麦角系 DA アゴニストであるブロモクリプチン発売直後の 1981 年には早くも Rinne[2] により肺線維症の報告が行われ，その後も同様の報告が続くことになった[3]．2002 年には麦角系 DA アゴニストであるブロモクリプチン[4]およびペルゴリド[5]によって生じた心臓弁膜症の報告がされた．次いで 2004 年には Lancet 誌上にペルゴリドの大量投与では心臓弁逆流が有意に高頻度に生じ，弁置換症例も併せて報告された[6]．この Lancet の論文を機に麦角系 DA アゴニストと線維症ことに心臓弁膜の線維症との関連が欧米で，そして，遅れて日本でも注目を浴びることになった．2007 年には米国からは PD 治療薬としてペルゴリドは自主撤退という形を取って市場から姿を消すことになった．しかし，このことは突然であり治療者側からすれば治療選択肢の制限という困った状態になったといえる．

本項では，始めに麦角製剤の歴史を振り返ると共にその薬理作用を概説する．そこには我々医師が本当に猛省をしなければならない多くの教訓が存在していることを強調したい．そして，この数年間に生じた DA アゴニスト，特に麦角系 DA アゴニストの副作用と関係した諸問題を併せて考える．この観点から，我々はもはや麦角系 DA アゴニストを使用すべきでないのだろうか，また，麦角系 DA アゴニストの役割はパーキンソン病治療において終わったのであろうかという点を中心に論じてみたい．

■A．麦角系薬物の歴史

麦角系物質は古代の人類の記録と共に存在してきた．麦角菌が穀類に寄生して多数の人たちを死に至らしめたことにより人類に大きな打撃をもたらした（図 2）[8]．人類は麦角系物質をある時

図 2　麦角はライ麦などのイネ科植物，すなわち多くの穀物に麦角菌（*Clviceps purpurea*）が寄生する（黒くなっている所）ことによって産生する（文献 7 より転載許可）

表1　麦角関連薬理作用と治療薬品

・ドパミン受容体刺激……ブロモクリプチン，ペルジリド，カベルゴリン
・脳・末梢循環改善……ジヒデルエルゴトキシン
・血管性頭痛……ジヒデルエルゴタミン
・片頭痛……カフェルゴット
・自律神経調整……ベレルガル
・子宮収縮止血……メチルエルゴメトリン

は毒物として，ある時には薬物として薬理作用は当然のことながら知ることはなかったが経験的にそのように使用してきた．最初，麦角は毒物として知られていた．古代から現代に至るまで，ヨーロッパ大陸の各地で散発的に起こった麦角病は，四肢の壊疽とけいれんを主症状として人々にとっては恐怖の的であったという[8]．

薬剤として認識して使用されたのは16世紀半ばであると記録されている[9]．子宮作用薬として使用されたとされ，それ以前には民間における麦角の陣痛促進作用効果が知られており，広く使用されていたと言われる（図3-a）．この麦角薬物は1824年にはニューヨークの医師会は「出産用散剤は，出産児からみれば死の散剤にほかならない」と言う警告を出したという[8]．麦角は分娩後の出血時に限って使用すべきことを勧告した．つまり，1世紀半以上前から麦角の適応と禁忌は正確に決められていたという[8]．このように麦角薬剤のリスクとベネフィットは正確に認識されていたのである．

1918年サンド社は最初の単一成分としてエルゴタミンを抽出した．その後各種のアルカロイドが抽出され（図4），多彩な薬理作用を利用して薬剤としてその後広く使用されていった（図3-b，表1）．しかし弁膜症を機に線維症に重大な関心が払われるようになり，他の薬剤（カフェルゴット）の販売中止も行われてきた．

B．麦角系薬物の薬理作用

麦角系物質は多彩かつ多様な薬理作用を有するために「薬理学の宝庫」と呼ばれてきた（図3-b，4）[8]．麦角系物質はドパミン，セロトニン，ノルアドレナリン類似の構造式を有するためにモノアミン系に作用する薬理作用を有する（図4，5）．物質によりこれらの作用の強さが異なり様々な疾患の治療薬として使用されてきた．これらの作用効果を表1に示す．

しかし，最初に登場した麦角系DAアゴニストに関しては麦角構造を有することによる多彩な薬理作用を有するにもかかわらず，ドパミン受容体刺激作用を強調するためであろうか，製薬企業からはその他の薬理作用情報は提供されなかった．実際はセロトニン受容体へも当然作用するものであったことは予想できたはずである．図6はDAアゴニストのセロトニン2B受容体への作用力価を示す[9]．これはDAアゴニストは薬理作用としては多彩な薬理作用も有する薬物（dirty drug）であることを示している．その結果は線維症という大きな重大な副作用を起こしてしまったのである（図7）．麦角系DAアゴニストによるこうした副作用は，1966年にすでにmethyser-

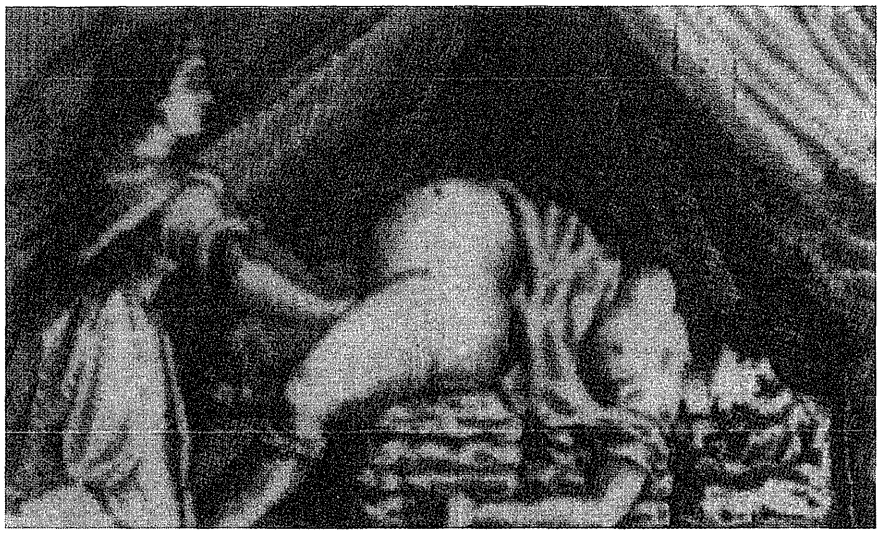


図 3-a　妊婦に麦角薬物を使用（文献 7 より転載許可）

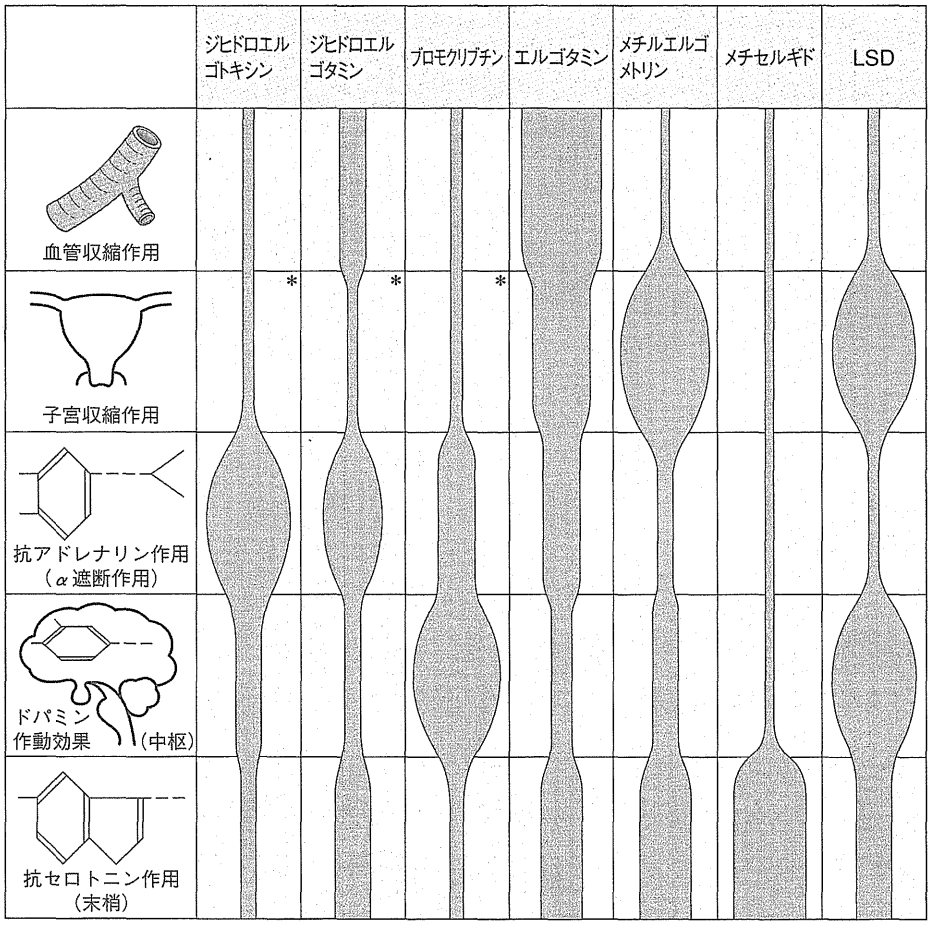
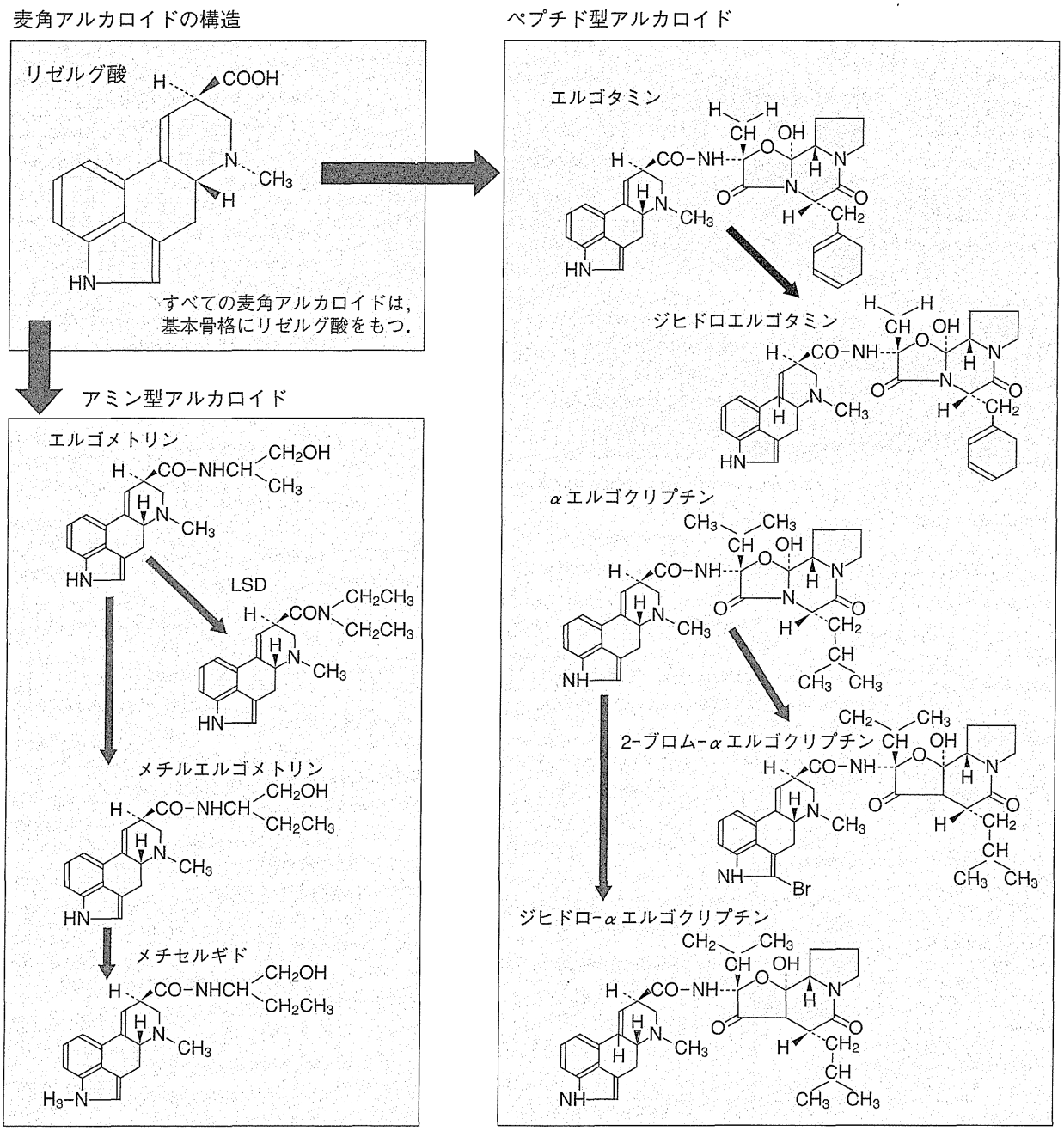


