200921001B

厚生労働科学研究費補助金（長寿科学総合研究事業）

高齢者の切迫性尿失禁に対する膀胱壁内A型ボツリヌストキシン注入療法の多施設臨床試験と腹圧性尿失禁に対する新規治療法の開発

（H19-長寿-一般-001）

平成19～21年度

総合研究報告書

平成22(2010)年3月

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総合研究報告書

高齢者の切迫性尿失禁に対する膀胱壁内A型ボツリヌストキシン注入療法の多施設臨床試験と腹圧性尿失禁に対する新規治療法の開発

研究代表者　岡村菊夫　国立長寿医療センター　手術・集中医療部長

研究要旨
高齢者の切迫性、腹圧性尿失禁に対する新規の治療法を開発することを目的とした。　切迫性尿失禁に対しては、非神経因性過活動膀胱に対するA型ボツリヌストキシン膀胱壁内注入療法と脊髄損傷による神経因性排尿筋過活動に対する A 型ボツリヌス毒素膀胱壁内注入療法の臨床試験を行い、中間解析の現時点では尿失禁の改善効果は極めて優れていると考えられた。自己骨格筋幹細胞を用いた再生治療の研究では、高齢者から摘出した微量の筋組織から筋幹細胞培養法の確立、培養の安全性を高めるための方法論の確立、ヒト細胞由来不死化細胞株の樹立、増殖ならびに筋細胞への分化の優れる細胞を培養に先立ち分取する方法、さらに培養により増殖させた筋細胞から選別する品質確保システムの確立、安全性に関する検定系を確立した。自己脂肪組織由来幹細胞を用いた再生治療の研究では、細胞分離装置（Cytori Therapeutics, Inc.）を用い臨床応用を果たした。5例に試み、1例が著効、3例が有効であった。今後、「幹細胞を用いる臨床研究に関する指針」に則ったトランスレーショナルリサーチに取り組んでいく所存である。

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A.　研究目的

高齢者の尿失禁には、切迫性、腹圧性、溢流性、機能性の4つのタイプが存在し、いずれのタイプの尿失禁も高齢者の QOL を大いに障害するとされている。これまでの治療法において切迫性尿失禁に対しては抗コリン薬内服が最も有効な治療法として行われているが、効果が不十分な症例も多い。また、認知症悪化、堪えがたい口内乾燥、便秘などの副作用があり、内服を継続できない高齢者も多い。こうした難治性の切迫性尿失禁に対しては、A型ボツリヌストキシンの膀胱壁内注入療法が有望視されている。この治療法が新たな治療法として確立できれば、抗コリン薬をもらうための通院が不要になるとともに Poly-pharmacy に対する対策にもなり、抗コリン薬が不適切な高齢者の治療が可能となろう。

一方、腹圧性尿失禁は咳・くしゃみ、重いものを持ち上げた時など腹圧が加わった時に生じ、女性に多く見られる。高齢男性においても前立腺肥大症、前立腺癌の手術後にかなりの頻度で発生することが知られている。骨盤底筋体操、TVT (Tension-free Vaginal Tape手術)手術があるが、前者は継続が難しく、後者は侵襲性・術後の尿排出障害の可能性を考慮すると高齢者には比較的行いがたい治療法となっている。コラーゲン尿道壁内注入療法は侵襲性が低く、短期的には有効とされているが、コラーゲンがいずれ吸収されるため再発率は極めて高い。しかし、注入物質が吸収されることもなく、その場所で膨隆（漏れに対する抵抗）となり、その上に括約筋が再生できるような注入治療であれば、有効性は極めて高くなることが予想される。そのような治療法として、自己骨格筋幹細胞や脂肪組織由来幹細胞を用いた再生治療が考えられる。現時点では、体外で培養した幹細胞を移植するような治療はGMPに基づいた設備や管理体制が必要とされるため、すぐに行えるわけではない。しかし、尿失禁を有する高齢者が今後ますます増加することを鑑みれば、高齢者に適した低侵襲治療の開発は急がねばならない課題であると考えられた。

B.　研究方法

1) 切迫性尿失禁に対するA型ボツリヌストキシン膀胱壁内注入療法

本研究では、抗コリン薬の効果が不十分であるか、副作用のため抗コリン薬の継続使用ができない非神経因性過活動膀胱と脊髄損傷による神経因性排尿筋過活動に対して、それぞれA型ボツリヌス毒素100単位と200単位の膀胱壁内注入療法の検討を多施設共同研究として行うこととした。

多施設（14施設）共同研究プロトコールは平成20年1月16日付で当院の倫理委員会で承認された後、「A型ボツリヌス毒素膀胱壁内注入による死亡例があること」が報道されたり、本省から研究費から入院治療にかかる費用を賄うよう指導があり、プロトコールの変更を余儀なくされた。調査の結果、死亡の原因は神経疾患を有する患者での誤嚥あるいは誤嚥性肺炎であり、A型ボツリヌス毒素との因果関係が明らかでないことがわかった。この研究では、嚥下困難のある症例は除外することとした。また、平成20年1月下旬に海外の研究者（ピッツバーグ大学 Michel Chancellor教授）からの神経因性排尿筋過活動に対しては投与量を200単位に減量した方がよいとのアドバイスがあり、投与量を300単位から200単位に減量することとした。また、平成20年3月25日付けで倫理委員会へ再審査依頼をしたが、9月24日まで倫理委員会が開催されず、承認を受けたのは10月に入ってからになってしまい、研究進行がおくれてしまう原因にもなった。

研究参加施設の中には当院より先に倫理委員会の再承認を得られた施設もあり、実際の登録は平成20年7月から始まった。しかし、ボツリヌス毒素の副作用として、「極めてまれだが死亡がある」との説明を聞いて研究参加をためらう患者も多く、症例登録がなかなか進まないため、2度に渡って、研究参加施設（新たに18施設）を拡充した。国立長寿医療センターホームページに研究に関する情報を掲載し、また、Uminの臨床試験情報にも掲載した。

注入方法は、脊髄麻酔、仙骨ブロック下に患者を砕石位として、尿道から膀胱鏡を入れ、三角部をさけて粘膜下層に注入する。非神経因性過活動膀胱症例ではBotox100単位を、神経因性排尿筋過活動症例ではBotox200単位を生理食塩水15mlで溶解して30箇所に注入する。

2) 自己骨格筋幹細胞を用いた再生治療

橋本が中心的役割を担い、上住（旧姓：池本)、宋、岡村がそれぞれの役割を分担した。橋本は自己骨格筋細胞移植による再生治療を可能とするために、効率がよく、安全性の高い細胞培養法の確立を目指して、分離・培養行程に関する安全性の見直しを行い、上住は移植細胞の品質管理システム（移植に適した筋細胞を識別し、移植に適した細胞のみを迅速かつ生きたまま単離する方法）の確立を目指し、宋は移植細胞の安全性検定系の確立を目指した。岡村は、これらの研究に使用されるヒト骨格筋組織を泌尿器科で何らかの開腹手術を行う患者から得た。

（倫理面への配慮）

動物およびヒト材料を用いた実験に関しては、国立長寿医療センターの動物実験倫理委員会、倫理委員会の承認を得、規定にしたがって実施した。

3) 脂肪組織由来幹細胞を用いた再生治療

山本が中心的役割を担い、丸山、松尾、後藤がそれぞれの役割を果たした。丸山はラット膀胱頸部へ脂肪組織由来間葉系細胞の移植・生着について経時的に検討し、松尾はラットを使った尿失禁モデル開発を行った。山本と後藤は、基礎実験データを根拠に、Cytori Therapeutics, Inc.の脂肪幹細胞分離装置を用いて細胞培養を行なわずに脂肪組織由来幹細胞の膀胱頸部注入手術が可能かどうかの基礎実験ならびに臨床応用を行った。

（倫理面への配慮）

動物実験については、名古屋大学医学部動物実験施設における規程に準じて行った。自己脂肪組織由来幹細胞を利用した腹圧性尿失禁治療の研究は名古屋大学倫理委員会承認を得ている。

