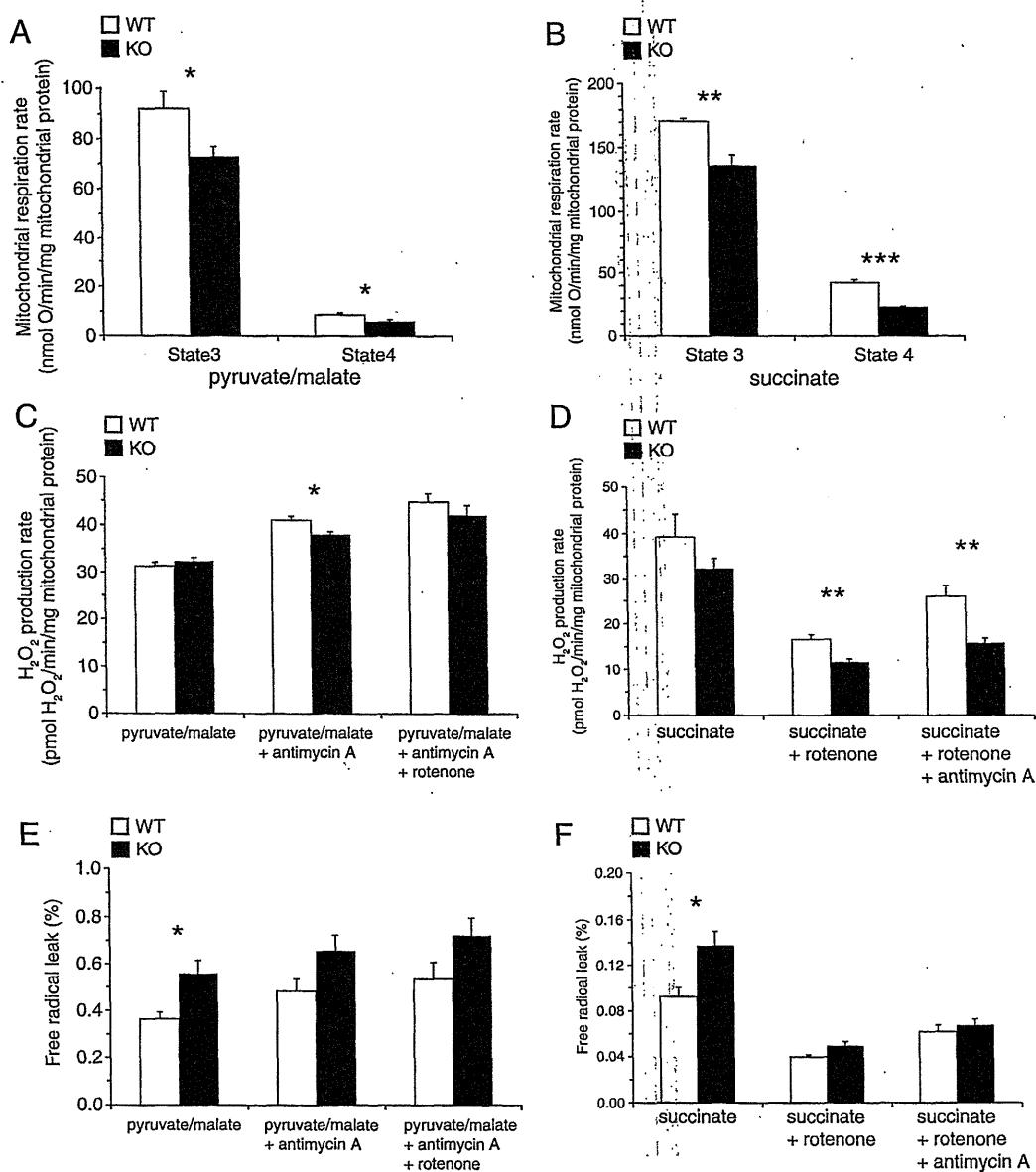
enhanced H<sub>2</sub>O<sub>2</sub> generation. During succinate respiration in the absence of respiratory chain inhibitors, ROS are generated mainly from the quinone binding site of complex I due to reverse electron flow from coenzyme Q to complex I. For succinate respiration, H<sub>2</sub>O<sub>2</sub> generation rate in the absence of respiratory chain inhibitors was higher in PINK1<sup>+/+</sup> mitochondria than in PINK1<sup>-/-</sup> mitochondria, but the difference was not significant (Fig. 4D). The addition of rotenone, which blocks reverse electron flow from coenzyme Q to complex I, attenuated H<sub>2</sub>O<sub>2</sub> generation.