図 3-b　麦角アルカロイドの作用比較（文献 7 より転載許可，改変）

gide による心弁膜症の報告があり[10]，事前に想定されたはずであり，注意して使用すれば重大な副作用に至る前に予防可能であったと思われる．



天然の麦角アルカロイドは，それぞれに構造の部分変更がなされ，その結果，高度な物理スペクトルが生まれた．

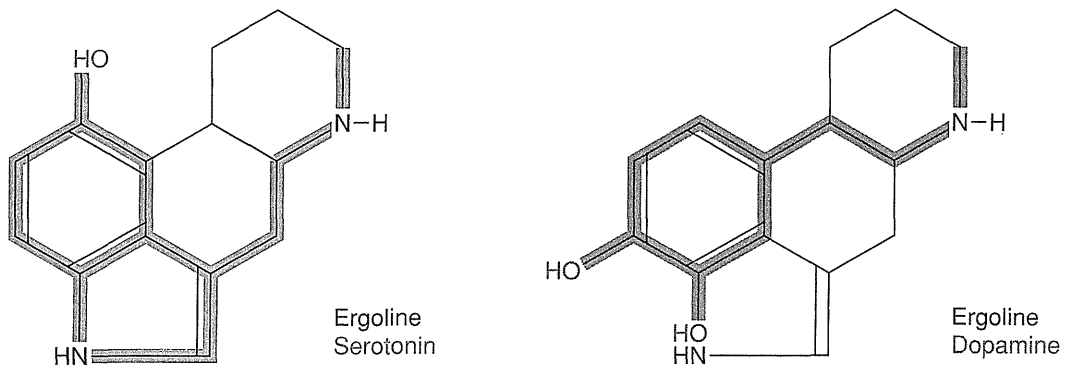


図4　麦角アルカロイドの構造

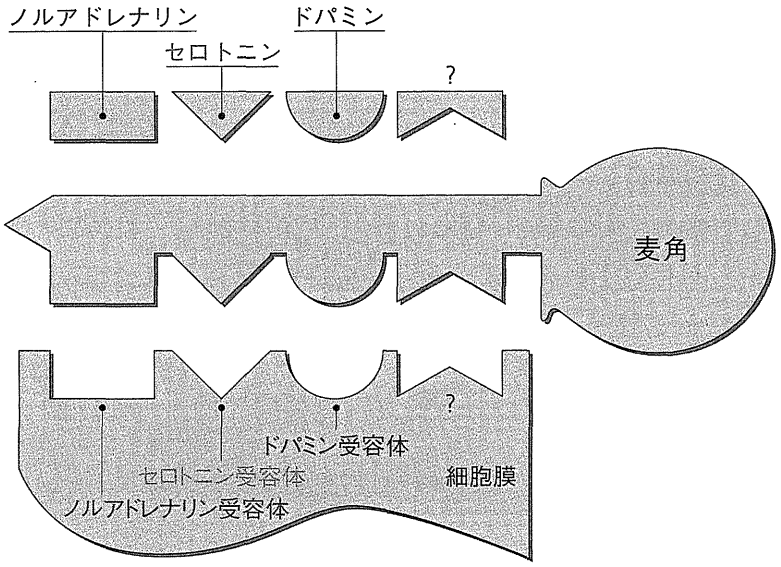


図5　モノアミン受容体と麦角アルカロイド分子の薬理学的特性

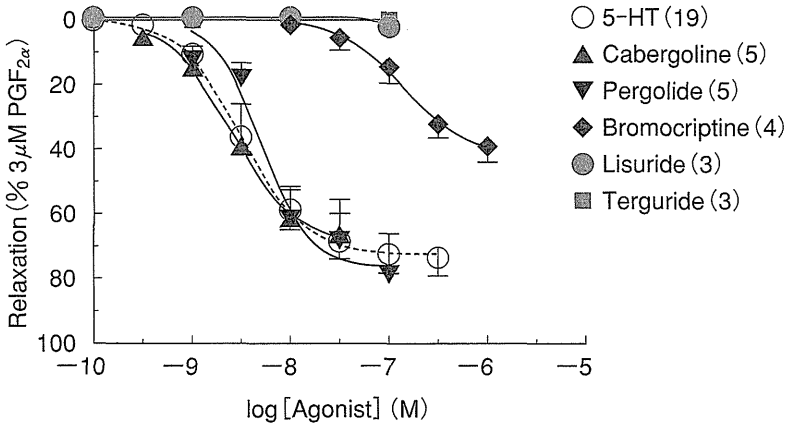


図6　5-HT2B 受容体に対する麦角系 DA アゴニストの親和性
（Jahnichen S, et al. Eur J Pharamacol 2005：513：225-8）

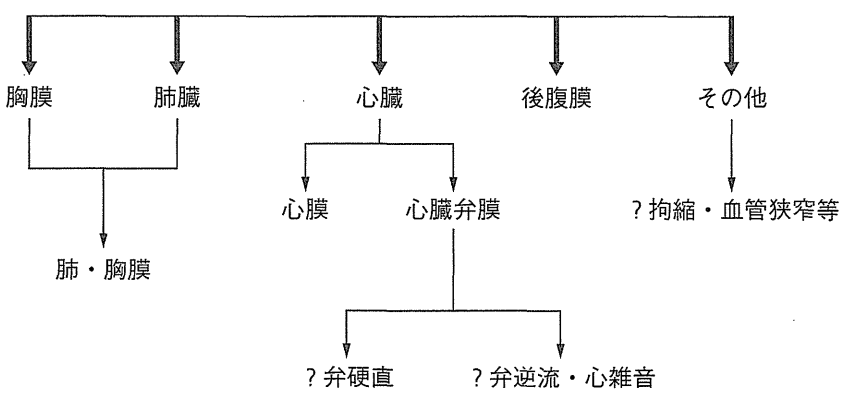


図7　DA アゴニストによる全身の線維症

C. 麦角系ドパミンアゴニストの薬理作用と副作用

麦角系 DA アゴニストはレボドパの半減期が短いという短所を補う治療薬として登場した．登場当初はもっぱらドパミン D2 受容体を刺激する作用があるために PD 症状を改善するということで DA アゴニストは PD 治療薬として強調されて発売が行われた．この段階では，一般的にいえば，麦角系薬剤がもつ多様な薬理作用に関しては製薬企業も PD 治療専門医や研究者すらも十分な注意を払うことはなかったと指摘できる．