C. 研究結果

① 切迫性尿失禁に対する A 型ボツリヌストキシン膀胱壁内注入療法

この分担研究は岡村が担当した。平成 22 年 2 月 22 日の段階で、非神経因性過活動膀胱には 18 症例が神経因性排尿筋過活動には 18 症例が登録された。この報告書では、前者 5 例、後者 9 例の中間解析を報告する。

A) 非神経因性過活動膀胱に対する A 型ボツリヌス毒素膀胱壁内注入療法

5 例はすべて男性で、年齢は 66～77(71.5±4.1)歳、術前の尿路感染症はなく、尿流動態検査では全例で排尿筋の不随意収縮（70±29cmH_2O）を認め、最大膀胱容量は 24～250(161±83)ml、最大尿流時排尿筋圧は 23～132(64±63)cmH_2O であった。合併症では中等度の排尿困難が見られた。術後 1 ヶ月目の最大膀胱容量の増加、不随意収縮消失、最大尿流時排尿筋圧の低下が認められた。1 ヶ月目には排尿の勢いが低下し残尿が増加したが、2 ヶ月目にはもとに戻るように思われた。毎月の 3 日間の排尿記録では、尿意切迫感、切迫性尿失禁回数の著明な低下が 6～8 ヶ月間認められた。国際尿失禁会議質問票ショートフォーム(ICIQ-SF)における困窮度では、およそ 6 ヶ月間困窮度は低下するように思われた。

B) 神経因性排尿筋過活動に対する A 型ボツリヌス毒素膀胱壁内注入療法

9 症例の年齢は 22～66(42±16)歳、男性 7 例、女性 2 例であった。麻痺のレベルは、頚髄損傷 3 例，胸髄損傷 5 例，腰髄損傷 1 例である。麻痺の程度は完全麻痺（ASIA:A）7 例，不全麻痺（ASIA:B）2 例であり、受傷後期間は 3 年 4 ヵ月～27 年 1 ヵ月（中央値 6 年 11 ヵ月）であった。尿流動態検査では、全例に排尿筋不随意収縮（84±47cmH_2O）を認め、最大膀胱容量は 126±137ml であった。治療の

合併症はほとんど認められず、術後１ヶ月目に尿流動態検査が行われた８例中４例で不随意収縮が消失し、４ 例で不随意収縮は残ったものの 28.0±11.4 cmH_2O へ低下し、最大膀胱容量は２倍以上に増加した。導尿・尿失禁記録（FVC）における尿失禁回数は、１ヶ月後、平均 4.9±6.2 → 1.1±2.2 回に、１日導尿回数も平均 10.0±5.1 → 7.9±4.4 回に劇的に減少し、１回導尿量は平均 97.7±44.7→184.0±78.3 ml/回に劇的に増加した。脊髄損傷患者では間欠導尿は避けることができないので、導尿回数が減少し、一回量が増加するのは望ましいことである。国際尿失禁会議質問票ショートフォーム（ICIQ-SF）の困窮度は 7.1±2.6→1.1±2.3 に劇的に低下した。

② 腹圧性尿失禁に対する自己骨格筋幹細胞を用いた再生治療

分担研究者の橋本は、2007 年４月から 2010 年２月の３年間に、59 歳から 86 歳までの男性 24 名、女性１名から、開腹手術時に摘出された開腹部位の筋肉約 1g を用いて、幹細胞化実験を行った。全例で幹細胞化に成功し、培養技術は確立できていると考えられた。動物由来成分の代替、変更を図り、安全性の高い細胞調整法の開発を試みた。その結果、20%ウシ胎児血清は代替困難であり、今後、安全性評価指標の確立を目指す。成長因子やコラーゲンコート培養皿も必要性が確認されたので、組み替えタンパク質、合成化合物への代替を目指す。プロテアーゼなどの酵素は組み替えタンパク質、合成化合物で代替可能であることがわかった。また、初代培養細胞を用いた解析で問題となる個体差および細胞老化の影響を排除するため、単一細胞に由来する初代培養筋細胞クローン Hu5（分担研究報告書参照）に、レンチおよびレトロウィルスベクターを用いて、ヒト telomerase 遺伝子、変異型ヒト CDK4 遺伝子（CDK4R24C）、およびヒト Cyclin D1 遺伝子を導入し発現させ、不死化ヒト筋細胞クローン KD3 を独自に樹立した。

分担研究者の上住は、前立腺全摘出手術時に摘出した高齢者の腹直筋あるいは錐体筋より分離、培養された骨格筋幹細胞を、CD56 及び骨・肝臓・腎臓型アルカリフォスファターゼ（BLK-ALP）を指標とし、フローサイトメーターを用いて品質評価する方法を検討した。その結果、CD56 および BLK-ALP の発現は、移植用筋細胞の「質」を移植直前に判定するための良い指標となることを確認した。また、高齢者の筋組織中での CD56 と BLK-ALP の発現を調べ、骨格筋幹細胞の増殖・分化能を反映するマーカーとして有用であることを示した。

分担研究者の宋は、移植用ヒト筋前駆細胞の安全性検定系として、免疫不全マウス骨格筋への移植実験系を用いて検討した。この系において上記の橋本が樹立した不死化ヒト筋前駆細胞は、マウスの筋芽細胞とキメラとなって再生筋線維を構成した。これまでの一般病理組織学的検索、細胞増殖に関わる Mcm4 の免疫組織化学的検索によって、移植した不死化ヒト筋前駆細胞の造腫瘍性は否定的であったが、この点をさらに、移植した不死化ヒト筋前駆細胞で発現している Venus 蛍光を手がかりとして、顕微鏡下に同定した再生筋線維を laser capture microdissection によって切り出し、そこから抽出した RNA を基に、定量的 PCR によって、細胞増殖にかかわる遺伝子発現を検討した。Ki67 mRNA の発現は、細胞増殖能をもつマウス筋芽細胞 Ric10 の 4.6%であり、対照となる周囲の正常マウス筋組織とは大差ないことがわかった。こうした遺伝子発現の面からも、移植したヒト筋前駆細胞が著しい増殖能を持ち、腫瘍化する危険性は否定的であると判断した。

③ 腹圧性尿失禁に対する自己脂肪組織由来幹細胞を用いた再生医療

分担研究者の丸山と松尾は、ラットを使った動物実験において、ラットから採取した脂肪由来幹細胞を括約筋に注入するモデルの開発し、移植した脂肪由来幹細胞群が真に増大し、平滑筋に分化するか否か検討した。GFP抗体、αSMA抗体を用いた免疫染色実験から、尿道内に注入した脂肪由来幹細胞が平滑筋に分化した可能性を示唆した。また、ラットを使って腹圧性尿失禁モデルを作成し、注入後、増大した移植細胞により尿道閉鎖圧が上昇することを示した。

臨床への応用として、分担研究者の後藤、山本が前立腺全摘除術後1年以上経過した70歳以上の難治性腹圧性尿失禁5症例に、吸引自己脂肪から分離した細胞群を内視鏡下に傍尿道周囲に注入する新規治療を施行した。名古屋大学医学部附属病院手術室にて、細胞分離装置(Cytori Therapeutics, Inc.)を用いて、腹部脂肪層から脂肪組織を吸引して、脂肪由来幹細胞を含む細胞成分を分離した。5症例のうち、1例が著効、3例が有効、1例が悪化であった。特に、症例1は尿失禁の程度は経過とともに軽快し、術後16週後にはパットテストは0mlと著効を示した。5症例のうち1例で、幹細胞注入後が一過性の尿閉があり導尿を2回行うこととなったが、その後自排尿可能となった。

山本は上記治療を施した5例の細胞分離成分のテストを行った。エンドトキシン、マイコプラズマニューモニエDNA(PCR)、培養好気性菌、培養真菌、培養マイコプラズマはすべて陰性であった。FACSによる分離直後の細胞表面マーカーの検索では、間葉系幹細胞マーカー、CD29およびCD44が陽性であることが示された。1次培養された細胞はFibroblasticな形態を示し、4日目には70-80%confluentとなっていた。脂肪への分化を誘導すると7日目には脂肪滴の形成が確認された。Oil-Redによる染色により脂肪への分化を確認した。また、平滑筋分化誘導2週間では、α-smooth muscleと細胞の核が全視野の細胞に染色され、平滑筋への分化誘導も確認した。今回、治療に用いた細胞分離装置により採取された脂肪由来幹細胞/間質細胞のviabilityは90%前後(n=4)であり、対象が高齢者であっても「活きのいい」細胞が得られることがわかった。