Figs. 4 C and D show a tendency for PINK1<sup>+/+</sup> mitochondria to generate more ROS than PINK1<sup>-/-</sup> mitochondria. However, their respiration rates were remarkably different (Figs. 4A and B). Therefore, we calculated the percentage free radical leak, which is the fraction of molecules of O<sub>2</sub> consumed that give rise to H<sub>2</sub>O<sub>2</sub> release by mitochondria (free radical leak) during either pyruvate/malate or succinate State 4 respiration (Figs. 4E and F). For pyruvate/malate respiration, mitochondria isolated from PINK1<sup>-/-</sup> cells had higher proportion of H<sub>2</sub>O<sub>2</sub> generation than PINK1<sup>+/+</sup> mitochondria. During succinate respiration without respiratory inhibitors, PINK1<sup>-/-</sup> mitochondria had also higher proportion of free radical leak mainly from complex I due to reverse electron flow from coenzyme Q to complex I. Because the differences disappeared with addition of rotenone, which inhibit reverse electron flow, ROS generation enhanced by loss of PINK1 was mostly from complex I.

#### Discussion

We produced an *in vitro* model of Parkinson's disease, immortalized PINK1<sup>-/-</sup> MEFs. Previously, impairment of mitochondrial respiration was observed in the brains of PINK1<sup>-/-</sup> mice (Gautier et al., 2008). PINK1<sup>-/-</sup> MEFs clearly showed a phenotype of mitochondrial dysfunctions, which is consistent with PD pathogenesis. This phenotype was apparent in a cell growth experiment using medium containing galactose instead of glucose (Fig. 1B). Mitochondrial fragmentation was observed when PINK1<sup>-/-</sup> MEFs grew in the galactose medium (Fig. 1C), which was consistent with previous reports (Exner et al., 2007; Grünwald et al., 2009). Our results have unveiled that the PINK1<sup>-/-</sup> MEF line could be a potential PD model, presenting growth retardation due to decreased mitochondrial respiration activity. Thus, the PINK1<sup>-/-</sup> MEFs are a useful tool for evaluating the role of PINK1 in mitochondrial dysfunction and relevant to PD.

In PINK1<sup>-/-</sup> MEFs, mitochondrial membrane potential was decreased compared with that in littermate wild-type MEFs (Figs. 2C and D), as reported previously for PINK1 knock-down HeLa cells (Exner et al., 2007) and stable PINK1 knock-down neuroblastoma cell lines (Sandebing et al., 2009). This is a key event during elimination of mitochondria. Mitochondrial fission followed by selective fusion segregates damaged mitochondria, which decreases their membrane potential, and permits their removal by autophagy (Twig et al., 2008). The PINK1-parkin pathway is thought to have a crucial role in this mitochondrial elimination mechanism (Geisler et al., 2010; Kawajiri et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010; Vives-Bauza et al., 2010). To clarify what caused the decrease in mitochondrial membrane potential, we performed a modular kinetic analysis using isolated mitochondria (Fig. 3). This analyzes the kinetics of the whole of oxidative phosphorylation divided into three modules connected by their common substrate or product, mitochondrial membrane potential ( $\Delta\psi$ ). The modules are include one  $\Delta\psi$ -producer (substrate oxidation) and two  $\Delta\psi$ -consumers (phosphorylating system and proton leak) (Brand, 1990). To decrease  $\Delta\psi$ , the  $\Delta\psi$ -producer should be down-regulated and/or  $\Delta\psi$ -consumers should be up-regulated. As cellular ATP levels were decreased compared with those in littermate wild-type MEFs (Fig. 2B), it is unlikely that the phosphorylating system is up-regulated. Indeed, the kinetics of the phosphorylation module were not altered (Fig. 3C). The other  $\Delta\psi$ -consumer, proton leak,