1981 年に早くも Rinne がブロモクリプチン投与による肺線維症を報告したときに，我が国ではまだ発売はされていなかったが，この報告を受けて，すぐに治験患者での調査が行われ結果は 1991 年に発表された[11]．その結果はブロモクリプチン治療患者での肺線維症の患者は認められなかった．その後は散発的に肺線維症などの報告が行われてきたが神経内科医の注目を集めるものではなかったし，製薬企業も医師に副作用情報提供を積極的に行わなかった．その後の線維症に関する関心は心臓弁膜症が大きく取り上げられるまで麦角系 DA アゴニストのセロトニン受容体刺激作用に関心が払われることはなかった．

2002 年に麦角系 DA アゴニストによる心臓弁膜症の報告が行われたが，前述のように，図 1 に示すように麦角系薬剤による心臓弁膜症の報告は 1966 年の片頭痛薬である methysergide を嚆矢とする[11]．しかし，その後はほぼ忘れ去られた線維症問題であり，弁膜症問題であったが，1997 年に，やせ薬として使用されていた fenfluramine-phentaermine 投与で心臓弁膜症が発生して改めて麦角系製剤が線維症を起こすということが報告され[12]，当該薬は薬剤としては米国内市場から姿を消した．

そして，2002 年に始まる，麦角系 DA アゴニスト問題は我々に，麦角系薬物が構造式から本質的に有する薬理作用の功罪を大きくしかも明確に認識させることとなった．2002 年にペルゴリドによる心弁膜症の 2 症例報告が行われた時に Mayo Clin Proceedings の editorial は「Drug-related Heart Valvular Disease: Here we go again: Will we do better this time?」「Patients currently taking or those who have taken pergolide」[13]と述べているが，我が国ではこの問題に注意を払うことはなかったし，このような意識さえなかったと言える．製薬企業も積極的に医師・薬剤師に対して啓蒙をしてきたとはいい難いように思われる．

米国ではペルゴリドの売り上げが少ないために早々と自主撤退の形をとって発売を中止してしまったが，ヨーロッパでは 2008 年 6 月に欧州医薬品審査庁（EMEA）は最高用量の制限を加えて第二選択薬としてその使用を認め（表 2）[14]，我が国でもヨーロッパに追従して全く同様の規制を同年 8 月に行った（表 3）[15]．

D. 麦角系ドパミンアゴニストはまだ必要なのか？

Rascol らは，麦角系 DA アゴニストの線維症，心臓弁膜症問題が脚光を浴びる前に，現在の PD 治療は有効性と安全性の観点からは理想的な治療法はなく不完全であると述べている[16]．PD 治療薬の種類は着実に増えてきているが依然として状況は Rascol の指摘したとおりである．

表2 欧州医薬品審査庁（EMEA）による麦角系ドパミンアゴニスト使用に関する新規制

European Medicines Agency
Press office

London, 26 June 2008
Doc. Ref. EMEA/CHMP/322395/2008

PRESS RELEASE
EMEA recommends new warnings and contraindications for ergot-derived dopamine agonists

The European Medicines Agency has recommended updating the product information for ergot-derived dopamine agonists with new warnings and contraindications in relation to the risk of fibrosis.

Ergot-derived dopamine agonists are mainly used to treat Parkinson's disease. The class comprises bromocriptine, cabergoline, dihydroergocryptine, lisuride and pergolide, all five of which are authorised at the level of the Member States.

At its June 2008 meeting, the Agency's Committee for Medicinal Products for Human Use (CHMP) finalised a review of the safety of ergot-derived dopamine agonists in relation to the risk of fibrosis (the formation of fibrous tissue in some body structures), particularly cardiac fibrosis, associated with their chronic use.

The development of the symptoms of fibrosis is a known side effect of ergot-derived dopamine agonists. However, the CHMP has reviewed new scientific data showing an increased risk of fibrosis in patients receiving ergot-derived dopamine agonists as chronic treatment, suggesting that fibrosis can start to develop far before the occurrence of symptoms.

Finalising the review of the new data the CHMP has concluded that the marketing authorisations for these medicines should be maintained, but that new warnings and contraindications should be added to their product information to reduce the risk of fibrosis.

As the risk of fibrosis is not equally established for all ergot-derived dopamine agonists, the CHMP recommended updating their prescribing information as follows:

- For cabergoline and pergolide, for which the prescribing information currently includes a contraindication for patients with evidence of valve problems and a restriction to second-line use in patients with Parkinson's disease:
  - a warning stating that patients must be monitored for signs of fibrosis with echocardiography before treatment is started and regularly during treatment;
  - a reduction of the maximum recommended dose to 3 mg per day;
  - inclusion of cardiac fibrosis as a very common side effect.
- For bromocriptine and dihydroergocryptine:
  - a contraindication for patients with pre-existing valve problems.
- For bromocriptine:
  - restriction of the maximum dose to 30 mg per day.
- For bromocriptine, dihydroergocryptine and lisuride:
  - a warning on the possible risk of fibrosis in patients taking these medicines at high doses for long periods.

Doctors should prescribe ergot-derived dopamine agonists according to the updated prescribing information and should monitor the development of fibrosis in patients in the heart and elsewhere in the body throughout treatment. Patients should speak to their doctor or pharmacist if they have any questions.

--ENDS--

NOTES

1. More information is available in a question-and-answer document.
2. The review procedure was initiated by United Kingdom under Article 31 of Directive 2001/83/EC, as amended, following studies suggesting that the risk of cardiac fibrosis associated with the chronic use of ergot-derived dopamine agonists was greater than previously identified. An article 31 referral may be initiated in specific cases where the interest of the Community is involved. The expression 'Community interest' has a broad meaning but it refers particularly to the interests of the public health in the Community, for example following concerns related to the quality, efficacy and/or safety of a medicinal product or new pharmacovigilance information.
3. This press release, together with other information about the work of the EMEA, may be found on the EMEA web site at http://www.emea.europa.eu.

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（文献 14）

麦角系 DA アゴニストは副作用の観点からは線維症という避けることのできない問題があることは誰もが認めざるを得ないが，非麦角系 DA アゴニストも眠気，下肢の浮腫，精神症状の増加の問題が指摘されている[17]．眠気は患者の QOL を阻害することになるし，仕事をしている患者では大きな支障になる．理想的な薬剤がない以上，一人一人の患者の生活状況を考えた治療が重要である．麦角系 DA アゴニストの線維症が問題だから非麦角系 DA アゴニストを使用するというだけでは適切な治療となり得ないことを認識しなければならない．この故に現時点では麦角系 DA アゴニストの市場からの退場は適切な決断であったとはいえず，PD 治療における一定の役割はまだ存在すると考えられる．

私たちには，安全性と有効性が十分な理想的な治療薬が登場するまでは，麦角系 DA アゴニストと非麦角系 DA アゴニストをうまく使い分ける十分な知識と経験が依然として必要とされてい

表3　カベルゴリンのパーキンソン病に係る用法・用量について

薬食審査発第 0829001 号
平成 20 年 8 月 29 日　　厚生労働省医薬食品局審査管理課長

記

1．カベルゴリンのパーキンソン病に係る用法・用量について

通常，成人にはカベルゴリンとして 1 日量 0.25 mg から始め，2 週目には 1 日量を 0.5 mg とし，以後経過を観察しながら，1 週間毎に 1 日量として 0.5 mg ずつ増量し，維持量を定めるが，最高用量は 1 日 3 mg とする．いずれの投与量の場合も 1 日 1 回朝食後経口投与する．

（参考）カベルゴリンのパーキンソン病に係る現在の用法・用量

通常，成人にはカベルゴリンとして 1 日量 0.25 mg から始め，2 週目には 1 日量を 0.5 mg とし，以後経過を観察しながら，1 週間毎に 1 日量として 0.5 mg ずつ増量し，維持量（標準 1 日量 2～4 mg）を定める．いずれの投与量の場合も 1 日 1 回朝食後経口投与する．

なお，年齢，症状により適宜増減する．

なお，今回の措置は，1 日 3 mg を超えて投与し，病状が安定している場合等にあっては，副作用の発現に十分留意する必要があるものの，速やかに減量することを求めるものではないことを，今回の用法・用量の変更に関する情報提供に併せて，医療機関へ情報提供を行うこと．

（文献 15）

る．

■E．麦角系ドパミンアゴニストの適正使用について

欧米のみならず我が国の規制当局（PMDA）は事後処理型かつ欧米追随型の規制であり，事前に想定されうる副作用を考えて行動しているとは考えられない．例えば，薬剤の効能効果・副作用等を記載した添付文書には，麦角系のみならず非麦角系 DA アゴニストの薬理作用の詳細は記載されていない．麦角系 DA アゴニストに対する心臓弁膜症を始めとした線維症の警告は大きく目を引くように記載されているが，なぜそのようなことが生じるのか，頻度はどの程度なのかという基本的なことの記載はないし，そのような記載に至ったエビデンスさえ示されていない．2008 年に EMEA は麦角系 DA アゴニストの使用量の規制を公表したが，日本はそれに追随しただけである．両者ともその規制となるエビデンスは示していないのである．カベルゴリンの 1 日最大使用量は 3 mg とされたが，我が国の心エコー検査によるエビデンスでは 3 mg では安全性は確保できないと考えられる[18,19]．心臓弁膜症を起こす原因としてセロトニン 2B 受容体に対する刺激が関与していると推定されているが，セロトニンおよびペルゴリドとカベルゴリンの受容体への親和性は同一であり（図 6）[20]，両薬剤の分子量は 1：1.1 とほぼ同一である（図 8）．我が国での最高規制用量はペルゴリド 1.25 mg とカベルゴリンは 3 mg であるが，これを考慮すればカベルゴリンの安全性は保証できないことになる．さらに，この規制用量よりも低用量で両薬剤では心臓弁膜症を生じた例が報告されており我が国の厚生労働省の規制はエビデンスに基づいていない無責任なものだと言えよう．そして，麦角系 DA アゴニスト使用時の規制決定に際して，規