D. 考察

尿失禁は、認知症、転倒・骨折、骨粗鬆症などとともに高齢者のQOLを著しく損なう問題であり、介護者にとっても、肉体的・費用的負担がたいへんに大きい問題である。新たな尿失禁の治療法が開発されれば、高齢者・介護者のQOLも向上し、健康長寿社会の確立に大いに貢献できるものと考える。切迫性尿失禁に対するA型ボツリヌストキシン膀胱壁内注入療法の中間検討ではたいへん有望であると思われた。

自己脂肪組織由来幹細胞を用いた腹圧性尿失禁治療の臨床試験では、尿失禁の減少、局所での血流増加を認め、1例では尿失禁消失、3例で失禁量が半減するなどの効果が得られ、今後期待できる治療になるものと思われた。尿失禁も次第に軽快していく経過も捉えられ、尿道括約筋機能が再生されていることが推測される。しかし、このことは松尾が指摘したように、検証が必要である。

骨格筋幹細胞を用いた腹圧性尿失禁に対する移植治療では、高齢者骨格筋組織から高い増殖・分化能を保持した筋細胞を、必要量(10^7細胞)調製するための分離培養方法を確立し、有効な細胞の品質および安全性評価系も確立することができた。しかし、臨床応用を実現するためには、前臨床研究で用いた全ての試薬・器具、作業環境および作業工程を、GMP

基準に適合したレベルのものに置き換えねばならない(「橋渡し研究」に相当する)。現在、国立長寿医療センターでは、GMP 基準に適合した施設の設置準備が進められている。当該施設完成と同時に臨床研究に移行できるよう、本研究を「橋渡し研究」に発展させるとともに、安全性を担保するための研究評価組織の構築および施設運用規則の整備など、ソフト面とハード面の両面から、再生医療実現のための体制作りを進める予定である。

E. 結論

非神経因性過活動膀胱に対する A 型ボツリヌストキシン膀胱壁内注入療法と脊髄損傷による神経因性排尿筋過活動に対する A 型ボツリヌス毒素膀胱壁内注入療法の臨床試験では前者に 18 例、後者にも 18 例が登録された。中間解析では、いずれに対しても注入に伴う合併症はほとんど認めず、尿失禁の改善は優れていると考えられた。治療後 1〜2 ヶ月程度、残尿の増加が認められたが、尿閉となった症例はいなかった。治療前に、膀胱出口閉塞を除外しておくことが重要であると考えられた。

自己骨格筋幹細胞を用いた再生治療の研究では、1) 高齢者から採取した微量な筋肉から筋幹細胞を分離し、体外で培養する技術は高齢者でも有効であった。2) CD56 と BLK-ALP を用いて、品質の高い移植筋細胞の採取が可能となった。CD56 と BLK-ALP が幹細胞の増殖・分化能を反映するマーカーとして有用である。3)安全性を高めるため、代替可能な動物由来の試薬を探索した。4)安全性検定系を確立し、ヒト筋前駆細胞移植によってできる再生筋線維が癌化する危険性は低いことを示した。5)初代培養細胞を用いた解析で問題となる個体差および細胞老化の影響を排除するため、不死化ヒト細胞株を樹立した。

自己脂肪組織由来幹細胞を用いた再生治療の研究では、1) ラットを使った腹圧性尿失禁治療モデルを作成し、尿道括約筋内自己脂肪組織由来幹細胞注入が有効であることを示した。しかし、動物実験系では平滑筋に分化したか否かはさらなる検討が必要である。2)吸引自己脂肪から分離した細胞群を内視鏡下に傍尿道周囲に注入する新規治療を 5 例に施行し、1 例が著効、3 例が有効であった、3) 吸引自己脂肪から分離した細胞群の FACS による分離直後の細胞表面マーカーの検索では、間葉系幹細胞マーカー、CD29 および CD44 が陽性であり、平滑筋への分化を誘導すると、2 週間目で α-smooth muscle と細胞の核が全視野の細胞に染色され、平滑筋への分化が確認された。

F. 健康危険情報

特になし

G. 研究発表

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G.　知的財産権の出願・登録状況

1.　特許出願

1)　脂肪組織由来間葉系幹細胞を含有する、前立腺癌治療用細胞製剤剤
山本徳則、小出直史、後藤百万、武井佳史
特許願人：名古屋大学
出願日：平成 21 年 12 月 7 日（特願 2009-277437）

2)　脂肪組織由来間葉系幹細胞を含有する、勃起不全または尿意障害の細胞製剤
山本徳則、後藤百万
特許願人：名古屋大学
出願日：平成 21 年 10 月 6 日（特願 2009-232068）

3)　脂肪組織由来多分化能幹細胞を含有する細胞製剤
尾崎武徳、安田香、丸山彰一、山本徳則、後藤百万、松尾清一、北川泰雄
特許願人　名古屋大学
出願日：平成 18 年 8 月 9 日
（日本特許　特願 2006−216234）
（国際特許 PCT/JP2007/065431）

2.　実用新案登録　なし

3.　その他　なし

研究成果の刊行に関する一覧表

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Autologous Transplantation of SM/C-2.6⁺ Satellite Cells Transduced with Micro-dystrophin *CS1* cDNA by Lentiviral Vector into *mdx* Mice

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Duchenne muscular dystrophy (DMD) is a lethal muscle disorder caused by mutations in the dystrophin gene. Transplantation of autologous myogenic cells genetically corrected *ex vivo* is a possible treatment for this disorder. In order to test the regenerative efficiency of freshly isolated satellite cells, we purified quiescent satellite cells from limb muscles of 8–12-week-old green fluorescent protein-transgenic (GFP-Tg) mice using SM/C-2.6 (a recently developed monoclonal antibody) and flow cytometry. Freshly isolated satellite cells were shown to participate in muscle regeneration more efficiently than satellite cell-derived myoblasts passaged *in vitro* do, when transplanted into tibialis anterior (TA) muscles of 8–12-week-old cardiotoxin-injected C57BL/6 mice and 5-week-old dystrophin-deficient *mdx* mice, and analyzed at 4 weeks after injection. Importantly, expansion of freshly isolated satellite cells *in vitro* without passaging had no detrimental effects on their regenerative capacity. Therefore we directly isolated satellite cells from 5-week-old *mdx* mice using SM/C-2.6 antibody and cultured them with lentiviral vectors expressing micro-dystrophin *CS1*. The transduced cells were injected into TA muscles of 5-week-old *mdx* mice. At 4 weeks after transplantation, the grafted cells efficiently contributed to regeneration of *mdx* dystrophic muscles and expressed micro-dystrophin at the sarcolemma. These results suggest that there is potential for lentiviral vector-mediated *ex vivo* gene therapy for DMD.

Received 22 February 2007; accepted 28 July 2007; advance online publication 28 August 2007. doi:10.1038/sj.mt.6300295

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by mutations in the dystrophin gene.[1] Dystrophin is a 427 kd large sub-sarcolemmal protein that forms the dystrophin/glycoprotein complex at the sarcolemma with α- and β-dystroglycans, α-, β-, γ-, and δ-sarcoglycans, and other molecules, and links the cytoskeleton with the basal lamina.[2,3] The lack of dystrophin in the sarcolemma causes instability of the muscle membrane, leading to muscle degeneration and myofiber loss. Although there is no effective treatment for the disease at present, cell therapy could be a promising approach. Satellite cells are quiescent mononucleated cells located external to the muscle membrane but internal to the basal lamina in adult skeletal muscle.[4] On muscle damage, they activate, proliferate, and then exit the cell cycle either to differentiate into mature myofibers or to renew the quiescent satellite cell pool. Because satellite cells have robust regenerative capacity,[5,6] they are expected to be a feasible source for cell therapy in DMD. Indeed, transplantation of myoblasts successfully restored dystrophin expression in dystrophin-deficient muscle under immunosuppression.[7,8] Nevertheless, in the early 90s, transplantation of satellite cell-derived myoblasts failed to improve muscle force in DMD patients.[9–11] The failure has been ascribed to poor survival[12–14] and limited distribution of the transplanted cells after injection.[15] The latter problem could possibly be partly overcome by using high-density injections of myoblasts.[16,17] On the other hand, the mechanisms by which grafted myoblasts are rapidly lost after injection have not been fully addressed.[12–14]

Many studies have employed crude cell preparations containing both satellite cells and non-myogenic cells[18,19] or satellite cell-derived myoblasts extensively amplified *in vitro*.[15,20–22] In a recent study, Montarras *et al.* directly isolated (Pax3)green fluorescent protein (GFP)-expressing satellite cells from the diaphragm of adult *Pax3*$^{GFP/+}$ mice by flow cytometry.[23] These cells constituted a homogeneous population and the majority were quiescent. When grafted into irradiated muscles of immunodeficient *nu/nu* dystrophin-deficient *mdx* mice, the freshly isolated satellite cells efficiently contributed to both fiber repair and the muscle satellite cell compartment,[23] thereby suggesting that fresh satellite cells are a potential source for cell therapy in DMD.