**Fig. 4.** Oxygen consumption rate and H<sub>2</sub>O<sub>2</sub> production rate of mitochondria isolated from PINK1<sup>+/+</sup> and PINK1<sup>-/-</sup> MEFs. Open bars, PINK1<sup>+/+</sup> MEFs; closed bars, PINK1<sup>-/-</sup> MEFs. (A) State 3 and State 4 respiration rate of mitochondria with pyruvate/malate as a respiratory substrate. (B) State 3 and State 4 respiration rate of mitochondria with succinate as a respiratory substrate. Data were derived from the results of modular kinetic analysis (Fig. 3). State 3 respiration rates were the kinetic start points of the  $\Delta\psi$ -consumers (the sum of the phosphorylating system and proton leak). State 4 respiration rates were average values of the respiration rates at the kinetic start points of substrate oxidation and proton leak. (C, D) Mitochondrial H<sub>2</sub>O<sub>2</sub> production rate with pyruvate/malate (C) or succinate (D) as a respiratory substrate. (E, F) Percentage free radical leak (FRL) for State 4 respiration with pyruvate/malate (E) or succinate (F) as a respiratory substrate. Error bars indicate SEM ( $n=5$  and 4 independent mitochondrial preparations for pyruvate/malate and succinate respiration, respectively). \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

which partially dissipates the membrane potential without ATP synthesis, was also not changed (Fig. 3B). Therefore, the decrease in membrane potential caused by loss of PINK1 is likely to have been caused only by lower activity of the  $\Delta\psi$ -producer, substrate oxidation (Fig. 3A). This is the first report showing that mitochondrial membrane potential decrease caused by loss of PINK1, which is the key event for the following mitochondrial elimination, was not due to proton leak, but to respiratory chain defects. We used only succinate (a complex II-linked substrate) as a respiratory substrate in the modular kinetic analysis for technical reasons. However, complex I-linked respiration (pyruvate/malate) was also decreased in PINK1<sup>-/-</sup> MEFs like succinate respiration (Fig. 4A).

The mitochondrial respiration rates in State 4 were decreased in PINK1<sup>-/-</sup> MEFs, and consequently, the proportions of free radical leak were significantly higher in PINK1<sup>-/-</sup> MEFs than in PINK1<sup>+/+</sup>

MEFs (Figs. 4E and F). Because the differences disappeared with addition of rotenone (complex I inhibitor, which inhibits reverse electron flow from coenzyme Q to complex I), ROS generation enhanced by loss of PINK1 was mostly from complex I. These results are partially consistent with those in previous reports, suggesting that MPTP and rotenone induce neuronal cell death by inhibiting complex I activity, leading to a PD-like phenotype (Dauer and Przedborski, 2003; Jackson-Lewis and Przedborski, 2007; Trojanowski, 2003).

In this study, we developed an *in vitro* PD model, the PINK1<sup>-/-</sup> MEF line, and established the experimental conditions for cell growth to detect mitochondrial dysfunction. This is the first report showing that complete ablation of PINK1 causes a decrease in mitochondrial membrane potential, which is not due to proton leak, but to respiratory chain defects.



Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2010.08.027.

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## Phenotype of the 202 Adenine Deletion in the *parkin* Gene: 40 Years of Follow-Up

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### ABSTRACT

**Background:** We describe the four decades follow-up of 14 *parkin* patients belonging to two large eight-generation-long in-bred Muslim-Arab kindreds.

**Results:** All patients had a single base-pair of adenine deletion at nucleotide 202 of exon 2 (202A) of the *parkin* gene (all homozygous, one heterozygous). Parkinson's disease onset age was 17–68 years. Special features were intractable axial symptoms (low back pain, scoliosis, camptocormia, antecollis), postural tremor, and preserved cognition.

**Conclusions:** The 202A deletion of the *parkin* gene causes early-onset Parkinson's disease with marked levodopa/STN-DBS-resistant axial features. Postural tremor and preserved cognition, even after 40 years of disease, were also evident. © 2011 Movement Disorder Society

**Key Words:** Parkinson's disease; genetics; *Parkin*; PARK2; follow-up

### Introduction

Mutations in the *parkin* gene (6q25.2-6q27, MIM 602544) are the most common cause of monogenic

autosomal recessive Parkinson's disease (PD).<sup>1,2</sup> The phenotype includes early onset of classic PD symptoms, but may vary with respect to additional atypical features.<sup>3–5</sup> Exonic deletions or multiplications and truncating or missense mutations have been described.<sup>1–6</sup> No reports point to ethnic clusters of specific *parkin* mutations. We describe four-decades follow-up of 14 *parkin* patients belonging to two large in-bred Muslim-Arab kindreds.