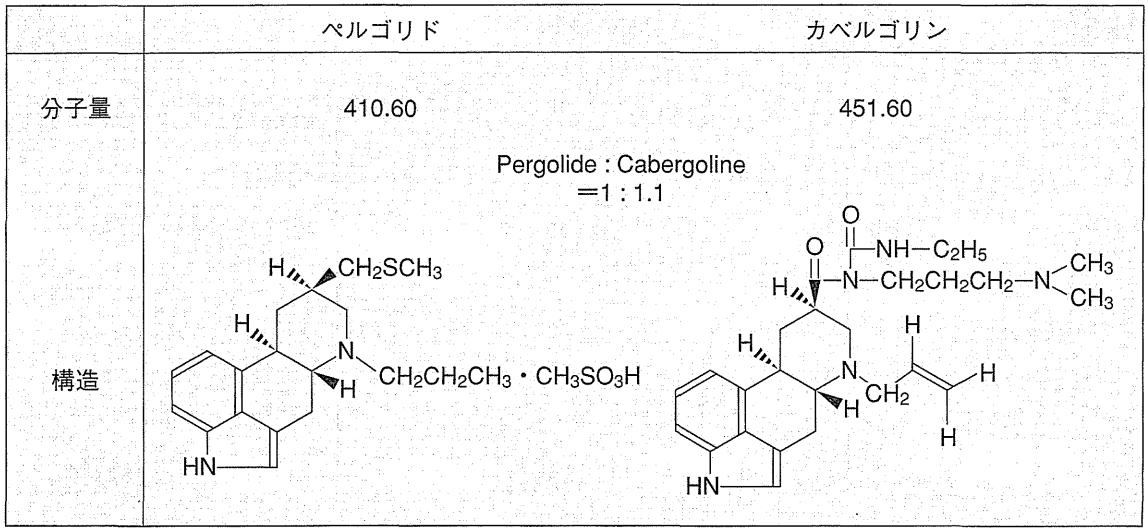

図8　ペルゴリドとカベルゴリンの分子量と構造式

制当局（PMDA）はこの領域の専門家を顧問としてもっていなかったし，専門家にコンサルトすることはなかった．

一方，麦角系 DA アゴニストによって生じた副作用による健康被害の補償に関して，補償が行われたか否かに関しては，製薬企業は現時点（2009 年 3 月）では把握していない．PMDA の web-site での検索では 1 例だけ申請があり，給付が認められていた[21]．医薬品副作用補償制度の機能は果たしていなかった．これは医師と製薬企業の両方に責任があるといえる．

製薬企業も心臓弁膜症の実態を規制当局による DA アゴニストの使用上の警告が示されるまで，心臓弁膜症が我が国で企業に報告されていた事実を医師の要請がない限り具体的な事例を明らかにしてこなかった．理由は報告した医師より公表の許可を得ていないと説明されてきたが，個人情報とは異なるし，公益性を考えると規制当局と製薬企業の態度は倫理的には許容の範囲外だと言えよう．こうした両者の行動パターンは現在でも観察されるところであり，非麦角系 DA アゴニストの市販後の副作用調査が公開された時には心臓弁膜症と同じ事態の繰り返しを確認できる可能性がある．

結局，副作用の最終責任は使用した医師の側に大きくのしかかる構図になっている限りは医師を始めとする医療者は薬剤情報に対する情報検索能力を高め，薬剤の薬理作用を十分に知ることが求められる．我が国の臨床薬理学の教育はないも同然であり，その専門家と称する人たちはしばしば製薬企業の立場に立つことも多い．専門家と称される人達の言う言葉には常に用心が必要だと言えよう．

結　語

麦角系 DA アゴニストと線維症問題から我々は多くのことを学ぶことができるし，学ばなければならない．以下に問題点を要約して指摘するが，規制当局と製薬企業は薬剤の副作用の責任を医師に転嫁することになってしまった．これに対して医師，および学会は守勢に回ってしまった．

以下に麦角系 DA アゴニストと線維症をめぐる事実を要約して今後に生かしたいと思う．この問題は近い将来，別の薬剤において生じる可能性がないとは言えないからである．

1）麦角系 DA アゴニストが登場した時点ですでに麦角系物質で線維症は報告されていた．麦角系 DA アゴニストにおいても同様にリスクがあることを製薬企業は知っていたはずである．

2）製薬企業は麦角系 DA アゴニストの多様な薬理作用を医師に十分に説明していなかった．ドパミン受容体刺激作用だけを強調していた．

3）医師は麦角系物質の薬理作用を十分に理解していたとは言えなかった．

4）外国で報告された副作用情報をもとに我が国で迅速に実態を調べる体制がなかったため，その後の副作用による使用の規制は欧米に倣うことになった．

5）製薬企業はこの規制が生じるまで各企業が有していた心臓弁膜症の報告を，情報提供者の承諾がないとの理由で医師からの要請がない限り積極的に公開することはなかった．

6）規制当局の一方的規制は我が国では薬剤使用の急激な変化を起こし，非麦角系 DA アゴニストの副作用（睡眠発作など）問題を生じることになった．全てがハッピーな事はないのである．

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Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy

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LETTER

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Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy

Mariko Taniguchi-Ikeda[1,2]*, Kazuhiro Kobayashi[1]*, Motoi Kanagawa[1], Chih-chieh Yu[1], Kouhei Mori[1], Tetsuya Oda[1], Atsushi Kuga[1], Hiroki Kurahashi[3], Hasan O. Akman[4], Salvatore DiMauro[4], Ryuji Kaji[5], Toshifumi Yokota[6], Shin'ichi Takeda[7] & Tatsushi Toda[1]

Fukuyama muscular dystrophy (FCMD; MIM253800), one of the most common autosomal recessive disorders in Japan, was the first human disease found to result from ancestral insertion of a SINE-VNTR-*Alu* (SVA) retrotransposon into a causative gene[1–3]. In FCMD, the SVA insertion occurs in the 3′ untranslated region (UTR) of the *fukutin* gene. The pathogenic mechanism for FCMD is unknown, and no effective clinical treatments exist. Here we show that aberrant messenger RNA (mRNA) splicing, induced by SVA exon-trapping, underlies the molecular pathogenesis of FCMD. Quantitative mRNA analysis pinpointed a region that was missing from transcripts in patients with FCMD. This region spans part of the 3′ end of the fukutin coding region, a proximal part of the 3′ UTR and the SVA insertion. Correspondingly, *fukutin* mRNA transcripts in patients with FCMD and SVA knock-in model mice were shorter than the expected length. Sequence analysis revealed an abnormal splicing event, provoked by a strong acceptor site in SVA and a rare alternative donor site in *fukutin* exon 10. The resulting product truncates the fukutin carboxy (C) terminus and adds 129 amino acids encoded by the SVA. Introduction of antisense oligonucleotides (AONs) targeting the splice acceptor, the predicted exonic splicing enhancer and the intronic splicing enhancer prevented pathogenic exon-trapping by SVA in cells of patients with FCMD and model mice, rescuing normal *fukutin* mRNA expression and protein production. AON treatment also restored fukutin functions, including *O*-glycosylation of α-dystroglycan (α-DG) and laminin binding by α-DG. Moreover, we observe exon-trapping in other SVA insertions associated with disease (hypercholesterolemia[4], neutral lipid storage disease[5]) and human-specific SVA insertion in a novel gene. Thus, although splicing into SVA is known[6–8], we have discovered in human disease a role for SVA-mediated exon-trapping and demonstrated the promise of splicing modulation therapy as the first radical clinical treatment for FCMD and other SVA-mediated diseases.

FCMD (incidence 1/34,000 births) shares phenotypic similarities with other severe muscular dystrophies, including muscle–eye–brain disease and Walker–Warburg syndrome. All show deficiencies in *O*-glycosylation of α-DG, an extracellular protein anchored on the plasma membrane. Insufficient *O*-glycosylation interferes with the ability of α-DG to interact with extracellular matrix proteins such as laminin[9,10]. For this reason, FCMD, muscle–eye–brain disease and Walker–Warburg syndrome are categorized as 'α-dystroglycanopathies (α-DGpathy)'[10]; so far, no effective treatments exist for these conditions. SVA is a hominid-specific, composite non-coding retrotransposon that contains SINE (short interspersed sequence), VNTR (variable number of tandem repeat), and *Alu* sequences. It is still active in humans, polymorphic and mobilized by the human LINE-1 *in trans*[6,11–15].

In previous work, we showed that *fukutin* mRNA (10 exons, 7.4- and 6.4-kilobase (kb) cDNAs in size with two poly-A sites, 461-amino-acid protein with calculated molecular mass of 53.7 kDa) was not detectable by northern blot analysis in patients with FCMD carrying the SVA insertion[2]. To investigate the aetiology of this decreased expression, we have now analysed whole *fukutin* mRNA in lymphoblasts from patients with FCMD using quantitative PCR with reverse transcription (qRT–PCR). PCR products corresponding to the protein-coding region of *fukutin*, as well as those including sequences in the distal part of the 3′ UTR (and thus downstream of the SVA insertion), were similar in abundance to those from an unaffected control (Fig. 1a). However, products located at sequence positions within the 3′ UTR were markedly decreased relative to the control. From these results and along with previous reports of many 3′ and 5′ splice sites within SVA elements[6–8], we hypothesized that abnormal splicing occurs somewhere between the end of the fukutin protein-coding region and the SVA insertion.