For transplanting autologous cells, which are expected to evade the host immune response to grafted cells, the lentiviral vector is a potential tool for introducing the therapeutic gene

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because it integrates into the host genome in a variety of dividing and non-dividing cells. Because the dystrophin complementary DNA is too large to be incorporated into a lentiviral vector, a truncated but fully functional version of the dystrophin complementary DNA[24–27] has to be used instead of the full-length one. When compared with conventional transfection of myogenic cells with large dystrophin-coding plasmids[28] or nucleofection in combination with ϕC31 integrase,[29] transfection by lentiviral vectors led to much more efficient expression of mini- or micro-dystrophin in *mdx* mice,[30] in non-human primate cells, and in human myogenic cells.[31] Lentiviral vectors were also used for introducing the therapeutic genes into other types of stem cells. Bachrach *et al.* reported expression of human micro-dystrophin in mdx^{5cv} muscles after systemic delivery of autologous side population cells modified with lentiviral vectors expressing micro-dystrophin.[32] Sampaolesi *et al.* reported intra-arterial delivery of autologous mesoangioblasts corrected by lentiviral vectors expressing α-sarcoglycan (α-SG), resulting in many α-SG-positive fibers, and morphological and functional recovery in downstream muscles of α-SG-null dystrophic mice.[33] A more recent study reported the autologous transplantation into skeletal muscle, of monkey muscle precursor cells transduced with micro-dystrophin by lentiviral vectors.[31] However, whether *ex vivo* gene therapy using lentiviral vectors expressing micro-dystrophin is indeed beneficial in large animal models such as dystrophic dogs, is still subject to controversy.[34]

Previously, Fukada *et al.* established a method of direct purification of quiescent satellite cells from adult mouse skeletal muscles, using fluorescence activated cell sorting (FACS) and a novel monoclonal antibody named SM/C-2.6.[35] The method is simple, and is expected to be applicable to the isolation of satellite cells from dystrophic (autologous) muscles for cell therapy.

In this study, we first directly isolated satellite cells from *mdx* mice using the SM/C-2.6 antibody and FACS. We showed that *mdx*-SM/C-2.6$^+$ cells transduced with lentiviral vectors expressing micro-dystrophin efficiently contributed to regeneration of *mdx* muscles and expressed micro-dystrophin at the sarcolemma when grafted. Our results indicate that the autologous satellite cell isolated by the SM/C-2.6 antibody and genetically corrected by a lentiviral vector is a feasible tool for cell therapy of DMD or of localized forms of muscular dystrophy.

RESULTS

Passaged SM/C-2.6$^+$ satellite cells show reduced regenerative capacity

We isolated satellite cells from the limb muscles of 8–12-week-old C57BL/6 mice using FACS and a novel monoclonal antibody, SM/C-2.6.[35] A previous study has shown that satellite cells are highly enriched in the SM/C-2.6$^+$ fraction.[35] Immediately after isolation by FACS, SM/C-2.6$^+$ cells expressed Pax7, but not MyoD, myogenin, or Ki67 (Table 1). After 4 days of culture, more than 95% of the cells expressed MyoD and Ki67 (data not shown). Pax7 marks quiescent, activated satellite cells and their progeny, myoblasts,[36] whereas MyoD marks activated satellite cells and myoblasts.[37,38] Ki67 is a marker of proliferating cells. It follows, therefore, that SM/C-2.6$^+$ cells are highly purified satellite cells in the G_0 phase immediately after isolation from muscle tissues.

Table 1 Expression of myogenic and proliferative markers of freshly isolated SM/C-2.6$^+$ cells from limb muscles of C57BL/6 or *mdx* mice

Marker	B6-SM/C-2.6$^+$ cells (%)	*mdx*-SM/C-2.6$^+$ cells (%)
Pax7	95 ± 1.4	94 ± 2.1
MyoD	0 ± 0	19 ± 2.8
Myogenin	0 ± 0	7 ± 1.3
Ki67	0.6 ± 1.0	34 ± 1.8

The expression level of each marker is shown as the percentage of positive cells in total cells stained with 4′,6-diamidino-2-phenylindole in three randomly selected fields. Data are represented as mean values ± SD.

To investigate the regenerative efficiency of SM/C-2.6$^+$ satellite cells when grafted into mouse skeletal muscles, three kinds of cells were prepared from limb muscles of 8–12-week-old GFP-Tg mice: (i) quiescent SM/C-2.6$^+$ cells freshly isolated by FACS (Figure 1a), (ii) expanded SM/C-2.6$^+$ cells *in vitro* with or without passaging (Figure 1a), and (iii) cultured primary myoblasts isolated by a conventional pre-plating method.[39] These cells were injected at 2×10^4 cells per muscle into the tibialis anterior (TA) muscle of 8–12-week-old C57BL/6 and 5-week-old dystrophin-deficient *mdx* mice. Twenty four hours before cell transplantation the recipient C57BL/6 muscles were injected with cardiotoxin (CTX) so as to induce regeneration. Four weeks after the injection, we investigated the contribution of each cell population to muscle regeneration by immunodetection of GFP-positive fibers. Freshly isolated SM/C-2.6$^+$ cells (Figure 1b) produced many more GFP-positive fibers than those produced by the same number of cultured SM/C-2.6$^+$ cells passaged once *in vitro* (Figure 1c, passage 1). We next examined the effects of expansion, without passaging and with repeated passaging, on the regenerative capacity of the cells. The number of GFP-positive myofibers derived from GFP-Tg SM/C-2.6$^+$ cells dropped considerably after first passage *in vitro* and then gradually decreased with subsequent passages in both CTX-injected C57BL/6 mice (Figure 1d) and in *mdx* mice (Figure 1e). Primary myoblasts prepared by the pre-plating method[39] also showed low regenerative capacity (Figure 1d). Surprisingly, the regenerative efficiency of cells expanded *in vitro* without passaging was comparable to that of freshly isolated cells (Figure 1d and e). These results suggested to us that it is possible to genetically correct dystrophin-deficient satellite cells *ex vivo* before transplantation without causing a reduction in their regenerative capacity.

In order to know why fresh or "expansion" cells gave rise to more myofibers when compared with cells passaged *in vitro*, we compared the colony formation ability of fresh satellite cells with that of passaged myoblasts (passage 1). The results showed that fresh satellite cells formed larger colonies than passage 1 cells, when plated at a density of 1 cell/well on 96-well plates, although the rate of colony formation was not significantly different between these two cells (fresh, 26% versus passage 1, 23%) (**Supplementary Figure S1**). In contrast, there was no difference in fusion index between fresh satellite cells and passaged myoblasts (data not shown). Collectively, a reduction in the proliferative ability of passaged myoblasts *in vitro* might partly explain their lower regenerative capacity *in vivo*.