### Methods

PD patients of Arabic-origin with age of disease onset < 50 years were recruited from the Sheba Medical Center Movement Disorders Clinic. The Institutional Review Board approved the use of human subjects for this study. All patients and family members signed informed consent for participating in the study.

Participants were examined by a movement disorders specialist at 2–12 months intervals. Asymptomatic family members were examined once at the time of DNA collection. DNA was extracted from blood leukocytes. All exons of the *parkin* gene were screened for deletions, insertions, or point mutations by direct sequencing of the PCR products, sequenced on both strands as previously described.<sup>4</sup>

### Results

Thirteen of 14 PD patients and 15 family members consented to genetic testing. Patient characteristics are summarized in Table 1 (10 men, 4 women; mean age 52 ± 10 years; range 35–73 years).

In all 13 patients, the same *parkin* mutation was found: a single base-pair deletion of adenine at nucleotide 202 of exon 2 (202A), causing an out-frame mutation with an early-stop codon (12 homozygous, 1 heterozygous) and one patient was not genotyped. The mutant *parkin* lacks a part of the Ubl domain and the entire region of the RING box, suggesting loss of activity of E3.

### Phenotype and Clinical Course

All patients belong to two large Muslim-Arab in-bred *hamulas* (kindreds). Each *hamula* can trace their ancestry to a few founders about eight generations ago. Family A traced back five generations and divided into three branches shown as Aa, Ab, and Ac Family B traced back eight generations.

Mean age ± SD at PD onset was 31 ± 15 years (range 17–68) and disease duration 21 ± 13 (median 19, range 1–41 years) (Table 1). The first patient (B-VII-22) was seen in our clinic in 1963, aged 27 years. She complained of “bent trunk” and slowing since the age of 23. Her first cousin (B-VII-25) was examined in 1989, aged 19 years due to scoliosis and

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**Table 1.** Demographic characteristics and motor features of Parkinson's disease (PD) patients with the *parkin* 202A deletion

Patient No.	<i>Parkin</i> 202A deletion	Gender	Age	Age of onset	PD duration	H&Y stage	Presenting sign	Rest tremor	Rig.	Asym	Brad.	Post. Inst.	Gait dist.	Post. tremor
Aa-II-3	HOM	M	58	17	41	3	Hand tremor	+	+	+	+	+	+	+
Aa-II-8	HOM	M	50	15	35	4	Leg dystonia	+	+	+	+	+	+	+
Aa-II-10	HOM	F	48	47	1	2	Leg tremor		+	+	+		+	
Aa-II-11	HOM	M	47	30	17	3	Leg tremor	+	+	+	+	+	+	+
Ab-IV-14	NG	M	73	68	5	3	Slow gait	+	+	+	+	+	+	
Ab-V-7	HOM	F	47	18	29	3	Hand tremor	+	+	+		+	+	+
Ac-IV-5	HOM	F	35	17	18	4	Hand tremor	+	+	+	+	+	+	+
B-VII-8	HOM	M	62	35	27	4	Leg tremor	+	+	+	+			+
B-VII-10	HOM	M	59	28	31	4	Hand tremor	+	+	+	+	+	+	+
B-VII-13	HOM	M	49	37	12	3	Leg tremor	+	+	+	+			+
B-VII-17	HOM	M	44	30	14	3	Hand tremor	+	+	+	+			+
B-VII-22	HOM	F	63	23	40	3	Camptocormia	+	+	+	+	+	+	+
B-VII-25	HOM	M	39	19	20	3	Hand tremor	+	+	+	+		+	+
Aa-II-6	HET	M	55	49	6	2	Hand tremor		+	+	+			
Mean±SD			52±10	31±15	21±13									

HET, heterozygous; HOM, homozygous; NG, not genotyped; Rig, rigidity; Asym, asymmetry; Brad, bradykinesia; Post Inst, postural instability; Dist, disturbance; Post, postural.

bradykinesia. The diagnosis of juvenile-onset PD was made in both.

The presenting symptom was hand tremor (n = 6), leg tremor (n = 4), foot dystonia (n = 1), camptocormia (n = 1), and gait disturbances (n = 1). Bradykinesia and rigidity were present in all patients and rest tremor in all but one. Eleven had postural hand tremor, three limb dystonia (two at PD onset) and three reported sleep benefit (Table 1).