We then performed long-range RT–PCR using primers that flank the region corresponding to decreased expression. In patients with FCMD, we detected a single 3-kb PCR product, which is shorter than the 5-kb product seen in the normal control (Fig. 1b). This observation was consistent in several tissue types from patients with FCMD (Supplementary Fig. 1). PCR from genomic DNA produced an 8-kb product in patients with FCMD, compared with a 5-kb product in the control (Fig. 1b). Sequence analysis of the 3-kb product from FCMD cDNA revealed a splicing event (Supplementary Fig. 2). This event generates a new donor-side breakpoint within the final coding exon (exon 10), located 116 base pairs (bp) upstream from the authentic stop codon. A rare alternative donor site at that position is activated and trapped by an alternative acceptor site located within the inserted SVA, creating an additional and aberrant exonic sequence (exon 11) (Fig. 1c). The acceptor-side breakpoint is located 274 bp downstream from the start of the SVA insertion, between ag and TC (Fig. 1c). The acceptor site has not been described in the previous reports of SVA splicing[6,7]. This location is preceded by a pyrimidine-rich stretch, the SVA $(TCTCCC)_{41}$ hexamer at the 5′ end of the SVA element, with a possible favourable branch point. Predicted exonic splicing enhancer sites occur around 70 bp downstream from the new acceptor site. We confirmed that the aberrant splicing event can be abolished by replacing AG with GG at the acceptor junction in cultured cells transfected with a *fukutin* construct carrying SVA insertion (Supplementary Fig. 3). *Fukutin* expression was not altered by cycloheximide treatment, indicating that the transcript was not subject to nonsense-mediated mRNA decay, possibly because this exon-trapping occurred within the last

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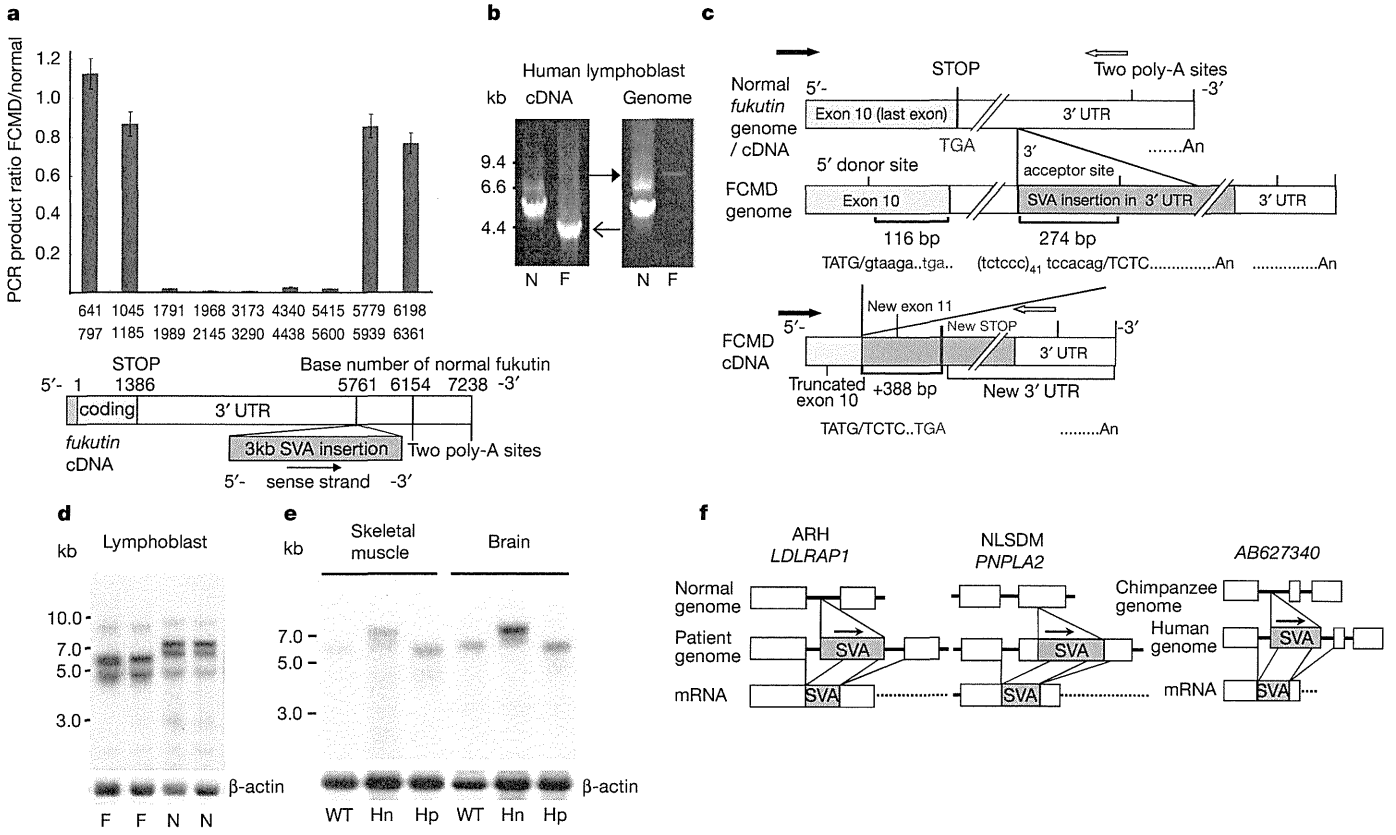

Figure 1 | An SVA retrotransposal insertion induces abnormal splicing in FCMD. **a**, Expression analysis of various regions of *fukutin* mRNA in lymphoblasts. Grey bar, the ratio of RT–PCR product in patients with FCMD relative to the normal control; numbers on the *x* axis, nucleotide positions of both forward and reverse primers in *fukutin*. Error bars, s.e.m. **b**, Long-range PCR using primers flanking the expression-decreasing area (nucleotide position 1,061–5,941) detected a 3-kb PCR product in FCMD lymphoblast cDNA (open arrow) and an 8-kb product in FCMD genomic DNA (filled arrow). In the normal control, cDNA and genomic DNA both showed 5-kb PCR products. The 8-kb band was weak, probably because VNTR region of SVA is GC-rich (82%). **c**, Representation of genomic DNA and cDNA in FCMD. Black and white arrows, forward and reverse sequencing primers. The intronic sequence in FCMD is indicated in lower case. The authentic stop codon is coloured red, and the new stop codon is coloured blue. **d, e**, Northern blot analysis of *fukutin* in human lymphoblasts (**d**) and model mice (**e**); F, FCMD; N, normal control. The wild-type mouse *fukutin* mRNA was detected at a size of 6.1 kb. Both skeletal muscle (left) and brain (right) showed smaller, abnormal bands in Hp/Hp mice. WT, wild type; Hn, Hn/Hn mice; Hp, Hp/Hp mice. **f**, Representation of genomic DNA and cDNA in ARH (*LDLRAP1*, left), NLSDM (*PNPLA2*, middle) and human (*AB627340*, right).

exon, and the new stop codon exists downstream of the new last exon–exon junction (Supplementary Fig. 4).

We have recently generated knock-in mice that carry a humanized fukutin exon 10, which either includes (Hp allele) or excludes (Hn allele) the SVA insertion, and bred these strains with heterozygous fukutin knockout mice to obtain compound heterozygotes (Hp/−)[16]. Knock-in mice that are homozygous (Hp/Hp) and compound heterozygous (Hp/−) are representative of the human FCMD alleles. These mice exhibit hypoglycosylation of α-DG in skeletal muscle, which is the most significant characteristic in α-DGpathy[16]. Quantitative RT–PCR in various tissues from Hp/Hp mice revealed an aberrant splicing pattern identical to that seen in human patients (Supplementary Fig. 5). Northern blot analysis detected abnormally spliced *fukutin* mRNA species at the expected sizes of 5.6 and 4.6 kb in patients with FCMD, whereas the normal *fukutin* mRNAs appeared at 7.4 and 6.4 kb (Fig. 1d and Methods). We replicated these results in the knock-in model mice (Fig. 1e and Supplementary Fig. 6a). The consistent observations between patients with FCMD and knock-in model mice lead us to conclude that a splicing abnormality underlies the pathogenesis of FCMD.

Abnormal splicing excises the authentic stop codon and produces another stop codon located 388 bp downstream from the 5′ side of the new exon 11 (Fig. 1c). The predicted protein lacks the C-terminal 38 amino acids of fukutin, instead containing 129 amino acids derived from the SVA sequence (Supplementary Fig. 7). Endogenous fukutin is scarce and difficult to detect; however, we were able to identify both normal and aberrant forms of the protein in human and mouse using immunoprecipitation followed by western blot analysis. The abnormal fukutin protein in FCMD displayed the predicted mobility shift (Fig. 2a–c and Supplementary Fig. 6b).

We introduced normal and aberrantly spliced *fukutin* cDNA constructs into mammalian cell lines. Whereas normal fukutin localized to the Golgi apparatus, the aberrantly spliced fukutin protein is displaced completely from the Golgi to the endoplasmic reticulum (Fig. 2d and Supplementary Fig. 8). Further examination showed that a *fukutin* construct lacking the C-terminal 38 amino acids also mislocalized to the endoplasmic reticulum (Fig. 2d and Supplementary Fig. 8), suggesting that the C-terminal domain of fukutin is important for localization to the Golgi. Thus, impairment of this domain may lead to fukutin dysfunction in FCMD. The mislocalization is unlikely to be toxic because FCMD is an autosomal recessive disease and heterozygous carriers of the SVA insertion have no symptoms.