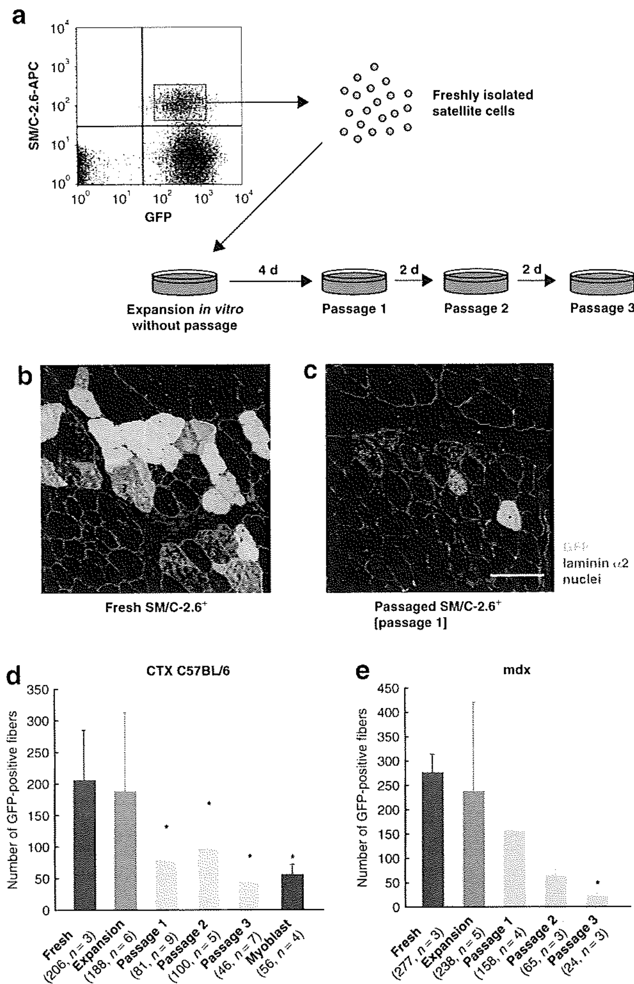
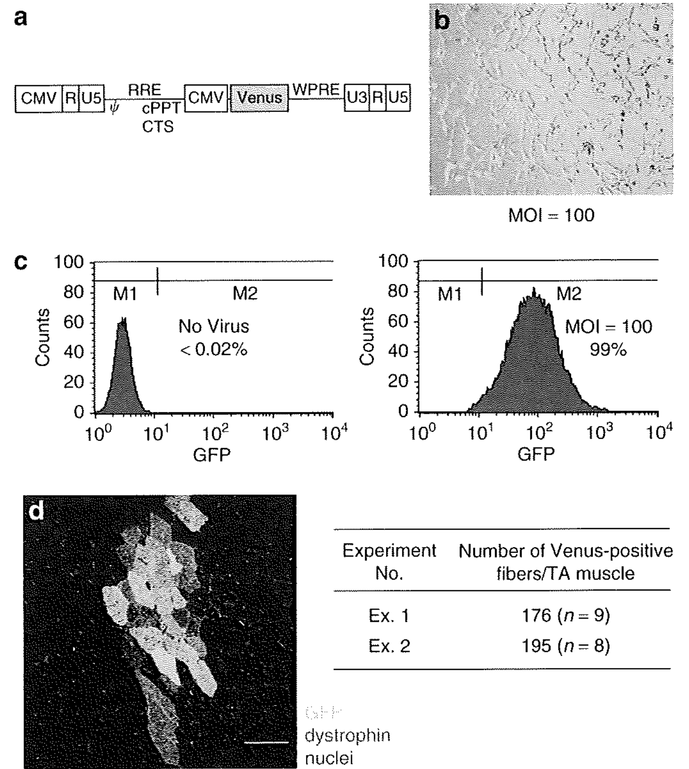


Figure 1 The regenerative capacity of SM/C-2.6$^+$ satellite cells isolated from adult mouse skeletal muscles by fluorescence activated cell sorting (FACS). (**a**) Flow cytometry of mononucleated cells derived from limb muscles of green fluorescent protein-transgenic (GFP-Tg) mice after staining with SM/C-2.6 antibody and culture conditions of sorted cells. SM/C-2.6$^+$ GFP$^+$ cells (red square) were sorted as the satellite cell fraction. These cells were cultured in proliferation medium for 4 days (expansion *in vitro* without passage) and then passaged up to three times at 2-day intervals. (**b**) Freshly isolated and (**c**) passaged SM/C-2.6$^+$ cells (passage 1) from GFP-Tg mice were injected into C57BL/6 tibialis anterior (TA) muscles. The muscles were treated with cardiotoxin (CTX) 24 hours before cell transplantation and then injected with 2 × 10^4 cells per TA muscle. Four weeks after the injection, cross-sections were stained with anti-GFP (green) and laminin α2 (red) antibodies. Nuclei were stained with TOTO3 (blue). Bar: 80 μm. (**d**, **e**) Comparison of muscle regenerative efficiencies of three kinds of cells prepared from GFP-Tg mice: (i) quiescent SM/C-2.6$^+$ cells freshly isolated by FACS (red bars), (ii) expanded SM/C-2.6$^+$ cells *in vitro* without passaging (orange bars) or passaged SM/C-2.6$^+$ cells (yellow bars with passage numbers), and (iii) primary myoblasts isolated by the preplating method (blue bar in **d**). The same numbers of cells (2 × 10^4 cells) were grafted into TA muscles of CTX-treated C57BL/6 (d) and *mdx* mice (**e**). The number of GFP-positive fibers per cross-section was counted after staining with anti-GFP antibody. Error bars represent SD. *$P < 0.05$ compared with freshly isolated SM/C-2.6$^+$ cells.

SM/C-2.6$^+$ satellite cells transduced with lentiviral vectors efficiently contribute to muscle regeneration

Successful gene and cell therapy for DMD requires sustained expression of the therapeutic gene in striated muscle. The



Experiment No.	Number of Venus-positive fibers/TA muscle
Ex. 1	176 ($n = 9$)
Ex. 2	195 ($n = 8$)

Figure 2 Lentiviral vector-mediated gene transfer into SM/C-2.6$^+$ satellite cells and transplantation of transduced cells into *mdx* mouse muscles. (**a**) Structure of the lentiviral vector expressing Venus under the control of a cytomegalovirus (CMV) promoter. (**b**) Fluorescence of Venus-expressing satellite cell-derived myoblasts. Freshly isolated SM/C-2.6$^+$ cells from C57BL/10 limb muscles were transduced with lentiviral vectors expressing Venus at a multiplicity of infection (MOI) of 100 for 16 hours, and cultured in proliferation medium for 3 days. (**c**) Flow cytometric analysis of non-transduced (left panel) and transduced (right panel) SM/C-2.6$^+$ cells 3 days after the transduction. M2 denotes the area of Venus-expressing cells. At a MOI of 100, 99% of the cells expressed Venus. (**d**) Venus- and dystrophin-positive fibers formed by SM/C-2.6$^+$ cells transduced with lentiviral vectors *in vitro*. Transduced cells (2 × 10^4) were injected into tibialis anterior (TA) muscles of *mdx* mice. Four weeks after the injection, cross-sections were stained with anti-GFP (green) and dystrophin (red) antibodies. Nuclei were stained with TOTO3 (blue). The number of Venus-positive fibers per cross-section was counted. Bar: 80 μm. cPPT, central polypurine tract; CTS, central termination sequence; GFP, green fluorescent protein; RRE, rev responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

lentiviral vector can carry a relatively large transgene and integrate it into the genome of non-dividing cells such as quiescent satellite cells. We therefore attempted lentiviral vector-mediated gene transfer into satellite cells. For this purpose, we used a human immunodeficiency virus-1-based lentiviral vector pseudotyped with vesicular stomatitis virus-G glycoprotein.[40] To start with, we used a vector that expresses Venus, a variant of yellow fluorescent protein[41] under the control of a cytomegalovirus (CMV) promoter (Figure 2a). Freshly isolated satellite cells from limb muscles of 8–12-week-old C57BL/10 mice, which are syngenic to *mdx*, were transduced with the lentiviral vectors at a multiplicity of infection (MOI) of 100 for 16 hours. After removal of free viral vectors and *in vitro* expansion of the cells for 3 days, numerous Venus-positive cells were detected (Figure 2b). Flow

cytometric analyses revealed that 99% of the SM/C-2.6$^+$ satellite cell–derived myoblasts expressed Venus when transduced at a MOI of 100 (Figure 2c, right panel). These transduced cells were injected into TA muscles of 5-week-old *mdx* mice at 2×10^4 per muscle. Four weeks after the injection, the muscle regeneration capacity of cells transduced with lentiviral vectors was investigated by immunodetection of Venus- or dystrophin-positive fibers. As in the case of the non-transduced cells (Figure 1d and e), grafting of transduced cells too led to many Venus- and dystrophin-positive fibers (Figure 2d). This serves to show that SM/C-2.6$^+$ satellite cell-derived myoblasts transduced with lentiviral vectors contribute efficiently to muscle regeneration.