Atypical motor features included prominent levodopa-resistant axial symptoms (n = 10): recurrent falls at onset (n = 1), gait disturbances at onset (n = 1), scoliosis (n = 1), camptocormia (progressive to fixed 90° trunk flexion, n = 2), antecollis (n = 1), lower back pain (LBP) (n = 8) (Table 2). Camptocormia and

antecollis 5 years after onset were observed in a heterozygous carrier with an intermediate PD phenotype (onset 49 years) and very slow disease progression.

Pain was a predominant symptom (painful dystonia = 2, LBP = 8). Two patients manifested autonomic dysfunction with complaints of constipation (Table 1).

None of the patients developed significant cognitive impairment or dementia during follow-up of up to 40 years (median 19 years). Seven patients had depressive symptoms but none developed hallucinosis or psychosis.

### Response to Treatment and Progression

Levodopa response was excellent for appendicular signs but only minor for axial signs. All patients devel-

**Table 2.** Nonmotor/atypical features and therapy-related features of Parkinson's disease (PD) patients with the *parkin* 202A deletion

Patient No.	Psych.	Cognitive decline	Sleep benefit	Autonomic features	Additional axial features	Response to levodopa	Wearing off	Levodopa induced dyskinesia	UPDRS III (on/off)	DBS (Yr after PD onset)
Aa-II-3		-				+	+	+	52	
Aa-II-8	DEP	-	+			+	+	+	20/27	+
Aa-II-10		-	+	constip	LBP	NA	NA	NA	17/NA	(30)
Aa-II-11		-				+			46	
Ab-IV-14		-		constip		+	+		38/41	
Ab-V-7	DEP	-	+		LBP	+	+	+	14	
Ac-IV-5	DEP	-				+	+	+	48/61	+
B-VII-8	DEP	-			LBP	+	+	+	16/22	(14)
B-VII-10	DEP	-			LBP	+	+	+	37/64	+
B-VII-13	DEP	-			LBP	+	+		4/20	(26)
B-VII-17		-			LBP	+	+		20/30	
B-VII-22	DEP	-			Camptocormia, LBP	+	+	+	37/48	
B-VII-25		-			Scoliosis, LBP	+	+	+	16/28	
Aa-II-6		-			Antecollis, Camptocormia	NA	NA	NA	NA	

DEP, depression; constip, constipation; LBP, Lower back pain; NA, not applicable.

oped motor fluctuations. Despite consistently very slow disease progression, three patients developed severe motor fluctuations and underwent deep brain stimulation (STN-DBS) 14, 26, and 30 years after PD onset. Response to STN-DBS was modest with improvement in appendicular symptoms but no change in axial features, particularly postural instability, gait disorders, and LBP.

### Genotype and Phenotype of Nonparkinsonian Family Members

Fifteen family members were genotyped. Five had no mutations and 10 were heterozygous, of whom nine were asymptomatic (age range 28–40 years). One family member, (70 year-old female) with postural tremor only (onset 60 years, B-VI-5), showed A-G heterozygosity for the 202A *parkin* deletion. Tremor remained her sole symptom during 10 years of follow-up and was levodopa unresponsive.

### Discussion

The marked axial nature is the most striking feature of the *parkin* 202A deletion phenotype. This includes camptocormia, antecollis, scoliosis, and low back pain. Dystonia in PD manifests mainly in the limbs as equinovarus foot, striatal toe, ulnar deviation or hemidystonia and in the mid-line as blepharospasm or oromandibular dystonia.<sup>7</sup> Scoliosis, if considered a form of dystonia, is also seen. Camptocormia is a rare feature of PD and is attributed to dystonia or imbalance in muscle activity.<sup>3,6–8</sup> We postulate that LBP is part of the dystonic phenotype. In our patients, axial symptoms were painful and levodopa/STN-DBS unresponsive.

A second main characteristic of the 202A *parkin* deletion phenotype was the preserved cognition after several decades since PD onset. Lack of dementia characterizes *parkin* disease.<sup>9</sup> To our knowledge long term follow-up up to 40 years has not yet been described. It has been suggested that the lack of dementia in *parkin* disease may be related to the absence of Lewy body pathology.<sup>9</sup> Hayashi et al. reported neuropathologic findings in a Japanese patient with a mutation in the *parkin* gene.<sup>10</sup> Loss of pigmented neurons and gliosis were most pronounced in the medial and ventrolateral regions of the SN pars compacta and in the locus ceruleus. There was no clinical evidence of dementia and no Lewy bodies were identified. Some neurofibrillary tangles and senile plaques were observed in the cerebral cortex. Another study also found diffuse tau pathology but no Lewy bodies.<sup>11</sup> Neuropsychiatric disturbances are reportedly common in carriers for *parkin* or *PINK1* mutations.<sup>12,13</sup> The 202A phenotype commonly included depression.