We next tested if exon-trapping occurs in other diseases with SVA insertion[6]. In a patient with autosomal recessive hypercholesterolemia (ARH), a 2.6-kb SVA was inserted within intron 1 of the *LDLRAP1* gene[4]. A patient with lipid storage disease with subclinical myopathy (NLSDM) also had a 1.9-kb SVA insertion in exon 3 of the *PNPLA2* gene[5]. We found abnormally spliced products induced by SVA exon-trapping in these patients' fibroblast (Fig. 1f left and middle panels, Supplementary Figs 9 and 10, and Supplementary Table 1). Cycloheximide treatment to fibroblasts from these patients increased expression of the genes (Supplementary Figs 9a and 10a), suggesting

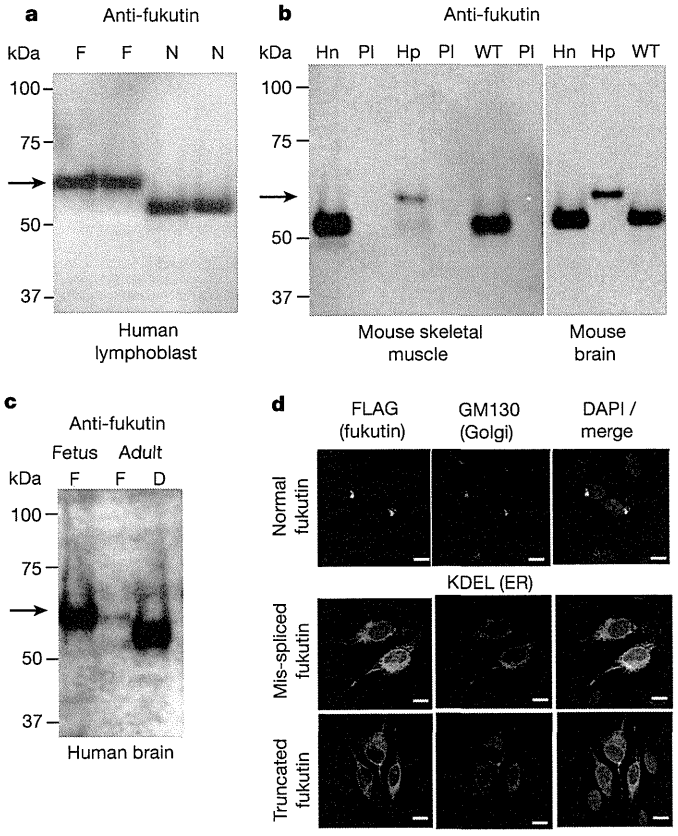

Figure 2 | Abnormal fukutin protein in FCMD. **a**–**c**, Immunoprecipitation analysis of fukutin protein in human lymphoblasts (**a**), both skeletal muscle and brain tissues from Hp/Hp mice (**b**) and brain tissue from patients with FCMD (**c**); filled arrow, abnormal fukutin; N, normal sample; F, sample from patient with FCMD; Hn, Hn/Hn mice; Hp, Hp/Hp mice; PI, pre-immune serum; D, patient with Duchenne muscular dystrophy. **d**, The subcellular localization of fukutin. Top, normal fukutin; middle, mis-spliced fukutin; bottom, truncated fukutin. Stained with anti-FLAG (left, to detect fukutin), anti-GM130 (middle, Golgi marker, top) and anti-KDEL (endoplasmic reticulum marker, middle and bottom), and merge (right, with DAPI stain). Scale bar, 10 μm.

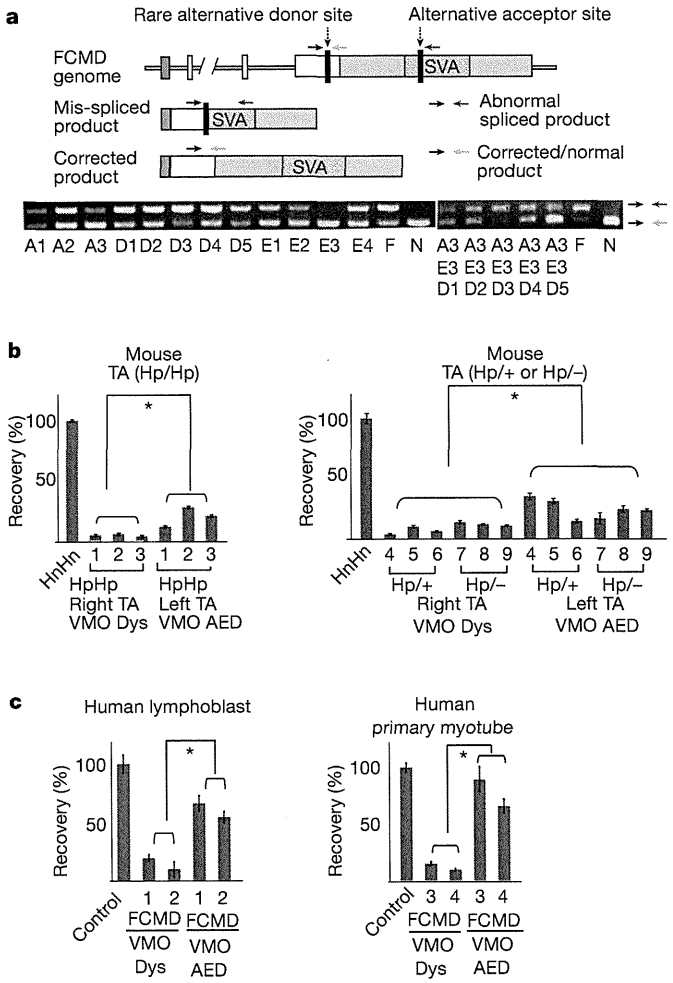

Figure 3 | AON cocktail rescues normal *fukutin* mRNA. **a**, RT–PCR diagram of three primers designed to assess normal *fukutin* mRNA recovery (upper). Black arrow, a common forward primer located on *fukutin* coding region; dark grey arrow, a reverse primer to detect the abnormal RT–PCR product (161 bp); light grey arrow, the other reverse primer to detect the restored normal RT–PCR product (129 bp). The effect on Hp/Hp ES cells treated with each single or a cocktail of AONs (lower). F, FCMD; N, normal sample. **b**, Rescue from abnormal splicing in VMO-treated Hp/Hp and Hp/− mice. Local injection of AED cocktail into tibialis anterior ($n = 3$). Dys, a negative control. **c**, Rescue from abnormal splicing in VMO-treated human FCMD lymphoblasts (left, $n = 2$) and myotubes (right, $n = 2$). The y axis shows the percentage recovery of normal mRNA (*$P < 0.01$ by Student's t-test). TA, tibialis anterior. Error bars, s.e.m.

that the SVA-trapped transcripts are likely to be subjected to nonsense-mediated mRNA decay[6,17]. In a search for the same events using the same acceptor site as FCMD in the human genome, we located two expressed sequence tags on human chromosome 4 (DA436529 and DA060755) that represent a spliced transcript induced by an SVA element. We found exonization in a human-specific insertion of SVA (AB627340) into a small gene (Fig. 1f right panel and Supplementary Fig. 11). The human-specific exon-trapping of SVA in the small gene might influence human evolution and development.

FCMD alleles of the *fukutin* gene contain a fully intact protein coding sequence, raising the possibility that FCMD could be treated by restoring translation of the full-length protein through splicing modulation with AONs. To identify promising target sequences in various cell lines, we produced 25-mer 2′-O-methyl phosphoramidite (2′OMePS) AONs targeted to the acceptor (A1–A3), donor (D1–D5) and exonic splicing enhancer sites (E1–E4) in *fukutin* pre-mRNA (Supplementary Fig. 12). We introduced the AONs into various cell types and assessed the recovery of normal processing and restoration of the authentic stop codon (Fig. 3a). Cells with A3 and E3 showed strong suppression of SVA-derived splicing. The greatest recovery of *fukutin* mRNA, to levels of more than 40% of the normal control, was achieved with a combination of A3, E3 and D5 (AED) (Fig. 3a). The D5 sequence overlaps with a predicted intronic splicing enhancer site within the aberrant intronic sequence; in normal *fukutin*, this sequence resides in exon 10 (Supplementary Fig. 12).

We injected octa-guanidine morpholino oligonucleotide (vivo-morpholino, VMO)[18] AED cocktail locally into skeletal muscle of knock-in mice and evaluated the therapeutic effect by calculating the percentage recovery of normally processed mRNA. In the AED-treated tibialis anterior and gastrocnemius of Hp/Hp and Hp/− mice, the amount of corrected *fukutin* mRNA increased significantly relative to mice treated with control VMO (Fig. 3b and Supplementary Fig. 13). We assessed fukutin protein recovery in injected skeletal muscle tissue from Hp/Hp mice. Consistent with the significant increase of restored normal mRNA, normal fukutin protein was rescued (Fig. 4a). We examined α-DG glycosylation in AED-treated Hp/− mice. Deficiently glycosylated α-DG, at the predicted smaller size, was reduced in abundance, whereas normal-sized α-DG increased after AED treatment (Fig. 4b). The signal intensity for glycosylated α-DG was clearly increased, and a shift in the α-DG core was observed, indicating that the rescued fukutin is functional. Laminin overlay assays revealed a marked increase in α-DG laminin-binding ability, indicating that α-DG