Direct isolation of SM/C-2.6$^+$ satellite cells from dystrophic muscles of *mdx* mice

In order to test whether autologous myogenic precursor cells genetically corrected to express a dystrophin gene represent a possible tool in DMD therapy, we next attempted to directly isolate SM/C-2.6$^+$ cells from limb muscles of 5-week-old *mdx* mice. Numerous inflammatory and fibroblastic cells reflecting the active cycles of the degeneration–regeneration process are found in dystrophic muscles. SM/C-2.6 antibody reacts with activated fibroblastic cells (Fukada *et al.*, unpublished data). Because satellite cells are negative for both Sca-1 and CD31,[35] we stained *mdx* muscle-derived mononuclear cells with a cocktail of CD45, CD31, Sca-1, and SM/C-2.6 antibodies and collected SM/C-2.6$^+$ CD45$^-$ CD31$^-$ Sca-1$^-$ cells as the satellite cell fraction (Figure 3a). When these cells were cultured in proliferation medium for 4 days, more than 95% of them expressed MyoD (Figure 3b). These results indicate that, using the SM/C-2.6 antibody, a pure population of satellite cells can be isolated, not only from wild-type muscle but also from dystrophic muscle of *mdx* mice. Immediately after isolation, the majority of satellite cells from C57BL/6 mice were negative for MyoD, myogenin, and Ki67. On the other hand, 19% of *mdx*-satellite cells were positive for MyoD and 34% of the cells were positive for Ki67 (Table 1). There was no difference between *mdx*- and B6-SM/C-2.6$^+$ cells with respect to expression of Pax7 (Table 1). This proves that a considerable fraction of satellite cells are in an activated, proliferative state in skeletal muscles of *mdx* mice.

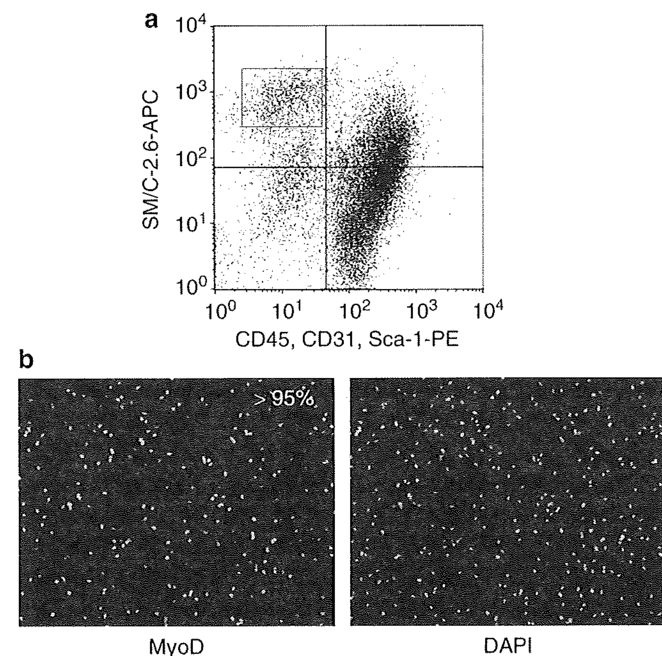
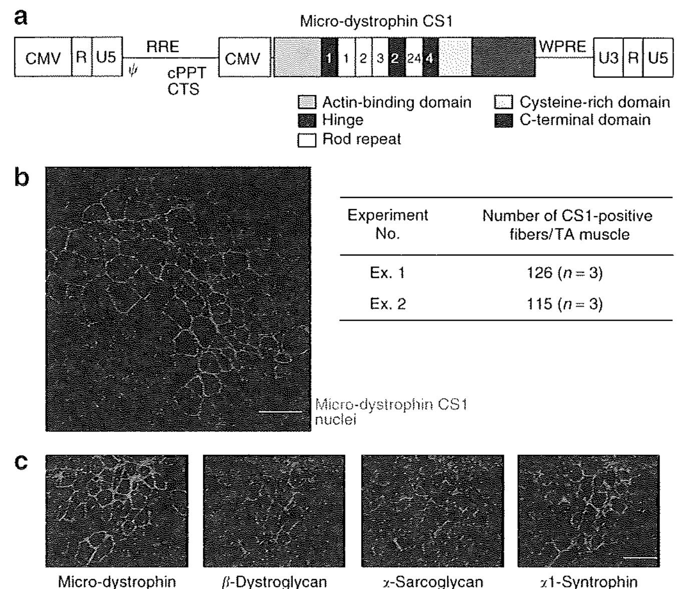


Figure 3 Direct isolation of SM/C-2.6$^+$ satellite cells from dystrophic muscles of *mdx* mice. **(a)** Flow cytometry of mononucleated cells derived from *mdx* mice, and stained with a cocktail of CD45, CD31, Sca-1, and SM/C-2.6 antibodies. SM/C-2.6$^+$ CD45$^-$ CD31$^-$ Sca-1$^-$ cells (red square) were sorted as the satellite cell fraction. **(b)** Sorted *mdx*-satellite cells were cultured in proliferation medium for 4 days and stained with anti-MyoD antibody (green) and 4′,6-diamidino-2-phenylindole (DAPI) (nuclei, blue). More than 95% of them expressed MyoD.

Successful micro-dystrophin gene transfer into *mdx*-SM/C-2.6$^+$ satellite cells

The full-length dystrophin complementary DNA, at 14 kilobase (kb), is too large to be incorporated into a lentiviral vector. In previous studies, we constructed a rod-truncated micro-dystrophin *CS1* and demonstrated that it effectively rescued



Experiment No.	Number of CS1-positive fibers/TA muscle
Ex. 1	126 ($n = 3$)
Ex. 2	115 ($n = 3$)

Figure 4 Lentiviral vector-mediated micro-dystrophin *CS1* gene transfer into *mdx*-SM/C-2.6$^+$ cells and transplantation of transduced cells into *mdx* muscles. **(a)** Structure of the lentiviral vector expressing micro-dystrophin *CS1*. *CS1* complementary DNA was inserted downstream of the cytomegalovirus (CMV) promoter. *CS1* has the N-terminal domain, a shortened version of the central rod domain with four rod repeats and three hinges, the cysteine-rich domain, and the C-terminal domain. The numbers of rod repeats and hinges are also shown. **(b)** Freshly isolated SM/C-2.6$^+$ cells from *mdx* dystrophic muscles were transduced with lentiviral vectors expressing micro-dystrophin *CS1* at a multiplicity of infection of 200 for 16 hours and cultured in proliferation medium for 2 days. Transduced cells (2×10^4) were injected into *mdx* tibialis anterior (TA) muscles. Four weeks after the injection, cross-sections were stained with anti-dystrophin antibody (red) and TOTO3 (nuclei, blue). The number of micro-dystrophin *CS1*-positive fibers per cross-section was counted. Bar: 80 μm. **(c)** Restoration of dystrophin-associated proteins at the sarcolemma of micro-dystrophin-positive fibers. Serial cross-sections were stained with anti-dystrophin, β-dystroglycan, α-sarcoglycan, and α1-syntrophin antibodies (red), and TOTO3 (nuclei, blue). Bar: 80 μm. cPPT, central polypurine tract; CTS, central termination sequence; RRE, rev responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

the dystrophic phenotypes of *mdx* mice when introduced as a transgene[24] or by adeno-associated viral vectors.[25] We therefore inserted a 4.9 kb micro-dystrophin *CS1* into the lentiviral vector as a therapeutic gene. Freshly isolated *mdx*-SM/C-2.6$^+$ cells were transduced with lentiviral vectors expressing micro-dystrophin *CS1* under the control of a CMV promoter (Figure 4a) at a MOI of 200 for 16 hours. In this condition, 97% of the cells expressed micro-dystrophin *CS1* (data not shown). These transduced cells were injected into TA muscles of 5-week-old *mdx* mice at 2×10^4 cells per muscle. Four weeks after the injection, the muscle regeneration capacity of the cells was investigated by immunodetection of micro-dystrophin-positive fibers. Many myofibers expressed micro-dystrophin *CS1* on the sarcolemma at an average of 120 fibers per muscle (Figure 4b). Further, we examined the restoration of the dystrophin-associated protein complex in micro-dystrophin-positive fibers by immunodetection of α-SG, β-dystroglycan, and α1-syntrophin. As shown in Figure 4c, all these proteins were expressed at the sarcolemma of micro-dystrophin *CS1*-positive myofibers, thereby suggesting the recovery of dystrophin-associated protein complex by the introduction of micro-dystrophin. These results indicate that *mdx*-SM/C-2.6$^+$ cells transduced with lentiviral vectors expressing micro-dystrophin *CS1* efficiently contribute to regeneration of dystrophic muscles of *mdx* mice and restore the expression of the dystrophin/dystrophin-associated protein complex. It therefore follows that transplantation of autologous myogenic precursor cells prepared using the SM/C-2.6 antibody and genetically corrected by a lentiviral vector, is a possible approach for cell therapy in DMD or in localized forms of muscular dystrophy.