There is controversy whether heterozygous carriers of *parkin* mutations are symptomatic and whether *parkin* is a susceptibility gene.<sup>14,15</sup> One of our heterozy-

gous patients (onset 49 years) developed a mild phenotype but with antecollis and camptocormia. Another heterozygous family member presented solely with postural tremor (onset 60 years) with no other parkinsonian features during 10 years of follow-up. While homozygous mutations in the *parkin* gene result in early-onset PD, heterozygous mutations in the *parkin* gene are identified in patients with later-onset disease, raising the possibility that heterozygous mutations may have a role beyond that of loss of function and confer an increased susceptibility to the disease.<sup>15</sup>

The risk of developing PD in individuals with mutations in the *parkin* gene is age-dependent and penetrance remains unclear. Our asymptomatic carriers were aged 28–40 years and need to be further followed. A recent study identified mutations in the *parkin* gene in 10% of probands with disease onset before age 50.<sup>16</sup> They estimated the cumulative incidence of PD to age 65 years in relatives of mutation carriers to be 7%, compared to 1.7% in noncarrier relatives, and 1.1% in relatives of controls.

Periquet and coworkers analyzed *parkin* gene inheritance to discriminate between single founder effects and independent recurrent events by the use of intragenic and tightly flanking markers of the *parkin* gene region.<sup>17</sup> By studying a variety of *parkin* mutations in 48 European families they showed that the majority of exon rearrangements result from distinct mutational events, whereas point mutations may have arisen from a limited number of founders suggesting different mechanisms underlying these two groups of mutations. According to this hypothesis, the frequency of exon rearrangements would be expected, in the absence of selection, to increase with the passage of time because of new mutational events, whereas the frequency of point mutations as in our patients would be expected to remain stable, because new mutations would be rare. In this context, the 202A deletion, being a point mutation, could be related to a founder effect which persisted because of high rates of consanguinity in this population.

The fact that all patients in our two kindreds carry the same mutation suggests that it might be common among young-onset PD patients of Arabic descent. Indeed, the 202A deletion has also been observed among Jordanian patients.<sup>17</sup> However, the exact phenotype in this population has not been specified. While PD prevalence above the age of 65 years in Arab villages in Israel is similar to that of Western populations,<sup>18</sup> neither the prevalence of *parkin* mutations nor its impact on PD prevalence in young adults is known. In our studied population of Muslim Arab villages with large in-bred kindreds, carrier frequencies may be high.

Our phenotypic observations might be useful for raising the suspicion and recognition of carriers of the

*parkin* 202A deletion mutation among young-onset PD patients with Arabic ancestry. Postural tremor, prominent and/or painful axial features, slowness of progression of motor symptoms and preservation cognitive function are strong clues to *parkin* disease.

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## LINGO1 Gene Analysis in Parkinson's Disease Phenotypes

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### ABSTRACT

**Background:** Parkinson's disease (PD) and essential tremor (ET) may share some etiopathogenic factors. A genome-wide association study has shown that *LINGO1* gene variants are associated with increased risk of ET. We hypothesized that *LINGO1* variants could increase susceptibility to PD. **Methods:** A large series of PD subjects and healthy controls were genotyped for rs9652490 and rs11856808 *LINGO1* single nucleotide polymorphisms (SNPs). **Results:** We found an increased frequency of the rs11856808<sup>T/T</sup> genotype in PD compared with controls (odds ratio = 1.46; corrected *P* value = 0.02). A recessive genetic model was the best fit for rs11856808 influence on PD (recessive gene action test: corrected *P* value = 0.01). Stratification analysis showed that rs11856808<sup>T/T</sup> genotype frequency was higher in the tremor-dominant PD and the classical PD (C-PD) subgroups (recessive gene action test for the C-PD subgroup: corrected *P* value = 0.004). **Discussion:** Our results indicate that *LINGO1* variants could increase risk of PD, specifically those presenting the non-rigid-akinetic phenotypes, which suggests that *LINGO1* may have a role in the etiology of tremor in PD at least in the Spanish population. ©2011 Movement Disorder Society

**Key Words:** Parkinson's disease; *LINGO1*; essential tremor; genetics; association study