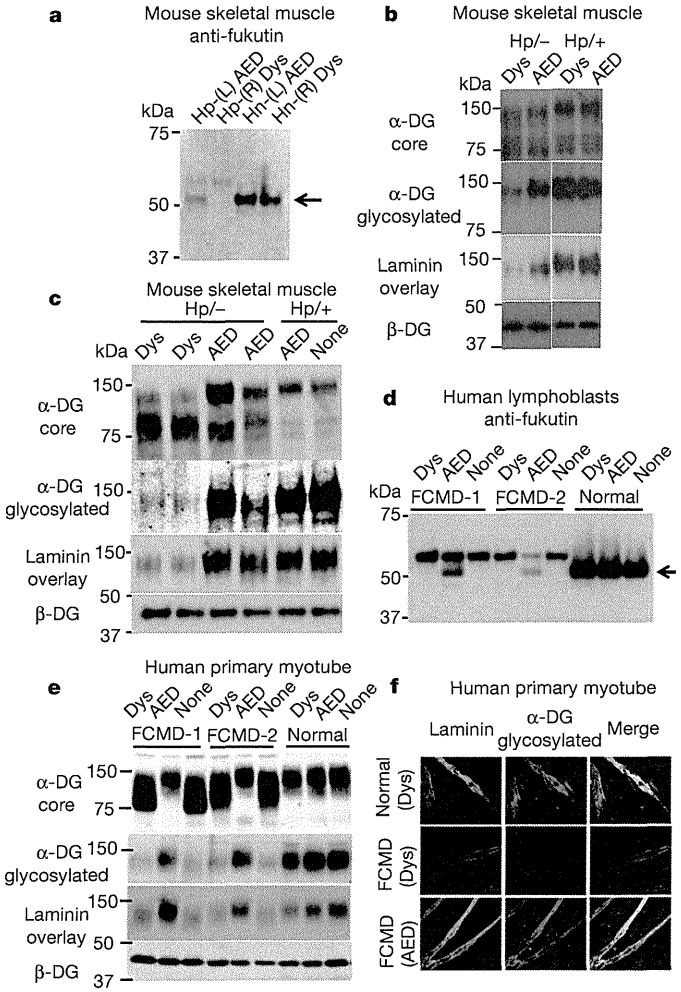

Figure 4 | AON cocktail treatment rescues normal fukutin protein and functional α-DG. a, d, Immunoprecipitation analysis of fukutin protein after local treatment with VMO (AED) in FCMD model mice (**a**) and human FCMD lymphoblasts (**d**). Arrow, normal fukutin protein. L, left tibialis anterior; R, right tibialis anterior; Dys, negative control. **b, c, e,** Tibialis anterior muscle after local (**b**) or systemic (**c**) treatment with AED and human FCMD lymphoblasts treated with the AED (**e**) were analysed by western blot using antibodies against α-DG core protein (top panel) and glycosylated α-DG (second), and by a laminin overlay assay (third). Bottom, β-DG (internal control). **f,** Laminin clustering assay. Left, anti-laminin; middle, anti-glycosylated α-DG; right, merged images. Upper, normal myotubes treated with control VMO; middle, FCMD patient myotubes treated with control VMO; bottom, FCMD patient myotubes treated with AED.

function also is recovered (Fig. 4b). We next tested systemic AED treatment by intravenous injection of Hp/− mice. This treatment also showed the recovery of normally glycosylated α-DG in AED-treated mice (Fig. 4c).

We administered the VMO AED cocktail to human lymphoblasts and myotubes. As in knock-in mice, we observed successful correction of the splicing abnormality. The corrected *fukutin* mRNA was restored to 50% or more of the levels seen in normal controls (Fig. 3c). We believe this to be sufficient recovery, considering that unaffected FCMD carriers have only 50% of normal *fukutin* mRNA. Finally, we tested recovery of the fukutin protein and the glycosylation of α-DG in the cells of patients with FCMD. Not only was normal fukutin protein expression significantly rescued in AED-treated lymphoblasts (Fig. 4d), but also we observed recovery of normally glycosylated α-DG in AED-treated myotubes (Fig. 4e). Immunofluorescence staining also showed immensely increased glycosylated α-DG (Fig. 4f). A laminin clustering assay showed increased laminin clustering ability, which is characteristically absent in α-DGpathy[19] (Fig. 4f). These data show that AED treatment effectively rescues normal fukutin, confirming our observation of abnormal *fukutin* splicing and raising the possibility of splicing modulation therapy as the first treatment for FCMD. To treat neuronal migration disorder of FCMD, prenatal treatment may be necessary, but it is currently difficult for ethical and technical reasons. Nevertheless, improving even only the muscular symptoms would greatly ameliorate quality of life of the patients as well as their families.

Retrotransposons account for nearly half of the human genome[20]. Increased numbers of reports have highlighted positive and negative contributions of retrotransposons to human health and disease[21,22]. In addition to being the causative factor for FCMD, ARH and NLSDM, SVA insertions have also been implicated in hereditary elliptocytosis, X-linked agammaglobulinemia, neurofibromatosis type 2 and X-linked dystonia-Parkinsonism[12,23–26]. It has been suggested that SVA insertions cause such diseases through genomic deletion, reduced mRNA expression or skipping of neighbouring exons[17,22]. Recently, SVA splicing has been suggested to generate variation within and across species by activating functional 3′ splice sites within SVAs across the human genome, controlling gene transcription, creating alternative splicing by exon-trapping, or inducing premature stop codons, and was experimentally demonstrated[6]. Our findings emphasize the importance of SVA functions in human disease and support the possibility of radical treatment against SVA-induced disease by splicing modulation therapy. AONs have become one of the most promising and practical candidate chemicals for splicing modulation therapy in cancer[27], infectious diseases[28] and Duchenne muscular dystrophy[29,30]. In demonstrating the ability of AONs to rescue fukutin function in FCMD, we introduce a novel clinical role for them in treating FCMD and other SVA-mediated diseases, while providing new insights about the influence of SVAs on human evolution, development and disease.

METHODS SUMMARY

For AON treatment, 25-mer 2′OMePS (GeneDesign and Invitrogen) and octaguanidine morpholino (VMO; Gene-Tools) were used. The knock-in mouse was produced as described previously[16].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions M.T.-I., K.K., M.K. and T.T. designed the study. M.T.-I. performed most of the experiments. K.K. developed a system to detect endogenous fukutin protein. M.K. performed biochemical analysis of VMO-injected mice. C.Y. produced the *fukutin* cDNA constructs for transfection experiments. K.M., T.O., and A.K. performed analyses of AON treatment in mice and various cell types. H.K., T.Y. and S.T. provided intellectual input. H.O.A., S.D. and R.K., provided patients' samples. M.T.-I., K.K. and T.T. wrote the paper.

Author Information The patient *fukutin* and a chimpanzee mRNA sequences are deposited in GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan under accession numbers AB609007 and AB627340, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to T.T. (toda@med.kobe-u.ac.jp).

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Endoplasmic reticulum stress response in P104L mutant caveolin-3 transgenic mice

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Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy 1C (LGMD1C). However, the precise molecular pathogenesis of caveolin-3-related muscular dystrophy remains uncertain. Here, we demonstrate the effect of gene dosage on the severity of the myopathic phenotype in P104L mutant caveolin-3 (mCav3^{P104L}) transgenic mice, a model of LGMD1C. We analyzed the endoplasmic reticulum (ER) stress response in the transgenic mice and found upregulated transcription of the molecular chaperone, glucose-regulated protein (GRP78). Moreover, signaling downstream of GRP78 in the myofibers was activated toward apoptosis. However, terminal transferase dUTP nick end labeling assays detected a few apoptotic nuclei in transgenic mouse skeletal muscle, probably due to the transcriptional activation of Dad1, an anti-apoptotic factor in the ER. These findings suggest that the ER stress response caused by mCav3^{P104L} plays a role in the pathogenesis of LGMD1C as a toxic gain of function effect.

INTRODUCTION

Caveolae are characterized as flask-shaped invaginations of the plasma membrane (1). Caveolin is a major structural component of caveolae (2) and it also functions as a scaffolding protein to concentrate and regulate many classes of signaling molecules. Distinct genes encode the isoforms, caveolin-1, -2 and -3. Caveolin-3 is specifically expressed in muscle cells (3). Co-expressed caveolins-1 and -2 form hetero-oligomers in non-muscle cells, whereas caveolin-3 forms homo-oligomers in muscle cells. Different mutations in the human caveolin-3 gene have been associated with several muscle diseases that are collectively called caveolinopathies and include limb-girdle muscular dystrophy, distal myopathy and rippling muscle disease (4,5). A mutant caveolin-3 with a single amino acid substitution from proline to leucine at amino acid residue 104 (mCav3^{P104L}) was originally identified from a genetic analysis of autosomal dominant limb-girdle muscular dystrophy 1C (LGMD1C) (6).

Since LGMD1C is inherited as an autosomal dominant trait, mCav3^{P104L} presumably has a dominant-negative effect on the molecular pathogenesis of caveolinopathy. Studies *in vitro* have shown that homo-oligomers of wild-type caveolin-3 translocate to the cell membrane via the endoplasmic reticulum (ER)–Golgi network, whereas mCav3^{P104L} does not target the cell membrane (7). The hetero-oligomers formed between wild-type caveolin-3 and mCav3^{P104L} in the ER–Golgi system are degraded by the ubiquitin–proteasome proteolytic pathway, which might lead to the loss of caveolin-3 in LGMD1C (7,8). Targeted downregulation of caveolin-3 gene in differentiating C2C12 myoblasts can inhibit myotube formation (9). Expression of mCav3^{P104L} can trigger a loss of caveolin-3 during C2C12 cell differentiation (10). These results suggest that the secondary loss of caveolin-3 due to mCav3^{P104L} is associated with the molecular pathology of LGMD1C. However, whether mCav3^{P104L} has a gain of function effect that contributes to the pathogenesis of LGMD1C remains uncertain.

The ER stress response to the accumulation of a mutant protein has recently garnered interest among those investigating the pathogenesis of neurodegenerative disorders. For instance, the cytoplasmic aggregation of alpha-synuclein that is a pathological hallmark of Parkinson's disease is accelerated in a form of familial Parkinson's disease caused by a dominant mutation of the alpha-synuclein gene (11). In addition, several genes responsible for autosomal recessive Parkinson's disease

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are involved in the ER-associated ubiquitination system (12). However, the ER stress response has not been intensively studied from the viewpoint of muscular dystrophy. We previously generated and studied mCav3^{P104L} transgenic mice as a model of LGMD1C (13–15). The present study investigates the ER stress response in mCav3^{P104L} transgenic mice as well as a possible gain of function effect of mCav3^{P104L}.