DISCUSSION

In vitro *passaging reduced the regenerative capacity of satellite cells*: In the present study, we directly isolated satellite cells from skeletal muscles of wild-type and *mdx* mice using SM/C-2.6, a novel monoclonal antibody,[35] and flow cytometry. Almost all satellite cells prepared from normal muscle are negative for MyoD, myogenin, and Ki67 immediately after isolation, thereby indicating that they are in a quiescent state. In contrast, approximately 20% of *mdx*-satellite cells are positive for MyoD and 35% are positive for Ki67 (Table 1). This result indicates that a fraction of *mdx*-satellite cells are already in an activated state.

Transplantation experiments showed that freshly isolated SM/C-2.6$^+$ satellite cells possess a higher capacity for muscle reconstitution when compared with SM/C-2.6$^+$ myoblasts passaged *in vitro* prior to transplantation. This result indicates that passaging and subsequent proliferation of satellite cells in culture reduce their intrinsic capacity for muscle reconstitution. In order to clarify the mechanisms of low myogenicity of passaged cells, we performed a colony-forming assay of freshly isolated satellite cells and passaged satellite cell-derived myoblasts (passage 1). When the cells were seeded at a density of 1 cell/well on 96-well plates, fresh satellite cells formed larger colonies than "passage 1" myoblasts (**Supplementary Figure S1**). In contrast, there was no difference in fusion index between these two cell populations (data not shown). Collectively, reduced efficiency of muscle fiber regeneration by passaged myoblasts can be partly explained by gradual loss of proliferative ability during passaging.

Importantly, we also found that satellite cells that were expanded *in vitro* without passaging showed regenerative capacity comparable to freshly isolated satellite cells. We therefore hypothesized that it might be possible to introduce therapeutic genes into satellite cells *in vitro* by a lentiviral vector before transplantation without causing any reduction in their regenerative capacity.

Comparison of regenerative capacity of SM/C-2.6$^+$ satellite cells with other reports: Previously, Montarras *et al.* directly isolated (Pax3) GFP-expressing satellite cells, which constitute a homogeneous population of small, non-granular, CD34$^+$ CD45$^-$ Sca-1$^-$ cells, from diaphragms of adult *Pax3*$^{GFP/+}$ mice by flow cytometry, and examined their regenerative capacity.[23] The researchers concluded that *in vitro* expansion of freshly isolated satellite cells for a few days prior to transplantation is a disadvantageous approach, because such satellite cell-derived myoblasts displayed considerably lower muscle regenerative efficiency than fresh satellite cells. In contrast, we observed no reduction in regenerative capacity as a result of *in vitro* expansion of freshly isolated satellite cells without passaging, although their capacity was remarkably reduced after passaging (Figure 1d and e). The discrepancy between the results of Montarras *et al.* and our results may be due to differences in the culture conditions employed. One possible explanation could be that our culture medium contained basic fibroblast growth factor. It has been reported that addition of basic fibroblast growth factor to culture medium improves transplantation efficiency.[42,43] The modification of culture conditions may enable maintenance of the intrinsic muscle regenerative capacity of satellite cells.

Previous muscle transplantation experiments utilized the progeny of satellite cells enzymatically dissociated from myofibers and extensively cultured to increase their numbers.[15,20–22] When 5×10^5 to 1×10^6 myoblasts taken from normal mice and prepared by the pre-plating method were transplanted into non-irradiated muscles of *mdx* mice, it resulted in fewer than 100 dystrophin-positive myofibers per muscle.[21] On the other hand, when 5×10^5 cells were injected into muscles of immunodeficient *mdx nu/nu* mice that had been pre-irradiated to ablate endogenous satellite cell function, they formed an average of 328 dystrophin-positive fibers.[18] Furthermore, grafting of 2×10^4 satellite cells freshly isolated from *Pax3*$^{GFP/+}$ mice into pre-irradiated TA muscles of *mdx nu/nu* mice led to dystrophin expression in an average of 587 fibers.[23] In our experiment, the same number (2×10^4) of satellite cells freshly isolated from adult normal mice gave rise to an average of only 277 myofibers in non-irradiated *mdx* muscles (Figure 1d and e). This shows that grafted muscle precursor cells form a far greater number of dystrophin-positive fibers in irradiated muscle than in non-irradiated muscle. The use of immunosuppressants such as FK506 also greatly improves the efficiency of transplantation.[7] In the present study, we injected myogenic cells into non-irradiated TA muscles of immunocompetent mice without any immunosuppressant. Therefore, in our experimental

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conditions, the intrinsic function of SM/C-2.6$^+$ satellite cells may be underestimated.

The use of the Lentiviral vector is feasible for ex vivo *gene transfer*: In this study we showed that, at a MOI of 200, lentiviral vectors can introduce the rod-truncated micro-dystrophin gene *CS1* into more than 97% of *mdx*-satellite cells without detrimental effects on cell viability and regenerative capacity. But at a MOI of 300 we observed cell toxicity, whereas at a MOI of 100, the transduction efficiency was below 80% (data not shown). When we injected the transduced autologous myoblasts into *mdx* muscle, the cells contributed to regeneration of myofibers and expressed micro-dystrophin and dystrophin-associated proteins at the sarcolemma. Our results therefore suggest that *ex vivo* gene transfer into autologous myogenic cells by a lentiviral vector is feasible. On the other hand, direct intramuscular injection of vesicular stomatitis virus-G glycoprotein–pseudotyped lentiviral vectors led to relatively low expression of the transgene in mouse skeletal muscles.[30,44] Because the lentiviral vector genome is inserted into the host genome, the transduction of cells other than the target cell could introduce the risk of mutagenesis. Further, *in vivo* administration could induce undesirable immune responses to exogenous viral proteins. In effect, direct *in vivo* administration of lentiviral vectors poses a safety problem for clinical application. In contrast, *ex vivo* gene transfer has the merit of minimizing the risks of introducing free lentiviral vectors into the host. Transduced cells can efficiently proliferate and differentiate *invitro* (data not shown).

Limitations of ex vivo *gene therapy in DMD, using satellite cells*: One of the demerits of our procedure, as compared to *in vivo* gene transfer, is that only a part of the genetically modified myogenic precursor cells contributes to regeneration of the host muscle, given the poor survival rate of these cells. In fact, by using real-time polymerase chain reaction on transcripts from the transgenic enhanced GFP gene, we found that more than 90% of the injected cells were lost within the first 24 hours after injection (data not shown). In addition, migration of the surviving cells is limited in the host muscle after injection. Furthermore, because *in vitro* passaging greatly reduces their myogenicity, it is difficult to obtain a sufficient number of satellite cells or their progeny from a small muscle biopsy of a DMD patient. Therefore current myoblast transfer might be more realistic for localized forms of muscular dystrophy, such as oculo-pharyngeal muscular dystrophy or facio-scapulo-humeral muscular dystrophy.[45] Surprisingly, however, Collins *et al.* transplanted a single intact myofiber into irradiation-ablated muscles and demonstrated that as few as seven satellite cells associated with one transplanted myofiber can generate over 100 new myofibers containing thousands of myonuclei.[5] Their observations suggest that proper isolation and handling of satellite cells might greatly improve their myogenic potential.

In this study we have demonstrated transplantation of autologous satellite cells genetically corrected by a lentiviral vector *ex vivo* into *mdx* muscle. For treating DMD patients, however, it is necessary to find the optimum *in vitro* culture condition that will enable human muscle precursor cells to maintain their intrinsic myogenic potential. It would also be useful to identify the factors that support survival and/or proliferation of transplanted cells in the host muscle.

MATERIALS AND METHODS

Animals. All procedures used on the experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight-to-twelve-week-old C57BL/6 mice were purchased from Nihon CLEA (Tokyo, Japan). C57BL/6-GFP Tg mice were kindly provided by Dr. Okabe (Osaka University, Japan). C57BL/10 mice and C57BL/10-*mdx* mice were maintained in our animal facility and propagated by allowing mating.

In order to induce muscle regeneration, 50 µl of CTX (10 µmol/l in saline; Wako Pure Chemical Industries, Tokyo, Japan) was injected into the TA muscle 24 hours before cell transplantation.

Cell preparation and FACS analysis. Freshly isolated muscle-derived cells were prepared from 8–12-week-old GFP-Tg mice, C57BL/6 mice, C57BL/10 mice, or 5-week-old *mdx* mice as previously described.[35] Hind-limb and fore-limb muscles were isolated and digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37 °C. The muscle slurries were filtered through 100 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ) and subsequently through 40 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were eliminated by treatment with 0.8% NH_4Cl in Tris-buffer. Mononucleated cells were stained with biotinylated SM/C-2.6 monoclonal antibody,[35] and labeled by allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA). Mononucleated cells derived from *mdx* muscles were stained with antibodies to additional surface markers, phycoerythrin-conjugated anti-CD45 antibody (clone 30-F11; BD PharMingen, San Diego, CA), phycoerythrin-conjugated anti-CD31 antibody (clone 390; BD PharMingen, San Diego, CA), and phycoerythrin-conjugated anti-Sca-1 antibody (clone D7; BD PharMingen, San Diego, CA). After being washed, stained cells were re-suspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (Trace Biosciences, New South Wales, Australia) and 2 µg/ml propidium iodide (BD PharMingen, San Diego, CA). Cell sorting was performed on a FACS VantageSE flow cytometer (BD Biosciences, Franklin Lakes, NJ). Debris and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. We used only propidium iodide-negative fractions for further experiments. We usually obtained approximately 1.5×10^5 sorted cells from 1 g of muscle of 8–12-week-old female C57BL/6 mice.

Cell culture and intramuscular transplantation. Freshly isolated SM/C-2.6$^+$ cells from GFP-Tg mice were seeded at a density of 1×10^5 cells per 35-mm dish coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) in a growth medium, Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum and 2.5 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA), and expanded for 4 days. Further, these cells were passaged up to three times at 2-day intervals. Primary myoblasts isolated by the pre-plating method[39] from GFP-Tg mice were also cultured in growth medium. Freshly sorted cells, expanded and passaged cells, or cultured primary myoblasts were injected into TA muscles of 8–12-week-old CTX-treated C57BL/6 mice or 5-week-old *mdx* mice that show active cycles of the degeneration–regeneration process. The number of injected cells was 2×10^4 per TA muscle. Four weeks later, the injected muscles were isolated and fixed in 4% paraformaldehyde for 30 minutes, immersed sequentially in 10% sucrose/PBS and 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry. Frozen muscle tissues were sectioned (6 µm) using a cryostat. The sections were blocked with 5% goat serum (Cedarlane, Hornby, Canada) in PBS and then reacted with anti-GFP antibody (1:100; Chemicon International, Temecula, CA) and/or anti-laminin $\alpha2$ antibody (1:100; Alexis, San Diego, CA), or anti-$\alpha1$-syntrophin antibody

(1:500)[46] at 4 °C overnight. Dystrophin (1:20; NCL-DYSB or DYS2; Novocastra, Newcastle, UK), α-SG (1:50; NCL-α-SARC; Novocastra, Newcastle, UK), and β-dystroglycan (1:50; NCL-β-DG; Novocastra, Newcastle, UK) were detected using monoclonal antibodies after blocking with a MOM kit (Vector Laboratories, Burlingame, CA). The sections were incubated with appropriate combinations of Alexa 488-, Alexa 568-, and Alexa 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. The nuclei were counterstained with TOTO-3 (1:5,000; Molecular Probes, Eugene, OR). The stained sections were observed under the confocal laser scanning microscope system TCSSP (Leica, Heidelberg, Germany).

Immunocytochemistry. Cells sorted using FACS were collected by Cytospin3 (Thermo Fisher Scientific, Waltham, MA). After being fixed with 4% paraformaldehyde for 10 minutes, the cells were blocked with 5% goat serum in PBS and then reacted with anti-Pax7 antibody (1:2; Developmental Studies Hybridoma Bank, Iowa, IA), anti-MyoD antibody (1:200; Dako, Glostrup, Denmark), anti-myogenin antibody (1:200; Developmental Studies Hybridoma Bank, Iowa, IA), and anti-Ki67 antibody (1:2, Ylem, Rome, Italy) at 4 °C overnight. Primary antibodies were detected by Alexa 488- or Alexa 568-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. Stained cells were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and observed with fluorescence microscopy IX70 (Olympus, Tokyo, Japan).

Generation of lentiviral vectors and* in vitro *transduction. The third-generation self-inactivated human immunodeficiency virus-1–based lentiviral vector, pCSII-CMV-IRES2-Venus, has been described previously.[47] The vector contains a CMV promoter; an internal ribosomal entry site (IRES) followed by *Venus*, which is a variant of yellow fluorescent protein[41]; and a woodchuck hepatitis virus post-transcriptional regulatory element. A rod-truncated micro-dystrophin *CS1* complementary DNA (four rod repeats, 4.9 kb) was excised from pCAG-*CS1*[24] and cloned into pCSII-CMV-IRES2-Venus, generating pCSII-CMV-*CS1*-IRES2-Venus. The lentiviral vectors expressing Venus only, or micro-dystrophin *CS1* followed by Venus, were generated by transient cotransfection of the pCSII-CMV-IRES2-Venus or pCSII-CMV-*CS1*-IRES2-Venus, respectively, with the packaging construct (pCAG-HIVgp), vesicular stomatitis virus-G protein, and Rev-expressing construct (pCMV-VSV-G-RSV-Rev) into 293T cells, using the calcium phosphate transfection method.[47–49] Two days after transfection, the vector-containing supernatant was collected, filtered through a 0.45-μm-pore-size filter (Thermo Fisher Scientific, Waltham, MA), and concentrated by centrifugation twice at 50,000*g* for 2 hours at 20 °C. The virus pellet was re-suspended in Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) and stored at −80°C until use. The titer of the concentrated virus was 5×10^8 to 1×10^9 infectious units/ml when assayed on 293T cells, and infectivity was determined by Venus expression as analyzed on a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

Sixty thousand freshly isolated SM/C-2.6$^+$ cells in 300 μl growth medium were seeded in each well of 24-well plates and cultured for 16 hours with viral vectors expressing Venus or micro-dystrophin *CS1* at MOI of 100 or 200, respectively. After removal of free viral vectors by changing the medium, the transduced cells were cultured for 2 or 3 days and trypsinized. A cell suspension containing 2×10^4 cells in 20 μl of PBS was injected into the TA muscles of *mdx* mice. The infection efficiency of the injected cells was evaluated using a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

ACKNOWLEDGMENTS

This work was supported by Research on Nervous and Mental Disorders (16B-2), and Health Science Research Grants for Research on the Human Genome and Gene Therapy (H16-genome-003), for Research on Brain Science (H15-kokoro-021, H18-kokoro-019) from the Japanese Ministry of Health, Labor and Welfare, Grants-in-Aids for Scientific Research (14657158, 15390281, 16590333, and 18590392) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the "Ground-based Research Program for Space Utilization" promoted by the Japan Space Forum.

SUPPLEMENTARY MATERIAL

Figure S1. Freshly isolated satellite cells give rise to larger colonies than passaged myoblasts *in vitro*.

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