RESULTS

Dose effect of mCav3^{P104L} transgene on the severity of the myopathic phenotype

We previously identified a severe myopathic phenotype in mCav3^{P104L} hemizygous transgenic mice (13). Homozygous mCav3^{P104L} mice were obtained by hemizygous intercrossing and southern blot analysis confirmed the wild-type, hemizygous and homozygous transgenic mouse genotypes (Fig. 1A). Western blot analysis detected less caveolin-3 protein in hemizygous than in homozygous mice (Fig. 1B). We then analyzed the effect of mCav3^{P104L} gene dosages on the myopathic phenotype. Consistent with our previous findings (13), hemizygous mice weighed less than wild-type mice, but the difference did not reach statistical significance. The difference in body weight between homozygous and wild-type mice was statistically confirmed from 4 and 12 weeks of age at all measured points (Fig. 1C). We also found that grip strength significantly differed between the groups from 4 and 12 weeks of age at most measured points (Fig. 1D). Central nucleation or mononuclear infiltration was essentially absent. We measured average areas of myofibers in quadriceps muscles from mice of each genotype (Fig. 1E). Small myofibers were more frequent in the order of homozygous > hemizygous > wild-type mice and non-parametrical statistical analysis confirmed significant differences among the groups (Fig. 1F). The frequency of small myofibers in the gastrocnemius muscle also significantly differed among the genotypes (Supplementary Material, Fig. S1A and B). These data showed that the dose of the mCav3^{P104L} transgene correlated with the severity of the myopathic phenotype.

Residual level of caveolin-3 protein and the myopathic phenotype

The residual amount of caveolin-3 protein in mCav3^{P104L} transgenic mice was <20% of that in wild-type mice (Fig. 1B). To confirm whether other caveolin isoforms counteract the loss of caveolin-3 in muscle cells, we analyzed the expression of caveolin-1 and -2 in mCav3^{P104L} transgenic mice. Messenger RNA levels of caveolin-1 and -2 were increased about 1.5-fold in the skeletal muscle of homozygous mice, whereas no significant change was detectable in hemizygous mice (Fig. 2A and B). Western blotting showed that these changes were consistent at the protein level (Fig. 2C and D). However, immunohistochemistry revealed that upregulated caveolins-1 and -2 were not localized to the sarcolemma, but to the muscle interstitial region containing blood vessels (Fig. 2E). These results indicated that caveolin-1 and -2 upregulation does not compensate for the lack of caveolin-3 function in skeletal muscle. Interestingly, less caveolin-3 protein was detected in hemizygous than in homozygous mice (Fig. 1B). Since the antibodies used for western blotting cannot distinguish between endogenous and mutant caveolin-3 protein, these results suggest that the increased amount of residual mCav3^{P104L} protein in homozygous mice led to more severe myopathy via a toxic gain of function effect.

Localization of mCav3^{P104L} protein and ER stress response

We investigated the subcellular localization of mCav3^{P104L} *in vitro*. Cosmids containing a fluorescent marker for the ER (DsRed-KDEL) or for the Golgi complex (DsRed-Golgi) were cotransfected with wild-type caveolin-3 or mCav3^{P104L} into C2C12 myoblasts. Immunostaining showed surface membrane localization of wild-type caveolin-3, whereas mCAV3^{P104L} did not target the surface membrane and tended to colocalize with the Golgi, but not the ER marker (Fig. 3). The Golgi marker and mCAV3^{P104L} also colocalized in COS7 cells (Supplementary Material, Fig. S2).

We investigated whether mCav3^{P104L} in muscle cells could induce the ER stress response in mCav3^{P104L} transgenic mice by analyzing the expression of genes related to ER stress. Messenger RNA levels of glucose-regulated protein (GRP78), a molecular chaperone in the ER (16), increased 1.5- and 2.5-fold in hemizygous and homozygous mice, respectively, compared with wild-type mice (Fig. 4A and C). Western blotting also confirmed GRP78 induction at the protein level (Fig. 4B and D). Eukaryotic initiation factor 2α (eIF2α) is phosphorylated in response to GRP78 induction under ER stress (17,18). Although the total amount of eIF2α did not significantly change, the amount of phosphorylated eIF2α was increased in transgenic compared with wild-type mice (Fig. 4B and E). We then evaluated the mRNA levels of C/EBP homologous protein (CHOP), which is a pre-apoptotic transcription factor that functions downstream of phosphorylated eIF2α (18,19), and found that they were significantly increased in mCav3^{P104L} transgenic mice (Fig. 4A and C). Overall, these results suggested that mCav3^{P104L} accumulates in the Golgi complex and induces the ER stress response mediated by the molecular chaperone GRP78 and its downstream pathway.

ER stress response toward apoptosis in myofibers from mCav3^{P104L} transgenic mice

We compared the consequences of the mCav3^{P104L}-induced ER stress response using terminal transferase dUTP nick end labeling (TUNEL) assays of quadriceps muscles from mCav3^{P104L} transgenic mice and other mouse models of muscular dystrophy, namely *mdx* and *dy* mice (Fig. 5A). Several TUNEL-positive nuclei of interstitial cells were located outside the myofibers of *mdx* mice and *dy* mice. To distinguish these cells from apoptotic myofibers, we counted the number of myofibers with TUNEL-positive nuclei. Unlike *mdx* mice and *dy* mice, TUNEL-positive nuclei in mCav3^{P104L} transgenic mice were mainly located inside myofibers. Although the average number of TUNEL-positive myofibers was increased in mCav3^{P104L} transgenic mice, the increase was modest compared with the amount in *mdx* mice and *dy* mice (Fig. 5B).

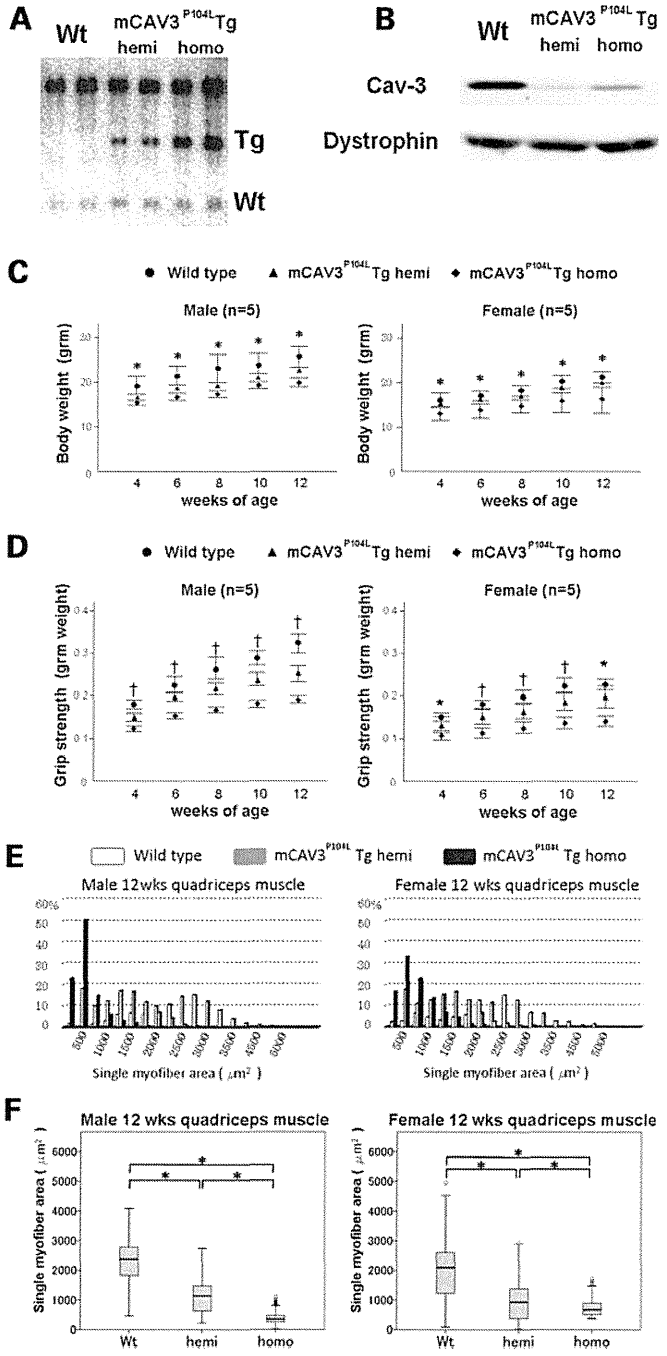

Figure 1. Effect of mCav3^{P104L} transgene dosage on the myopathic phenotype of LGMD1C model mice. (**A**) Genotyping of wild-type, hemizygous and homozygous transgenic mice by Southern blot analysis. A caveolin-3 DNA probe detected both endogenous caveolin-3 gene and mCav3^{P104L} transgene. Left to right, two independent samples of wild-type, hemizygous and homozygous transgenic mice. (**B**) Western blot analysis of caveolin-3 in skeletal muscles of wild-type and mCav3^{P104L} transgenic mice. Levels of caveolin-3 protein obviously decreased in the transgenic mice. Residual caveolin-3 was detected in homozygous mice. Temporal changes in body weight (**C**) and grip strength (**D**) of wild-type and transgenic mice between 4 and 12 weeks of age ($n = 5$ per group). Differences within each group were statistically determined using Scheffe's test. *Significant difference between wild-type and homozygous mice ($P < 0.05$). †Significant difference between each group ($P < 0.05$). Bars indicate standard error. (**E**) Histograms of individual myofiber areas in quadriceps muscle on transverse sections. Values were determined from 1000 myofibers per group. Bar = 100 μm. (**F**) Box plot represents non-parametric statistical analysis of myofiber areas of each group in (E). Significant difference between two groups (Mann–Whitney *U* test, *$P < 0.005$).

We immunohistochemically analyzed cleaved caspase-3 and cytochrome *c* to further elucidate the ER stress response toward apoptosis in myofibers. Caspase-3 is activated by proteolysis in the signaling cascade toward apoptosis (20). Cleaved caspase-3, which is an active form of caspase-3, is increased in response not only to ER stress but also to